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New insights of RA-V cyclopeptide as an autophagy inhibitor in human COLO 320DM cancer cell lines

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Colon cancer is the leading cause for the malignancy in the gastrointestinal tract. Autophagy is a self-degradation process of the unnecessary, injured and aged organelles and proteins in the cell, which is followed by recovering of degraded products. Apoptosis is a programmed cell death which is characterized by membrane blebbing, chromosome condensation and nuclear fragmentation. Apoptosis and autophagy can occur frequently in a cell, predominantly in a series preceding apoptosis through autophagy by the formation of autophagosomes. In current research, the impact of autophagy inhibition and apoptosis activation were found to be the targeted strategies to treat colon cancer. This study is focused on the apoptotic potential of RA-V, a natural cyclopeptide through the inhibition of protective autophagy in colon cancer cells. Growth inhibitory properties were observed in the RA-V treated (125 μM) colo 320DM cells using cell viability assay. RA-V induced apoptosis of colo 320DM cells at the maximum concentration of 125 μM, which was observed using DAPI and Annexin - PI staining methods. In this study we also examined the mechanistic role of RA-V (125 μM) in and colo 320DM cells in the presence of Rapamycin (mTOR inhibitor) and chloroquine (autophagy inhibitor) using MDC and AO staining methods.

Keywords: Apoptosis, Autophagosome, Chloroquine, Colon Cancer, Cyclopeptide, Rapamycin

Colon cancer is the major cause of the malignancy in the gastrointestinal tract and is the world's third-largest cause of death[1]. Peptides are recognized for their potential to effect anatomical and biochemical role of life, through metabolism and reproduction, or during the cell- cell communication upon interaction with receptors[2]. They are able to inhibit to specific destination *in vivo* without significantly affecting the immune system as their equivalents in endogenous proteins. In recent decades, they have been the focus of an escalated number of studies as they have a wide complexity of their component building blocks and their decreased toxicity render them attractive therapeutic opportunities in diverse fields of science and technology. Peptides have also been considered as bad drug molecules for different reasons in the past, with their rapid metabolism and degradation probably the most important disadvantages and the reality that many peptides do not normally cross the cells as some tiny molecules do[3-6]. Plant cyclic peptides have several properties such as target selectivity, immunosuppressive activity, good binding affinity, anti-inflammatory activity, cytotoxic activity, tyrosinase inhibitory activity, target selectivity and anti-malarial activity which is used for the development of therapeutics as it makes them an attractive modality[7].

RA-V is a cyclopeptide isolated from the roots of the Chinese medicinal plant *Rubia Cordifolia* L. of the family *Rubiaceae*. In the Yunnan region of China, this plant is used as an anti-cancer herb as it shows anti- cancer activity, anti- angiogenesis activity and anti- inflammatory activity[8,9].

Autophagy is a self-degradation process of unnecessary, injured and aged organelles and proteins in the cell, which is followed by recovering of degraded products. During invasion and progression, cancer cells require ample amount of energy and oxygen for maintaining their rapid growth and differentiation. Autophagy is the key regulator for cellular homeostasis, which has a cytoprotective role and is linked to the survival of the cells[10,11].

Apoptosis is programmed cell death, which is characterized by membrane blebbing, chromosome condensation and nuclear fragmentation. There are two signalling pathways which regulates the apoptosis such as intrinsic pathway and extrinsic pathway. The cell undergoes apoptosis in the intrinsic way, as the cell experiences stress. In extrinsic pathway, the cell undergoes apoptosis because it gets signals from the other cells. Caspase-9 is the mitochondria-mediated

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commencer caspase in the intrinsic pathway and Caspase-8 is the death receptor mediated initiator caspase in the extrinsic pathway[12].

Autophagy has been reported to be closely associated with apoptosis: (i) autophagy is one of the upstream processes required for apoptosis, and inhibition of autophagy can effectively inhibit apoptosis (ii) autophagy can reduce the rate of apoptosis *via* inhibition of apoptosis (iii) autophagy and apoptosis can synergistically promote cell death[13-16].

Mammalian target of rapamycin (mTOR) is a vital signalling pathway involved in many cellular processes. It regulates many intracellular signalling molecules to provide energy resources to the cell. The involvement of mTOR signalling in Colo rectal cancer (CRC) is well known. Similarly, the inhibition of mTOR signalling leads to the inhibition of CRC cell growth *in vitro* and adenoma formation *in vivo*[17]. The present study, aims to find the efficacy of RA-V cyclopeptide as an autophagy inhibitor to enhance apoptosis in Colo 320DM cancer cell lines.

Material and Methods

Materials and Reagents

RA-V, Roswell Park Memorial Institute medium1640 (RPMI-1640), 50X Penicillin and Streptomycin Antibiotic, Fetal bovine serum (FBS). Monodansylcadaverine (MDC), Rapamycin (RAPA) and Chloroquine was purchased from Santa-Cruz. Acridine Orange was purchased from SRL. Annexin-V FITC kit was purchased from Bio-vision.

Cell Culture

COLO 320DM cells were obtained from NCCS, PUNE and cultured at room temperature (37°C) in an atmosphere having 5% CO_2. Cells were maintained with RPMI 1640 media with 5% fetal bovine serum (FBS) and 1% antibiotic (penicillin and streptomycin cocktail).

Cell Viability (Sulforhodamine B) Assay

Cell viability assay was performed using Sulforhodamine B (SRB) assay kit from Himedia. COLO 320DM cells were grown in 96- well plates with a density of 1×10^5 cells/well and incubated for 24 h. After the treatment of RA-V, the cells were fixed with the fixation solution. After washing four times with the washing solution, sulforhodamine dye was added to each well and stained for 30 min. Then washed four times with the washing solution and stained cells were dissolved in the solubilization solution. The absorbance was measured at 565 nm using microtiter plate reader[18].

DAPI Staining

DAPI staining was performed to determine the morphological changes of COLO 320DM cells. The cells were seeded at a density of 1×10^5 cells/well. COLO 320DM cells were grown on cover slips in 6-well plates and incubated for 24 and 48h. COLO 320DM cells were treated with 100 μM and 125 μM of RA-V for 24 and 48 h. The cells were washed with PBS after incubation and fixed by acetone/methanol (1:1) at room temperature (37°C) for 10 min. DAPI staining was done for 10 min and washed with PBS. The cells were mounted and images were taken by Fluorescence microscopy[19].

Annexin-PI staining assay

Annexin-PI staining was performed to examine the effect of cellular apoptosis in COLO 320DM cells which were seeded in 6-well plates and grown for 24 h. The cells were treated with 100 μM and 125 μM of RA-V with and without Rapamycin (1 μM), also with and without Chloroquine at the concentration of 25 μM for 24 and 48 h. Doxorubicin was used as a Positive control. After the treatment, the cells were centrifuged at 1200 rpm for 10 min at room temperature. The pellet was washed with PBS and centrifuged again. Pellet was dissolved in 500 μL of binding buffer and 5 μL of Annexin-V and PI were added. Flow-Cytometry analysis was performed using BD FACS Caliber[20].

Staining of autophagic vacuoles by Monodansylcadaverine (MDC)

Monodansylcadaverine staining was performed to determine the formation of autophagic vacuoles. COLO 320DM cells were grown on cover-slips with 6 well plates after the treatment with RA-V (100 μM, 125 μM), Rapamycin (1 μM) and Chloroquine (25 μM). For MDC staining, cells were exposed to MDC stain (50 μM) in the dark for 15 min. Confocal microscopy analysis was performed after washing the cells with PBS[21].

Staining of autolysosomes of Acridine-Orange (AO)

Acridine-Orange staining was performed to determine the formation of autolysosomes. COLO 320DM cells were grown on cover slips in 6-well plates after the treatment with RA-V (100 μM, 125 μM), Rapamycin (1 μM) and Chloroquine (25 μM). For AO staining, cells are exposed to AO

stain (100 μM) for 15 min. Confocal microscopy analysis was performed after washing the cells with PBS[22].

Statistical Analysis

Statistical analysis was carried out using Graph-pad Prism 6 software. The data were analysed using One-way Anova. Cases with $P< 0.0001$ were considered to be significant.

Results and Discussion

Cell Viability Assay

Cell viability was evaluated by Sulforhodamine B Assay in COLO 320DM cells. RA-V inhibited growth of COLO 320DM cells in a time and dosage dependent manner (Fig. 1A & B). Increased growth inhibition of COLO 320DM cells was found at the maximum concentration of 125 μM RA-V cyclopeptide as compared to the control.

RA-V induces apoptosis in COLO 320DM cells

DAPI Staining method

Using DAPI staining, nuclear morphological changes of COLO 320DM cells treated with the concentrations of 100 μM and 125 μM of RA-V cyclopeptide were analysed and photographed with fluorescence microscopy. RA-V treated cells at 125 μM, led to a substantial increase in the number of apoptotic cells than 100μM concentration of the cyclopeptide which is inferred by fragmented nuclei and condensed chromatin which appeared as bright blue fluorescence (Fig. 2A-D). DAPI staining of (control) untreated cells depicted the absence of chromatin condensation and nucleus shrinkage which indicated the absence of apoptosis in these untreated cells (Control) for 24 and 48 h. Similar results were obtained on NSCLC cells treated with A. *macrocephala* which led to an increase in the number of apoptotic cells indicated by bright blue fluorescence [23].

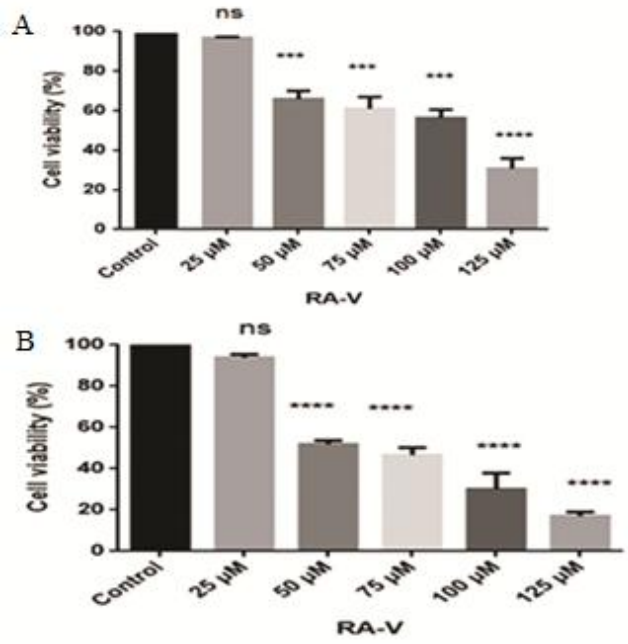

Fig. 1 — Dose dependent inhibition of RA-V on the growth of COLO 320DM cells (A) 24 h; and (B) 48 h. The data are presented as mean ± SEM (****$P< 0.0001$, *vs* Control)

Annexin-PI Staining method

Annexin-V- PI staining was used to examine the effect of RA-V on the cellular apoptosis of COLO 320DM cells to determine the percentage of apoptotic cells. Our results indicated that in untreated cells, (Fig. 3A-H), the percentage of the non-apoptotic cells was 99.23% and 98.82% which were found to be viable for 24 h and 48 h. RA-V treated COLO 320DM cells at 100 μM showed that there was an increase in the late apoptotic cells of about 7.68% and 9.26% as compared to that of positive control, for 24 and 48 h. RA-V treated COLO 320DM cells at 125 μM showed an increase in the late apoptotic cells which was about 18.28% and 20.28% as compared to the positive control for 24 and 48 h, respectively.

Rapamycin has been shown to be immunosuppressive and an essential signal mediator for PI3 kinase[24]. The Rapamycin target (TOR) in yeast and animal cells has also been established[25]. In RA-V treated COLO 320DM cells along with Rapamycin (a mTOR inhibitor) at 100 μM, the percentage of dead cells for 100 μM was found to be 5.67% and 6.65% for 24 and 48 h. In RA-V treated cells with Rapamycin at 125 μM, the percentage of dead cells was found to be 6.67% and 8.71% for 24 and 48 h, respectively. The results indicated that RA-V inhibited protective autophagy and also attenuated the activity of Rapamycin, a mTOR inhibitor, thereby promoting apoptosis of COLO 320DM cells.

Chloroquine (CQ) mainly inhibits autophagy by impairing autophagosome fusion with lysosomes[26,27]. Chloroquine is a poor hydrophobic base that diffuses through the cell lysosomes, where the cell becomes protonated and resulting in an increase in the pH of the lysosome.It serves as lysosomotropic agent in addition to its anti-cancer effects[28]. This chloroquine filled lysosomes are no longer capable of fusing autophagosomes, hence, blocking autophagy in the

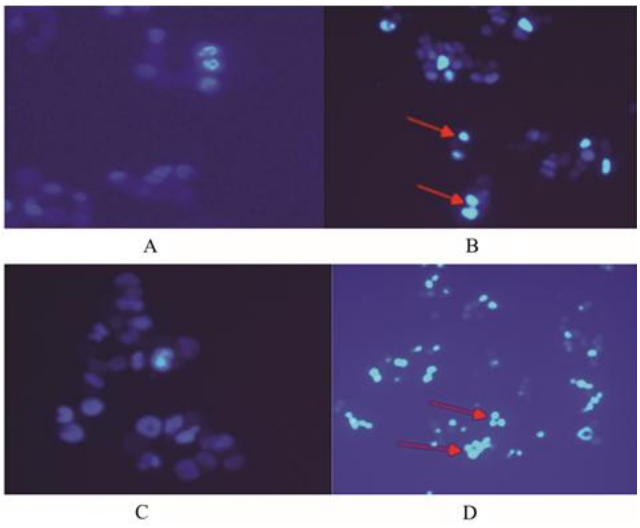

Fig. 2 —Photomicrographs of Nuclear staining using DAPI of human Colo 320DM cells treated with RA-V for 24 and 48 h. (A) Control- untreated cells; (B) 125 μM RA-V for 24 h; (C) Control- untreated cells; and (D) 125 μM RA-V for 48 h. (Arrows indicate cell shrinkage, nuclear fragmentation, and margination of the nucleus, all associated with the apoptotic mode of cell death)

late stage. In RA-V treated COLO 320DM cells along with Chloroquine (an Autophagy inhibitor) at 100 μM, the percentage of the dead cells for 100 μM was found to be 76.49% and 79.39% for 24 and 48 h. In RA-V treated COLO 320DM cells with Chloroquine at 125 μM, the percentage of the dead cells was found to be 80.01% and 88.07% for 24 and 48 h, respectively. These results indicated that Chloroquine inhibited autophagy and contributes to enhanced apoptosis of COLO 320DM cells.

RA-V suppresses autophagy

The aggregation of autophagic vesicles and autolysosome formation are two autophagy markers that could be stained by monodansylcadaverine (MDC) and acridineorange (AO), respectively. RA-V treatment of 125 μM resulted in a significant concentration-dependent reduction of autophagic vesicles stained with MDC. The mTOR signaling pathway was blocked with a special mTOR inhibitor rapamycin (RAPA) to elucidate the role of mTOR activation in RA-V-inhibited protective autophagy. RA-V treatment at the concentration of 125 μM in the presence of RAPA showed increased number of autophagic vesicles stained with MDC and AO than 100 μM of the cyclopeptide relative to RA-V-treated (125 μM) cells alone (Fig. 4A-D).

Similar results have been obtained from bright-red autolysosomes stained with AO. Reduction in the number of autophagosomes may either be related to an impaired autophagy or to an unnecessary deterioration[29]. Autophagy was studied in the vicinity of an inhibitor of autophagy, chloroquine (CQ), which prevents lysosome acidification and blocks downstream of autophagy. As shown in (Fig. 5A-D), combined treatment of RA-V with Chloroquine (CQ) inhibited a significant increase of autophagosome compared with RA-V treatment alone.

This research is primarily concerned with the anti-cancerous effects of RA-V on human colon cancer cells, which could provide a basis for drug development and colon cancer treatment. TAK1 inhibition of RA-V prevents protective autophagy with TAK2/AMPK/ mTOR and TAK1/P70S6K pathways in non-small-cell lung carcinoma (NSCLCs) cell -lines that are Kras-dependent[30]. The cyclopeptide RA-V, isolated from *Rubia Cordifolia* L. exhibits strong anti-inflammatory and anti-cancer properties in a variety of celltypes[31,32].

In investigating the effect of this cyclopeptide on colon cancer cells, RA-V has been found to induce apoptosis in colon cancer cells. Throughout growth and aging, natural apoptosis happens as a homeostatic process in order to maintain tissue cell populations. Autophagy is a retained catabolic technique requiring cellular homeostasis and is important under stressful conditions for preserving normal cell physiology[33]. It is responsible for combating carcinogenic, contagious, degenerative and deleterious agents in the preservation of the homeostasis of body and control healthy life processes[34]. Autophagy could be a selective or non-selective lysosomal degrading mechanism, and is triggered through regulatory signals by stresses such as starvation or rapamycin[35,36].

Apoptosis-inducing mechanism, along with chemo resistance, tumor growth and metastasis are recognized to play an important role in cancer pathogenesis[37]. The anti-tumor activity is partly due to the initiation of apoptosis. The main anti-cancer mechanism of chemotherapy is commonly considered to be apoptosis and may be activated by various tumor stimulation[38]Many cancer therapy strategies suppress tumors by inducing the apoptosis of cancer cell[39].

Autophagy was proposed as a tumor suppressing method that might reverse or postpone tumorigenesis[40,41]. Autophagy is a mechanism of cell death through which cytoplasmic organelles and proteins are cloistered in vacuoles and carried to lysosomes for recycling and degradation[42]. Autophagy

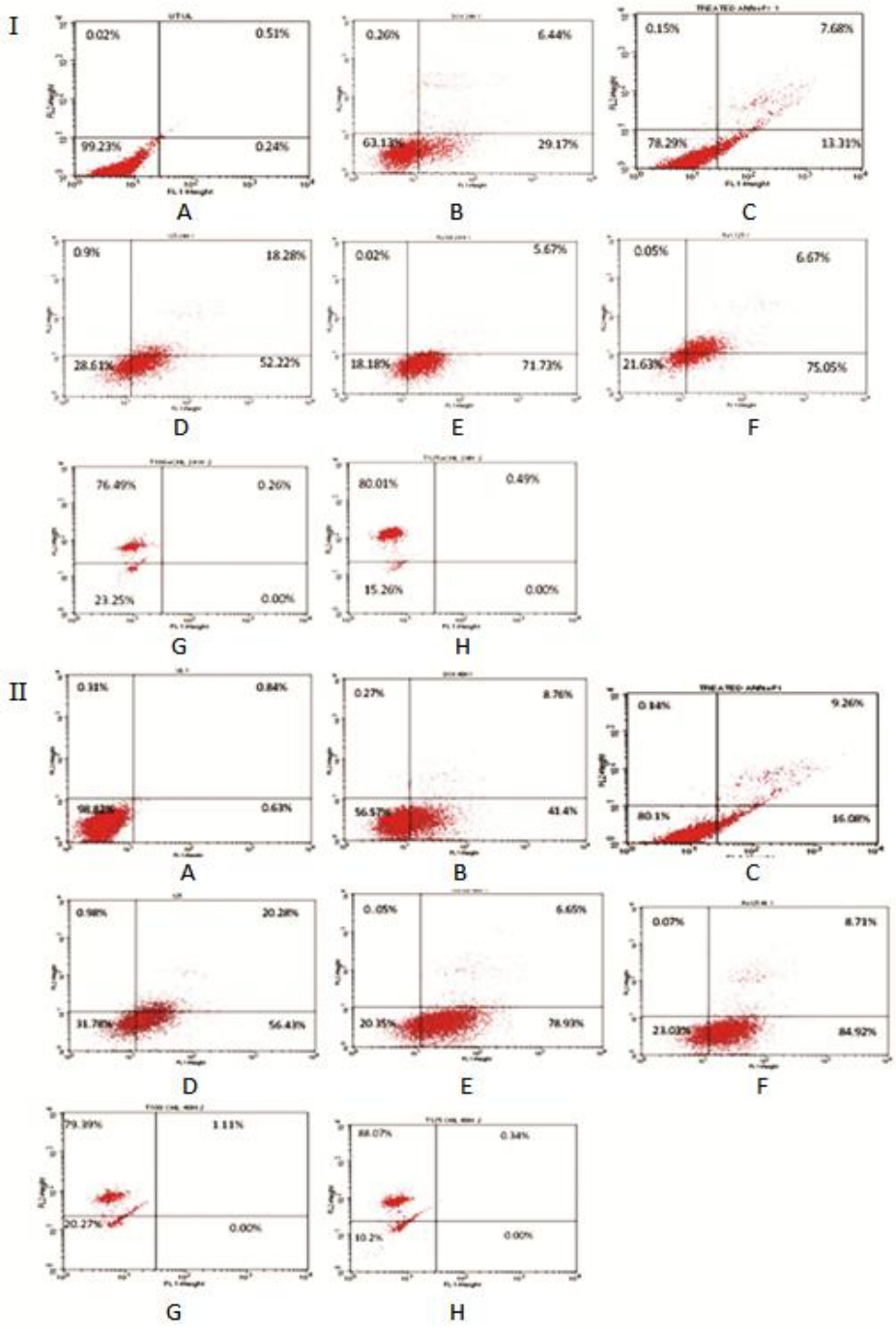

Fig. 3 — Annexin V -PI staining of COLO 320DM cells for 24 h (I) and 48 h (II): (A) Control- untreated cells; (B) Positive control; (C) 100 µM RA-V; (D) 125 µM RA-V; (E) 100 µM RA-V with inhibitor-1 µM Rapamycin; (F) 125 µM RA-V with inhibitor- 1 µM Rapamycin; (G) 100 µM RA-V with inhibitor-25 µM Chloroquine; and (H) 125 µM RA-V with inhibitor-25 µM Chloroquine

is not necessarily pro-death, but can proceed under cellular stress conditions, comprising that inhibited by chemotherapy or nutrient deprivation[43].

RA-V showed cytotoxicity activity in the COLO 320DM cells. The maximum cytotoxicity was found at 100 µM and 125 µM. The results showed that RA-V inhibits the proliferation of COLO 320DM cells in a dosage and time dependent manner (Fig. 1A & B). Similar results were found on the cytotoxicity of RA-XII on HepG2 cells at the concentrations of 2.5 and 5 µM[44].

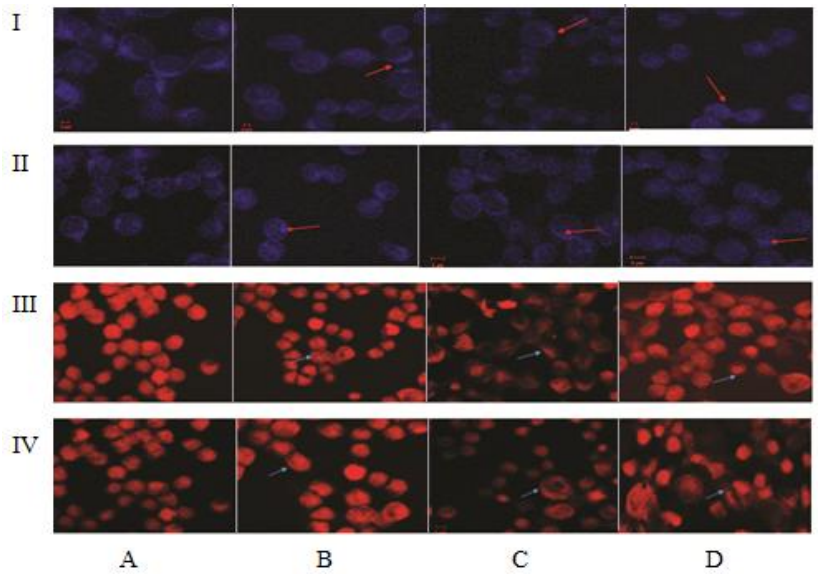

Fig. 4 — Confocal microscopy analysis of COLO 320DM cells treated with RA-V using MDC staining (I & II) and Acridine Orange (III & IV) after 24 h (I & III) and 48 h (II & IV). (A) Control-untreated cells; (B) Inhibitor- 1 μM Rapamycin; (C) 125 μM RA-V; and (D) 125 μM RA-V with inhibitor-1 μM Rapamycin

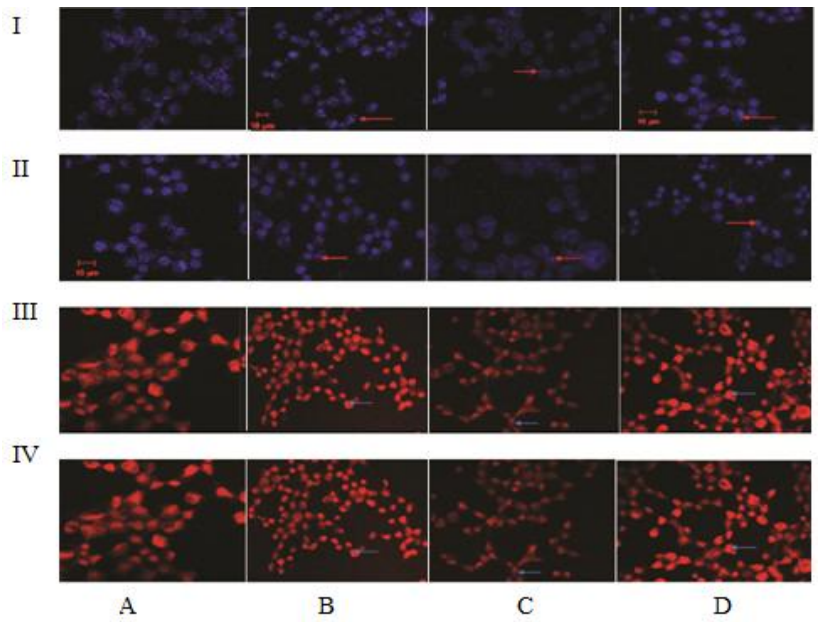

Fig. 5 — Confocal microscopy analysis of COLO 320DM cells treated with RA-V using MDC staining (I & II) and Acridine Orange (III & IV) after 24 h (I & III)and 48 h (II & IV). (A) Control-untreated cells; (B) Inhibitor-25 μM Chloroquine; (C) 125 μM RA-V; and (D) 125 μM RA-V with inhibitor-25 μM Chloroquine

RA-V induced apoptosis in COLO 320DM cells. The results showed that nuclear morphological changes were represented as chromatin condensation and fragmented nuclei (Fig. 2 A-D). Similar results were obtained on the inhibition of autophagy and induction of apoptosis through the activation of mTOR pathway by RA-XII on HepG2 cells[45]. The apoptotic cells in each phase were determined (Fig. 3 A - H). Similar results were found on the induction of apoptosis on HT-22 cells[45].

MDC and AO results indicate that the development of autophagic vesicles and autolysosomes by RA-V is significantly reduced (Figs. 4 & 5). Also, it suppressed protected autophagy and induced apoptosis in the COLO 320DM cells.

Conclusion

The purpose of this study was to elucidate the autophagy inhibitory mechanisms underlying RA-V in COLO 320DM cells which can impart a scope for the innovation of drugs and treatment of colon cancer.

RA-V substantially decreased the growth of COLO 320DM cells in a dosage and time- dependent manner. The determination of probable mechanism of RA-V to induce apoptosis was observed by DAPI staining and Annexin-PI staining of COLO 320DM cells. In DAPI staining, nuclear morphological changes were observed which exhibited as bright blue fluorescence. Annexin- PI staining of COLO 320DM cells differentiated between the live cells, early apoptotic cells, late apoptotic cells and dead cells.

RA-V also suppresses autophagy and has been identified as a powerful autophagy inhibitor. The staining results of Monodansylcadaverine and Acridine- Orange specified that the autophagic vesicles and autolysosomes were significantly reduced by RA-V. The experimental findings obtained in this study suggest that RA-V can be developed as an autophagy blocker for effective treatment of colon cancer.

Conflict of interest

The authors declare no conflict of interest.

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