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Anticancer Potential of Triaryl Methane

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Abstract

A derivative of Triaryl methane CRR- 638 was evaluated for their anticancer potential in lung cancer (A549) cells as *In vitro* model. CRR- 638 inhibited lung cancer cell in the dose- dependent manner. The maximum inhibition of 70.9 ± 0.98 µM was observed at 50 µM. The IC_{50} value of CRR- 638 in lung cancer cells was found to be 8.3 ± 2.5 µM. The CRR- 638 showed non- toxic to the normal cells (PBMCs). On phase contrast microscope, the CRR- 638 treated lung cancer cells showed dead and floating cells. The morphological feature was observed by AO/EB- staining of CRR- 638 treated lung cancer cells which showed induction of apoptosis. The cell cycle distribution was done using flow cytometer by propidium iodide staining, showed induction of apoptosis with significant reduction of cells in S and G2/M- phase in the CRR- 638 treated lung cancer cells. In conclusion, CRR- 638 selectively inhibited the lung cancer cells without any toxic effect on the normal cells.

Keywords: Triaryl methane, anticancer, PBMCs, CRR- 638

Contents

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1. Introduction

Cancer is a fatal, complex genetic disease in which uncontrolled clonal expansion of somatic cell kills by invading and eroding of normal tissues. Malignant tumors are commonly called as cancers are motile and spread to other parts (metastatic) and are very harmful. Whereas, Benign tumors are mainly localized and do not spread to other parts of the body. Hence, it is less harmful (Lee *et al* 2011). Cancer cells are characterised by multiple structural, molecular and behaviour features. There are six hallmarks of cancer-Sustaining proliferative signalling, evading growth supressors, activating invasion and metastases, enabling replicative immortality, inducing angiogenesis and resisting cell death (Hanahan *et al* 2000, Hanahan *et al* 2011). These six hallmarks of the cancer are shared by all types of cancers. Lung cancer continued to be most common cancer diagnosed in men (accounts 16.5 % of all new cases) and breast cancer in women (accounts 23% of new cases) (Jemal *et al* 2010) . The aim and goal of the chemotherapeutic drugs is to shrink primary tumor, slow down the tumor growth and kill cancer cells without spreading to other body parts. But chemotherapeutic drugs have a high rate of failure because they usually kill only specific types of cancer

cells in tumor or cancer cells mutate and become resistant to the drugs. And they also kill both cancer and normal cells. (Dorai *et al* 2004, Saunders *et al* 2010, and Goyal *et al* 2012).

2. Materials and Method

2.1 Cell Line:

A549 (adenocarcinoma lung cancer) cell line was obtained from NCCS (National Centre for Cell Science) was maintained as monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 10 U/ml of penicillin, 10 mg/ml of streptomycin, and 0.25 mg/ml of amphotericin B.

2.2 Maintainance and Storage of Cell Lines:

Lung cancer cell showed doubling time of 12 to 24 h. The cells reached confluence in 3 to 4 days and these cells were passaged to get the cells for the experiments and also to store in liquid nitrogen. Passaging was done as follows: The culture media was removed from 25 ml tissue culture flasks by aspiration inside the laminar air flow chamber, and the cells were rinsed with trypsin solution to remove the trace of serum in media, which may inhibit action of trypsin. 2 ml of trypsin solution was added to the flask containing cells and incubated at 37°C for few minutes. As soon as cells are dislocated from the surface, flask was rinsed with 5 ml of serum containing media to arrest the trypsinization. The suspension of cells was collected in the 15 ml sterile centrifuge tubes and cells were pelleted at 1500 rpm for 3 min. The pellet was suspended in fresh media and a part of the cells were seeded back into the flask. For cryopreservation, cells were suspended in cryopreservation media in cryo vial and frozen at -80°C for a day then transferred to liquid nitrogen.

2.3 Anti-Proliferative Assay:

Cell viability was measured by conventional MTT reduction assay, as described by Mossman et al (1983). Briefly, A549 cells were seeded at a density of one million cells per well in 96 well plates for 24 h, in 200 µl of DMEM supplemented with 10% Foetal bovine serum. Then culture supernatant was removed and 200 µl of DMEM containing different concentrations of compounds (10 to 100 µM) were added and incubated for 24 h at 37°C in 5% CO_2 incubator. After treatment cells were incubated with MTT (10 µl; 5 mg/ml) at 37°C for 3 h and then with DMSO at room temperature for 15 min. The plates were read at 570 nm on a scanning multi well spectrophotometer

2.4 Isolation of PBMC and Determination of Cytotoxicity:

In order to evaluate the effect of the CRR-638 on human normal cells, human PBMC were isolated (Gayathri *et al,* 2007).To isolate the PBMC, blood samples in sterile heparinised tubes were collected under medical and ethical committee control from healthy volunteer donors (aged 18-35). Peripheral blood mononuclear cells were isolated using standard Ficoll-Histopaque density centrifugation. The interface lymphocytes were harvested and washed twice with sterile phosphate buffered saline (PBS). Then PBMC were suspended in RPMI media and cells were counted $2x10^3$cells/200 µl/well were seeded in 96 well plates and incubated overnight. Different concentrations of compound were treated in triplicates. After 24 h of incubation, cells were treated with MTT (10 µl, 5 mg/ml) at 37°C for 3 h and then formazan crystals formed was dissolved in 100 µl of DMSO at room temperature for 15 min. The plates were read at 595 nm on a scanning multiwell spectrophotometer.

2.5 Phase Contrast Microscopic Observations:

To observe the morphological changes of CRR-638 treated lung cancer cells, lung cancer cells was plated in 12-well plates and treated with IC_{50} concentration of CRR-638 for 24 h. After treatment, the cells were observed in 40 X objective of phase contrast microscope.

2.6 Apoptosis Assay Adopting Acridine Orange/ Ethidium Bromide Double Staining:

Cells were cultured in 12-well plates and treated with IC_{50} concentration of CRR-638 and incubated for 24 h. After incubation period, cells were washed once with PBS, cells were stained with 200 µl of a mixture (1:1) of acridine orange-ethidium bromide (4 µg/ml) solutions. The cells were washed once with PBS and viewed using an inverted fluorescent microscope.

2.7 Cell Cycle Analysis:

In order to determine cell cycle distribution, A549 cells at cell density of $3X10^4$ cells was plated on six well plates and treated with IC_{50} concentration of CRR-638 for 24 h. After treatment cells were trypnised and fixed in 70% ethanol for overnight at 4°C. The cell were pelleted out, resupended in PBS and treated with RNase a (10 mg/ml) and incubated for 3 h at 37°C and then centrifuged at 6000 rpm for 10 min. To the pellet a 200 µl of PBS containing triton X100 (0.01%) and propidium iodide (100 µg/ml) was added and incubated for 15 min in dark. The stained cells were analysed by flow cytometry.

3. Results and Discussion

3.1 Anti-Proliferative Activity of CRR-638 in Lung Cancer Cells:

Lung cancer (A549) cells is a human alveolar basal epithelial adenocarcinoma cells isolated from 58 years old causcian male was used for assess the anti-proliferative activity of CRR-638. The dose response studies of CRR-638 on A549 cells showed maximum inhibition of 70.9±0.98% at 50 µM. The dose response curve of CRR-638 on A549

cells revealed CRR-638 inhibited the proliferation of the lung cancer cells in dose-dependent manner (fig 1). The IC_{50} concentration of CRR-638 (concentration of compound which inhibits 50% of cells) was found to be 8.3±2.5 µM.

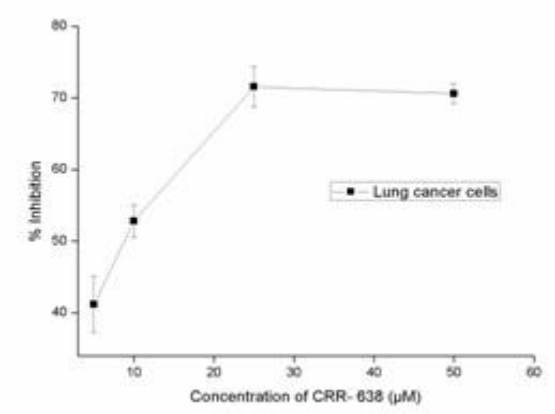

Figure 1: Anti-proliferative activity of CRR-638 in lung (A549) cancer cells

3.2 Inhibitory Activity of CRR-638 In Normal Cells (PBMCS):

In order to determine the effect of CRR-638 in normal cells, PBMCs was isolated from a human healthy volunteer and treated with different concentrations of CRR-638. The derivative, CRR-638 showed minimsum (5.75±1.6%) inhibitory activity in PBMCs (fig. 2).

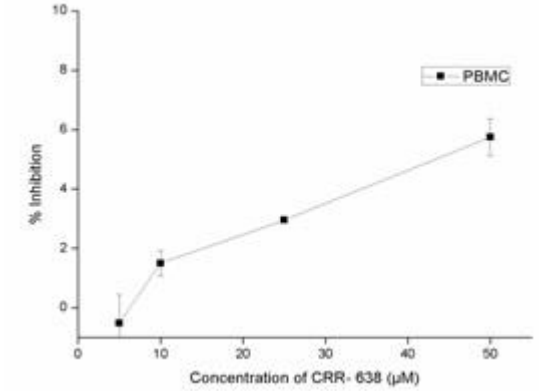

Figure 2: Inhibitory activity of CRR-638 in normal cells

3.3 Phase Contrast Microscopic Observations:

In order to determine the morphological changes on CRR-638 treatment, lung cancer cells after treatment with IC_{50} concentration of CRR-638 and observed under inverted phase contrast microscope. The phase contrast microscopic observations of CRR-638 treated lung cancer cells showed irregular shaped cells, dead and floating cells. This suggested that CRR-638 induced cell death in lung cancer cells.

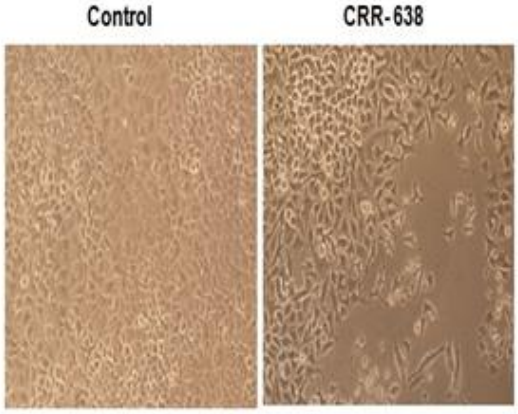

Figure 3: Morphological changes of lung cancer cells treated with CRR-638 by phase contrast microscope (40X**)**

3.4 Acridine Orange Ethidium Bromide Double Staining:

The principle of this staining is Acridine orange (AO) permeates all cells and makes the nuclei appears green. Ethidium bromide (EB) is only taken up by cells when cytoplasmic membrane integrity is lost, and stains the nucleus red. Thus live cells will have intact green nucleus; early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange chromatin; necrotic cells will have a structurally normal orange nucleus. In the control cells, the cells exhibited intact nucleus with green color in contrast to cells treated with CRR-638 showed typical characteristics of apoptosis such as cell shrinkage, nuclear fragementation and formation of apoptotic bodies. This also confirmed the CRR-638 induced apoptosis in lung cancer cells.

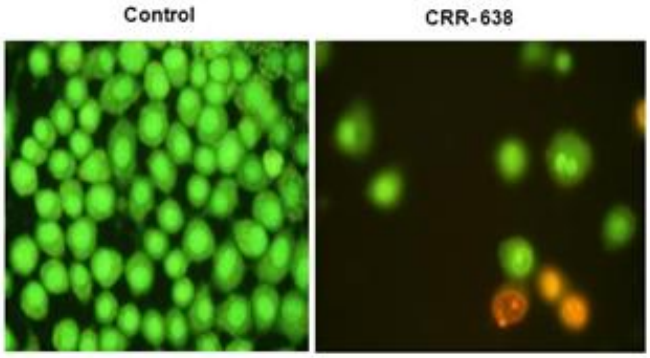

Figure 4: Acridine orange-ethidium bromide double staining of lung cancer (A549) cells treated with CRR-638(40x)

3.5 Cell Cycle Analysis:

Cell cycle analysis exploits the of DNA content of the cells. Cells have the DNA content of 2N in G1 phase, more than 2N in S-phase and 4N in G2/M-phase. The apoptotic cells will have less than 2N (Sub-G phase) due to DNA fragmentation. The DNA content can be determined using flow cytometer by propidium iodide staining of the cells. The induction of Sub-G population of cells from 0.3% in control cells was found to be increased to 15.9% in CRR-638 treated lung cancer cells was observed. Simultaneously, S- phase and G2/M phase population of cells were also decreased significantly. This also confirmed the induction of apoptosis in lung cancer cells.

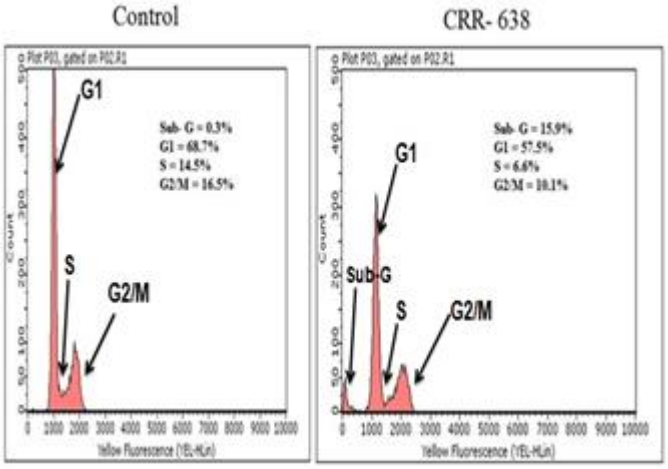

Figure 5: Cell cycle distribution of lung cancer cells (A549) treated with CRR-638 by flow cytometer

4. Conclusion

In vitro anti-proliferative activity of triaryl methane derivative (CRR-638), was done by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reduction(MTT) assay in lung cancer (A549) cells. A significant dose-dependent anti-proliferative effect was observed in lung cancer cells. Based on the dose-response curve, the IC_{50} (concentration of drug to inhibit 50% of cells) was found to be 8.3±2.5 µM. For the further analysis IC_{50} concentration was used to investigate the effect of drug on lung cancer cells. Peripheral blood mononuclear cells (PBMC) are frequently used as a model for cytotoxicity testing in normal cells. Upon treatment, CRR-638 exhibited minimum inhibitory activity in normal PBMC at the testing concentrations, suggesting that CRR-638 selectively

inhibited the cancer cells but not the normal cells. The cells treated with CRR-638 exhibited the structural and biochemical features of characteristic of apoptosis such as loss of cell viability, cell shrinkage and nuclear condensation. The DNA binding dye AO/EB was used to find apoptosis and necrotic cells due to loss of membrane integrity from intact membrane. The CRR-638 treated cells induced cell shrinkage, cytoplasmic condensation, nuclear condensation and formation of apoptotic bodies, which is the hallmark of apoptosis. The morphological and fluorescent staining confirmed the induction of cell death due to apoptosis in A549 cells. Flow cytometric studies further confirmed the induction of apoptosis.

5. References

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