International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 11, Issue 6, 2019

Short Communication

IN VITRO EVALUATION OF ANTI-CANCER POTENTIAL OF A3 ADENOSINE RECEPTOR AGONIST ON A549 HUMAN LUNG CANCER CELL LINE

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Received: 19 Nov 2018 Revised and Accepted: 24 Apr 2019

ABSTRACT

Objective: The objective of this study was to evaluate the cytotoxic potential of A3AR agonist (ABMECA) against human lung cancer cell line A549 by using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay.

Methods: Adenocarcinoma cell line A549 was used to assess MTT based cells viability. *In vitro* cytotoxic activity was evaluated for 3 different concentration of doxorubicin and A3AR by MTT cytotoxicity assay. Cytotoxicity assay carried out for 3 consecutive days that involves culturing cells into Dulbecco's MEM medium modified with 10% FBS for 24 h then treatment with different dose of standard and test drug with incubation period of 24 h followed by treatment with MTT for estimation of cytotoxicity and finally, optical density (OD) was measured at 570-630 nm.

Results: Different concentration of doxorubicin (1, 5, 10 µM) and ABMECA (10^{-6}M, 10^{-5}M and 10^{-4}M) shown dose-dependent cytotoxicity. There was a significant decrease ($p<0.05$) in cell viability in both doxorubicin and ABMECA concentration in a dose-dependent manner. This study may guide further for *in vivo* evaluation of test drug in the lung cancer model.

Conclusion: A_3 Adenosine Receptor agonist could be potential moiety for the treatment of lung cancer and it would require *in vivo* study for further research.

Keywords: A549 cell line, Adenosine, Doxorubicin, MTT assay, Lung cancer

DOI: http://dx.doi.org/10.22159/ijpps.2019v11i6.30863

According to WHO, Cancer is a leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018. The most common cancer is Lung cancer (2.09 million cases) and death due to Lung cancer expected to 1.76 million. Lung cancer is the leading cause of cancer death across the world. Although science shown dramatic advancement in the field of cancer biology, occurrence and mortality of lung cancer shown continuously rise globally. The worldwide incidence and mortality from various cancers in the year 2008 have been estimated for more than 100 countries under the GLOBOCAN series that later published by the International Agency for Research on Cancer. According to this report, the global prevalence of lung cancer is 12.7% (1.61 million). According to ICMR Cancer Registry, a total of 41,000 lung cancer cases would have been diagnosed as per data from the July 2002. The survival rate of lung cancer improved only 14% in contrast to the five-year survival of 52% in comparison to other cancers [1, 2]. All these data indicating sharply rising the incidence of cancer through worldwide.

Different therapeutic approaches for lung cancer have been evaluated, including the treatment with a cytotoxic agent called as chemotherapy, radiation and biologic therapies also called as Immunotherapy, both as monotherapy and in combination for its synergism and reducing toxicities [3], but it is not effective. Recently, adenosine receptors have been emerged as novel cancer cell-specific targets [4]. Adenosine is a purine nucleoside found within the interstitial fluid of tumor at high concentrations compare to normal cell. By affecting stimulation or inhibition of adenylyl cyclase, agonist of various receptor scans modulates tumor growth by interacting with all four G-protein-coupled adenosine receptors (AR) designated as A_1, A_{2A}, A_{2B} and A_3. The availability of specific agonists and antagonists enabling the evaluation of these ligands as an immunomodulator and anticancer agents. Interestingly, up regulation of A_3AR had been found in various tumor cells. This finding may suggest that the specific AR may work as a biological marker. If we could target this with specific ligands, it may be leading to cell growth inhibition in cancer [5].

A3AR agonists exert a differential effect on normal and tumor cells. Compare to normal cells in which the A3AR agonists induce the production of growth factors, apoptosis and tumor growth inhibition takes place in tumor cells [5]. There is much evidence support the link in between cancer and inflammation, and A_3AR had been identified as a link for the same [6]. Arrests of cell cycle and induction of apoptosis are main pharmacological action that result of activation of A_3AR by its agonist which ultimately inhibits the proliferation of human lung cancer cells. A_3AR have also been found to modulate TNF-α and VEGF. Moreover, the modulation of Akt-mediated signaling pathway is involved in the anti-proliferative effect [6, 7].

In this study, we have evaluated the cytotoxicity of a novel molecule ABMECA and doxorubicin by MTT assay. MTT assay working based on the principle of that yellow MTT (tetrazole) is reduced to purple formazan in the mitochondria of living cells. Reduction reaction depends on the functioning of the mitochondrial enzyme that can be directly correlated to living cells. The formed crystal of formazan later dissolved in suitable solvent like Dimethyl sulfoxide. Then absorbance of this colored solution can be quantified with a spectrophotometer at a wavelength range between 500 and 600 nm. After that to determine the cytotoxicity of test drug, the amount of formazan produced by untreated control cells can be compared. The cytotoxicity of the test drug can be inferred, through the production of a dose-response curve and comparing significance in between group. An increase in dead cell number results in an increase in the amount of MTT formazan formed that can be directly correlated by an increase in absorbance at specific wavelength [8]. There is certain limitation of MTT assay to determine cytotoxicity, like depends on physiological state of cells results, may be varies, mitochondrial dehydrogenase activity varies in different cell types, it may be possible that exposure to MTT and its conversion of formazan crystals cause cytotoxicity and abnormal alteration to cellular morphology take place. Also, it is less sensitive compares to fluorescent or luminescent assays in case of cells with low proliferation rate or with metabolically poor active and few chemical compounds like coenzyme A, polyphenols and vitamin A interfere with the reduction of MTT to formazan [9].

A549 (adenocarcinoma) cell line was obtained from the National Centre for Cell Science (NCCS), Pune. A549 cells are cultured in Dulbecco's MEM medium modified with 10% FBS. Maintained in a humidified atmosphere of 5 % CO2 in air at 37 °C in cell culture lab. All the chemicals purchased from good quality manufacturing companies. It involves ABMECA (1 mg) from Sigma Aldrich, doxorubicin (10 mg vial) from Pfizer, Phosphated buffered Saline from HIMEDIA, DMEM from HIMEDIA, dimethyl sulfoxide (DMSO) from HIMEDIA, MTT-salt from HIMEDIA.

MTT was weighed with proper handling precaution and dissolved in sterile phosphate-buffered saline in Eppendorf tube with aluminum foil covering at 5 mg/ml and cannot stored for more than 3 w in the dark at 4' C temperature. Whole assay the procedure was divided in to three parts. On day 1, separation and counting of cells from incubation flask was carried out. After counting 50000 cells were transferred to each well with fresh media. Than the plate was incubated for next 24 hrs. Then on day 2, drug solutions were applied to the each well according to the previously decided protocol along with fresh media. Than the plate was incubated for next 24 hrs. At last on day 3, 40µl of MTT solution (5 mg/ml) was administered to each well. Then the plate was incubated for 3-4 h at 37°C temperature. After incubation, the media was removed. After removal of media 200 µl of DMSO (Hi Media) solution was added to each well. Again, the plate was incubated at room temperature for 15-30 min. Finally, OD was taken at 570-630 nm [10].

After deriving absorbance of control, standard, test and blank, the absorbance reading of the blank must be deducted from control and all samples. Then, Absorbance readings from test samples divided by those of the control and multiplication with 100, give a percentage of cell viability or proliferation. If absorbance value is greater than the control, then it indicates cell proliferation and if lower than control values it suggests cytotoxicity or inhibition of proliferation.

$$\%\text{Viable cells} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100$$

The results were expressed as mean±SEM A value of $p<0.05$ was considered statistically significant.

In this study different concentration of doxorubicin (1, 5, 10 µM) and ABMECA (10^{-6}M, 10^{-5}M and 10^{-4}M) were used to determine its potential cytotoxicity on A549 Human lung Cancer Cell line by MTT assay (fig. 1). Result indicated that a decline in absorbance at 540 nm in the cells treated with increasing concentration of the both doxorubicin and ABMECA in comparison to the control cells that suggest cytotoxicity of both chemicals. Doxorubicin and ABMECA both shown significance difference ($p<0.05$) compared to normal control (fig. 2).

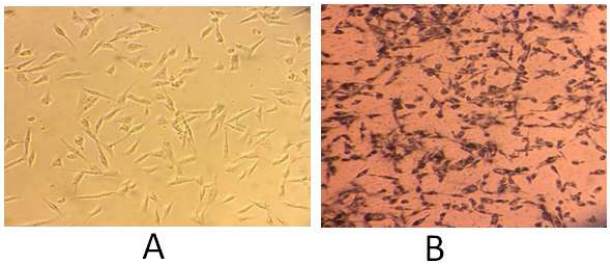

Fig. 1: (A) Normal cells, (B) Cells after MTT application

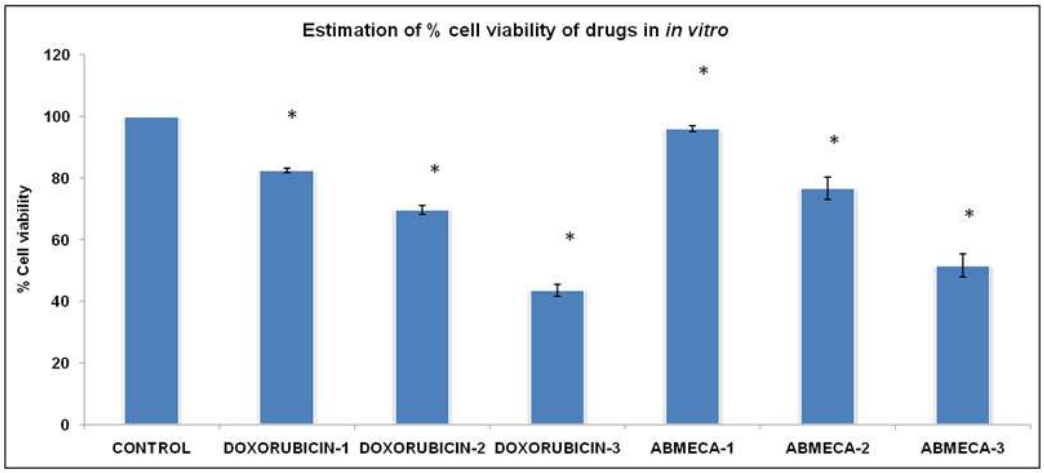

Fig. 2: Shows the % cell viability after drug treatment *in vitro* by performing cell cytotoxicity assay. The graphs show a significant decrease in cell viability in doxorubicin-3 concentration ($p<0.05$). There is a significant decrease in cell viability in A3AR AGONIST-3 concentration ($p<0.05$)

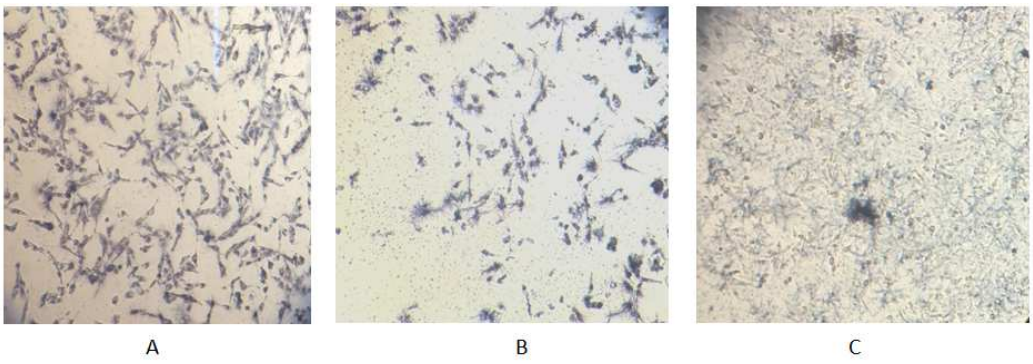

Fig. 3(A): (A) Photomicrograph of MTT assay of the cells treated with doxorubicin (1µM) (B) Photomicrograph of MTT assay of the cells treated with doxorubicin (5µM) (C) Photomicrograph of MTT assay of the cells treated with doxorubicin (10µM)

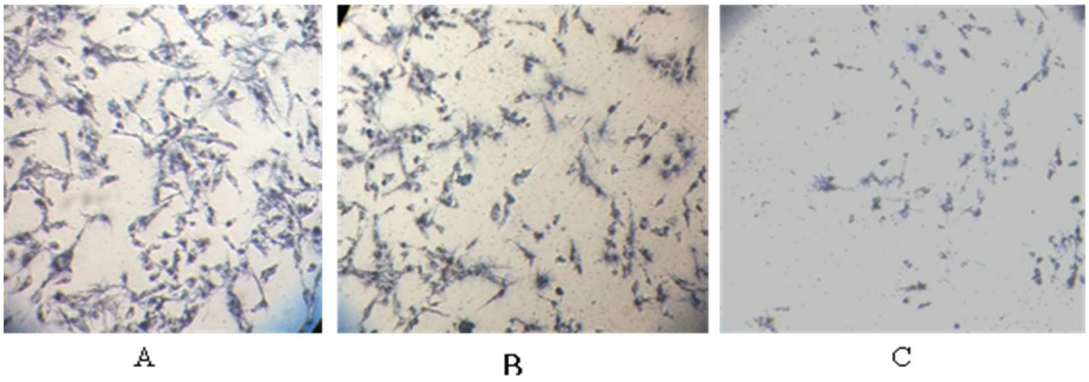

Fig. 3(B): (A) Photomicrograph of MTT assay of the cells treated with ABMECA (10^{-6}M) (B) Photomicrograph of MTT assay of the cells treated ABMECA (10^{-5}M) (C) Photomicrograph of MTT assay of the cells treated with ABMECA (10^{-4}M)

Cell line study provides good initiative in the beginning of drug discovery to check the cytotoxicity assay of unknown lead molecules. MTT assay used to assess the cytotoxic potential of most of the anti-cancer chemicals *in vitro*. The principle behind the assay is that mitochondrial reduction of MTT tetrazolium salt to formazan of living cells by succinate dehydrogenase. Then, compound dissolved in DMSO for colorimetric measurement. There are many drawbacks link to MTT assay that cannot overlook and need to consider while preparing for *in vivo* study [12]. Reduction of MTT tetrazolium not only restricted to mitochondria but also takes place on plasma membrane, in the cytoplasm, in lysosomes, phase of cell cycle and gets affected by pH. It has been observed that MTT assay is significantly influenced by many compounds that disturb cell metabolism by elevating NADPH level, lactate dehydrogenase activity, which leads to false-negative result [8, 10]. *In vitro*, results indicate cytotoxicity and anti-proliferative activity of Adenosine receptor agonist ABMECA that work as backbone to evaluate its activity for *in vivo* animal models. AR are therapeutic targets of many disorders like cardiovascular, immunological, and inflammatory and sleep [13]. Although MMT based cytotoxicity study depends on disturbance of basic function of cell line, further investigation of test drug in *in vivo* can be done to translate this result in living organisms. Cell line study definitely shows the path for anti-cancer drug discovery and development [14]. During this research, we found that ABMECA has shown more potent cytotoxic activity compared to doxorubicin (fig. 3). Both molecules have shown significant difference compare to cell line without any treatment. The hidden potential of test drug was evaluated in the future with the properly designed Pre-clinical study.

ACKNOWLEDGMENT

The authors gratefully acknowledge the financial support provided by Ramanbhai Patel College of Pharmacy, Charusat University, Changa

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

All the authors of this manuscript declared no conflict of interest

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