RESEARCH ARTICLE

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Induction of Apoptotic Signaling Pathways by 3' methyl ATP in Different Malignant Cells: *in vitro* Study

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Abstract

Extracellular ATP is a dynamic signaling molecule that modulates myriad of cellular functions through P2 purinergic receptors activation and is cytotoxic to a variety of cells at high concentration. But the mechanism of this extracellular ATP/ATP analogs- elicited cytotoxicity is not fully understood. In this study we aim to investigate whether there is differential sensitivity towards induction of apoptosis by ATP analogs (2'-Me ATP and 3'-Me ATP) and its effect on receptor mediated or extrinsic and mitochondria mediated or intrinsic apoptotic signaling pathways. Our findings demonstrated that the IC50 values for 2'-Me ATP and 3'-Me ATP were 3mM and 2mM, respectively, in Hep2, and SiHa cells. The downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL, along with a significant increase in the expression of the pro-apoptotic protein Bax ($p<0.05$), indicated the involvement of both pro- and anti-apoptotic factors in HeP2 cells, whereas in SiHa cells, a downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL was observed, whereas the expression level of the pro-apoptotic protein Bax remained unaffected. Furthermore, an upregulation of p53 and apoptosis-inducing factor (AIF) was observed in HeP2 cells ($p<0.05$) whereas, an upregulation of p53 was observed while no change was seen on the level of apoptosis inducing factor (AIF) was observed in SiHa cells. Additionally, there was a notable rise in caspase-3 and -9 activities, PARP cleavage, and the release of cytochrome c ($p<0.05$) from the mitochondria to the cytosol in both cells. Collectively, our study suggests that 3'-Me ATP induces apoptosis in Hep2 and SiHa cells through the intrinsic mitochondrial pathway.

Keywords: Apoptosis- cancer- ATP analogs- cytochrome c- mitochondria

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Introduction

Cancer is a major public health problem globally and second leading cause of mortality across the globe. Worldwide, GLOBOCAN 2020 estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths in 2020 [1]. Cancer is a group of diseases that undergo unregulated cell growth and proliferation without pausing. Inspite of an overall increase in survival due to early detection, cancer-related mortality is still the second biggest cause of death worldwide [2]. The occurrence of cancer initiation and progression mechanisms is not yet fully understood. One of the hallmarks of cancer is the ability to avoid apoptosis [3]. Apoptosis is recognized as the most important form of cell death, which is finely regulated at gene level resulting in the orderly and efficient removal of damaged cells such as those occurring following DNA damage or during development [4]. The machinery of apoptosis is complicated and involves many other signaling pathways. Identification of apoptotic mechanisms is critical for the understanding of the pathogenesis of a disease as a result of dysfunctional apoptosis. This, in turn, may assist in developing new drugs that target specific apoptotic pathways or genes involved in carcinogenesis.

Apoptosis can be triggered in a cell through either the rceptor-mediated extrinsic or mitochondria-mediated intrinsic pathways. Upon triggering of either pathway, caspases, the final executioners of apoptosis, are activated which causes degradation of cellular proteins and disassembly of the cell, leading to typical morphological changes such as chromatin condensation, nuclear shrinkage, and the formation of apoptotic bodies [5]. The ratio of the pro-and anti-apoptotic proteins plays an important role in the regulation of apoptosis, and disruption in the balance of pro- and anti-apoptotic proteins has been established to contribute to the process of cancer progression by reducing apoptosis. This balance between the pro- and anti-apoptotic protein regulators is a critical key point to determine whether a cell undergoes apoptosis or not.

Nucleoside and nucleotide analogs were among the first chemotherapeutic agents to be used for the treatment of cancer and viral diseases [6-8]. These antimetabolites include a variety of purine and pyrimidine nucleoside derivatives with activity in both solid tumours and

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hematological malignancies. A significant proportion of current chemotherapeutic treatments for cancer involve the use of these anti-metabolites, particularly modified nucleoside analogues that possess a capability to mimic native purine or pyrimidine nucleosides which can disrupt metabolic and regulatory pathways [9].

The potency of these nucleoside/-tide analogs as antitumor drugs was well established in the 1960s with the antimetabolites 5-fluorouracil [10] and the thiopurines [11]. Studies from over the past four decades have shown that ATP and its derivatives exert a strong cytotoxic effect on various cancer cell types [12]. Nucleotides are composed of a sugar moiety, nucleobase and at least one or more phosphate group. In nucleotide analogs, they are often altered at the sugar moiety to mimic the natural nucleotides structurally. Due to their structural modification the nucleotide analogs leads to disruption or termination of DNA replication or other critically important biological processes [13]. In this study, we used two ATP analogs, 2'Me ATP [9H-(2-methyl-β-D ribofuranosyl) adenine 5'-triphosphate] and 3'Me ATP [9H-(3-methyl-β-D ribofuranosyl) adenine triphosphate], which inhibit ribonucleotide reductase (RNR), a key enzyme in DNA synthesis, thereby inhibiting deoxyribonucleotide (DNA) synthesis. The thrust of the study was to investigate the mechanism of induction of apoptosis by ATP analogs using human malignant cell lines representing different organ origin. An understanding of the mechanism of induction of apoptosis with ATP analogs (2'Me ATP and 3'Me ATP) is of interest since this may to develop a novel approach to treat cancer.

Materials and Methods

Materials

2'-Me ATP and 3'-Me ATP were obtained from late Prof. H. N. Jayaram, Indianapolis, USA. Substrates for caspase-8 and caspase-9 were obtained from Genotech, USA and Assay kit for Caspase-3 was obtained from Pharmingen, Germany. Western-blot kit was purchased from Promega Corporation, USA. Bcl-2, Bcl-xL, Bax, p53, AIF, and cytochrome c antibodies were obtained from Santa Cruz, USA. Antibody for PARP was purchased from Neo Markers, USA.

Cell culture and treatments

Human malignant cell lines SiHa (human cervical cancer cell line), H520 (Non-small cell lung cancer) and Hep2 (human laryngeal cancer cell line) were grown in DMEM medium. The media was supplemented with 10% fetal calf serum and antibiotics in a humified atmosphere of 5% CO2 in air, at 370C. Logarithmically growing cells were used for all experiments. ATP analogs were dissolved in autoclaved DDW. The IC_{50} of 2' -Me ATP and 3' -Me ATP had been studied on the basis of MTT assay and flow cytometry. The calculated IC_{50} has been used for all subsequent experiments. Treatment of the cell lines with cisplatin was used as positive control. Normal human lymphocytes were used as controls.

MTT (cell viability) assay

The growth inhibitory effect of 2' -Me ATP and 3' -Me ATP was assessed by the assay. Briefly, 10,000 cells were seeded in a 96-well microtiter plate. Cells were then treated with different concentrations (0.5, 1, 2 and 3 mM) of ATP analogs (2' -Me ATP and 3' -Me ATP) for 24 h. Hundred microliters of 5 mg/ml of MTT was added followed by incubation for 4 h at 370C. Formazan crystals are formed after this incubation and are dissolved in DMSO. The absorbance was measured at 570 nm using an ELISA reader with the reference wavelength of 620nm. IC_{50} of 2' –Me and 3' –Me ATP was found to be 3mM and 2mM respectively on the basis of MTT assay and flow cytometry for all the cell lines.

Detection of apoptosis

Morphological analysis

Apoptotic cell death was evaluated by observing the morphological changes typical of apoptosis, such as cell shrinkage, chromatin condensation, membrane blebbing, and formation of apoptotic bodies, by light microscopy [14].

Flow cytometry

Briefly, 2×10^6 cells were washed once in phosphate buffered saline (PBS) and fixed in 70% ethanol at -200C overnight. Fixed cells were washed and resuspended in a buffer containing 5 mg/ml propidium iodide (PI), 0.1% sodium citrate, and 1% Triton-X 100. PI stained cells were analyzed using a FACScan cytometer (Coulter) equipped with an argon laser using Win MDI 2.8 software [14].

Western blot analysis

The levels of expression of Bcl-2, Bcl-xL, Bax, p53, AIF, PARP, and cytochrome c were determined in control and treated cells by western blotting as described previously [14]. Appropriate positive and negative controls were used. The bands were analyzed and quantitated using Alphaimager scanning densitometer (Alpha Innotech, USA). The protein expression is expressed in relative units (RU). The density of the control was taken as 1 and the results of the treatments were expressed in relation to the control.

Measurement of cytochrome c release

For cytochrome c determination, cytosolic fraction was obtained by differential centrifugation. Cytochrome c was detected by western blotting as described earlier [15].

Caspase-3, -8, and -9 activity assay

Caspase-3, -8, and -9 were measured by the direct assay for caspase enzyme activity in the cell lysate using synthetic fluorogenic substrate (Ac-DEVD-AMC; substrate for caspase-3; Pharmingen, Germany; Ac-LETD-AFC, substrate for caspase-8 and Ac-LEHD-AFC, substrate for caspase-9; Genotech, USA) as described by the manufacturer. Briefly, the cells were washed with PBS and lysed in lysis buffer on ice for 20 min. Aliquots of cell lysate (50–100 µl) were then added to the reaction buffer along with 250 µM of fluorogenic substrate, and the reactions were performed for 1 h at 370C. Amounts of

fluorogenic AMC/AFC moiety released were measured using a spectrofluorimeter (ex.380 nm, em.420–460 nm for caspase-3; ex.400 nm, em.490–520 nm for caspase-8 and -9). The results were expressed as Arbitrary Fluorescence Units/mg protein [16].

Statistical analysis

Statistical analysis of the samples was done using the SAS software. Paired t-test was used to analyze the difference in the parameters between control and various treatments. A 'P' value of less than 0.05 was considered to be statistically significant.

Results

In order to explore the cytotoxicity of 2' -Me ATP and 3' -Me ATP, we started our study with the cell viability assay to determine the IC_{50} value in SiHa, Hep2, and H520 cells. Figure 1 shows the dose response study in SiHa, Hep2, and H520 cells that were treated with 0.5, 1, 2 and 3 mM concentration of 2' -Me ATP and 3' -Me ATP for a period of 24 h. The IC_{50} value of 2' -Me ATP and 3' -Me ATP was found to be 3mM and 2mM respectively for Hep2 and SiHa cell lines. 2' -Me ATP and 3' -Me ATP at the respective dose of 3mM and 2mM induced apoptotic features such as chromatin condensation and formation of apoptotic bodies in all the three cell lines as revealed by light microscopy at the magnification 200x (Figure 2). Besides the morphological changes, apoptosis was also quantitated by measuring the sub-diploid population of cells by flow cytometry. 3'-Me ATP-treated cells showed 45.23, 50.15, and 33.23% apoptosis in SiHa, Hep2, and H520 cells in comparision to 7.12%, 7.81% and 8.23% in untreated cells, respectively (Figure 3).

Among the three cell lines Hep2 cells were most responsive to the ATP analogs followed by SiHa cells. Therefore we proceeded our further experiments with Hep2 and SiHa cells treated with 2mM 3' Me-ATP, as we had limited amount of ATP analogs (a kind gift from late Prof. HN Jayaram, Indianapolis, USA). Since the anti-apoptotic and pro-apoptotic proteins play an important role in the regulation of apoptosis, hence we analyzed their expression in control as well as treated cells. 3'-Me ATP downregulated Bcl-2 and Bcl-xL expression with an increase in Bax expression level in HeP2 cells. We found that 3'-Me ATP significantly downregulated the expression of the antiapoptotic protein Bcl-2 by 1.61-fold and Bcl-xL by 2.08-fold in Hep2 cells ($p<0.05$) with a 3-fold upregulation in the expression level of Bax ($p<0.001$) whereas Bcl-2 and Bcl-xL level was significantly dwnregulated by 1.93 and 1.6 fold ($p<0.05$) and Bax remained unaffected ($p=0.23$) in SiHa cells (Figure 4).

An increase of 1.92- and 2.52-fold in p53 level was observed in ATP-treated Hep2 and SiHa cells respectively

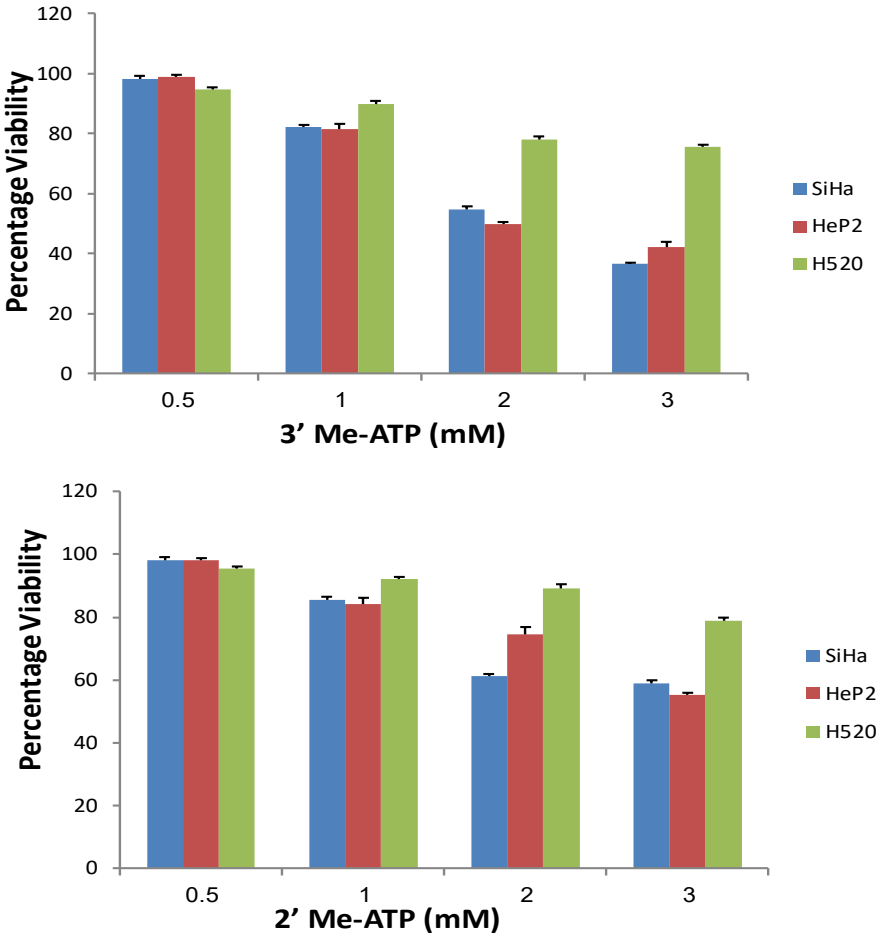

Figure 1. Cell Viability assay of SiHa, HeP2 and H520 Cells as Measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) after 24h treatment with 2'--Me ATP (3mM) and 3'-Me ATP (2mM) at their respective doses.

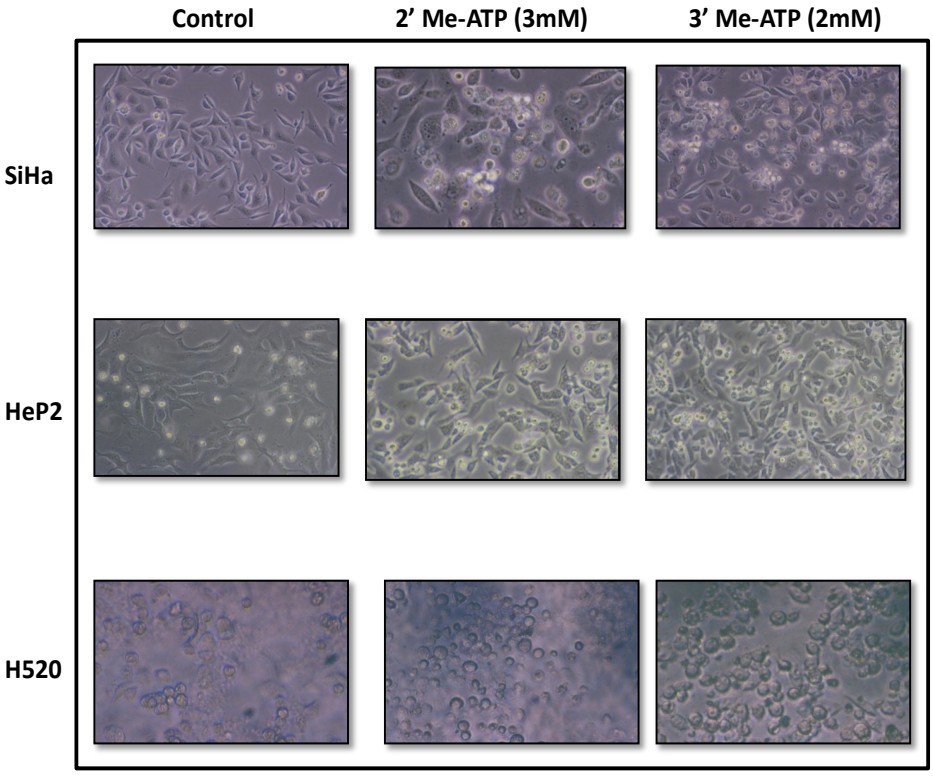

Figure 2. Morphological Changes in a SiHa. b HeP2. c H520 Cells as Revealed by Light Microscopy (200X).

($p<0.05$) (Figure 4), whereas a 1.56-fold significant increase was observed in AIF levels in Hep2 but no significant change was observed in AIF levels ($p=0.35$) in SiHa cells (Figure 4). An increase of 1.92-and 1.71-fold increase in cytochrome c level was seen in cytosolic extracts after 3'-Me ATP treatment in Hep2 and SiHa cells ($p<0.05$) (Figure 4) suggesting the involvement of mitochondria in ATP analog-induced apoptosis.

Since caspases are the key players in apoptotic cascade, we investigated the effect of 3'-Me ATP on initiator and the effector caspases. 3'-Me ATP caused 3.92-fold increase in caspase-3 activity ($p<0.001$), whereas an increase of 2.85-fold in caspase-9 activity ($p<0.05$) was seen after 3'-Me ATP treatment in the Hep2 cells. However, no significant increase in caspase-8 level ($p>0.05$)was seen after 3'-Me ATP treatment (Figure 5).

We also investigated the cleavage of poly (ADP-ribose) polymerase (PARP) in our study and measured

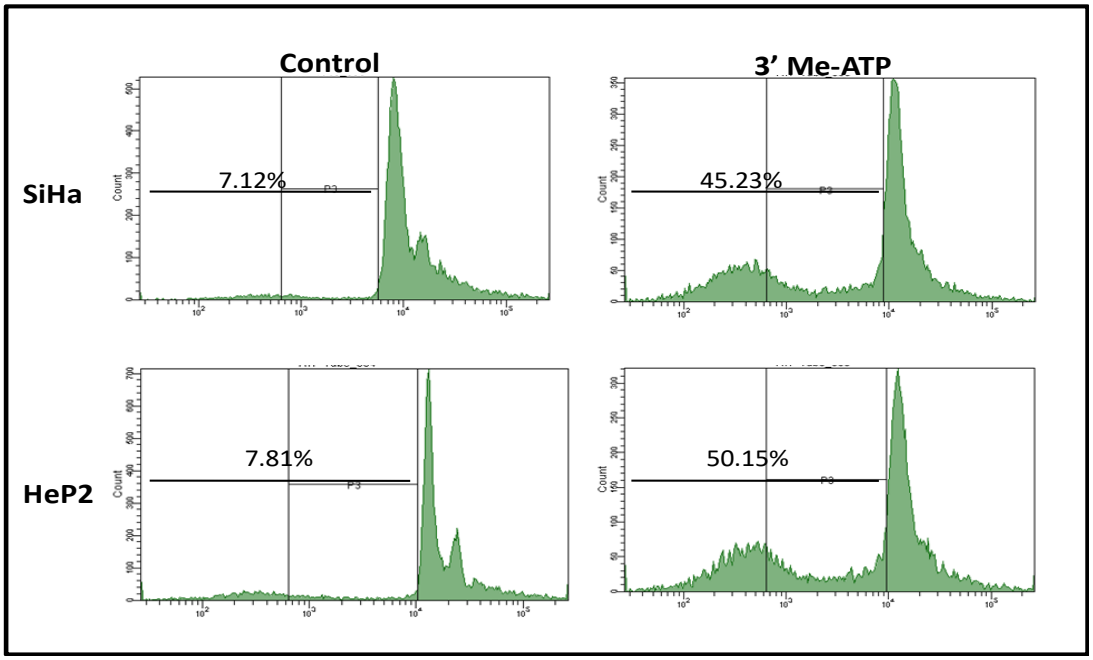

Figure 3. Percentage Apoptosis in a SiHa. b HeP2 as Measured by Flowcytometry. Flowcytometry graph is representative of a typical experiment.

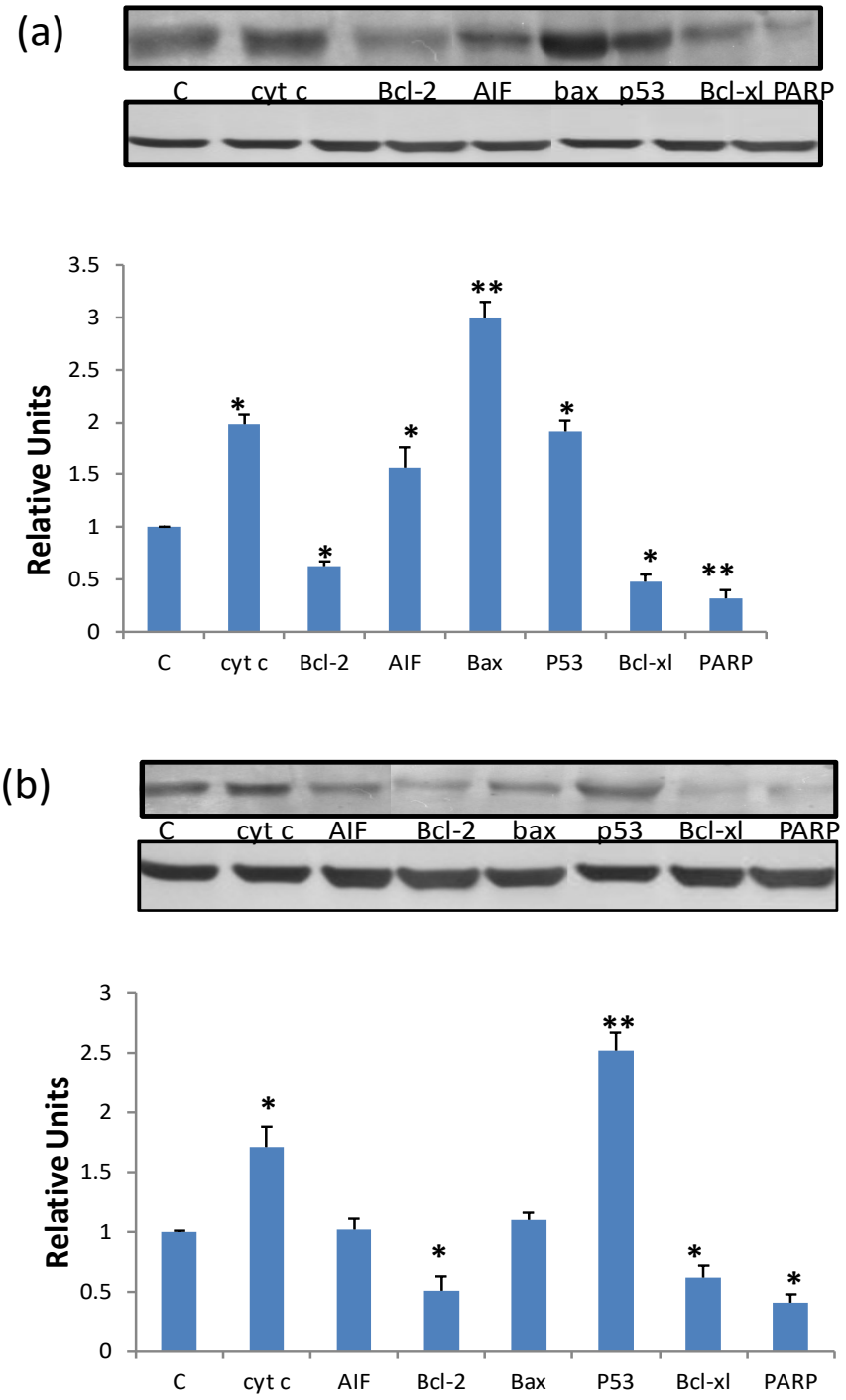

Figure 4. Densitometric Analysis of Protein Expression of Proteins Cytochrome c (cytosolic fraction), Bcl2, AIF, Bax, p53, Bcl-xL and PARP in control and treated cells (a) Hep2 and (b) SiHa cells as measured by western blot analysis. The bars represent the mean of the three independent experiments ± S.D. *Significantly higher/lower than control, $p<0.05$, **Significantly higher/lower than control, $p<0.001$

it by western blotting since PARP cleavage is one of the biochemical hallmarks of apoptosis. After 3'-Me ATP treatment, a 3.12-fold decrease was seen in PARP 116 KD band in Hep2 cells ($p<0.001$) (Figure 4).

Discussion

Cancer arises from the accumulation of genetic and epigenetic alterations, leading a normal cell to transform into a malignant one [17]. A distinctive characteristic of cancer is the evasion of apoptosis, a form of programmed cell death that occurs without causing inflammatory responses [3, 18]. Understanding the mechanism of apoptosis is crucial for comprehending pathogenesis of the disease and developing effective treatment strategies. Cancer cells employ various schemes to evade apoptosis, such as overexpression of antiapoptotic proteins, suppression of pro-apoptotic genes, and alteration of signaling pathways, thus hindering apoptosis induced by therapy [19].

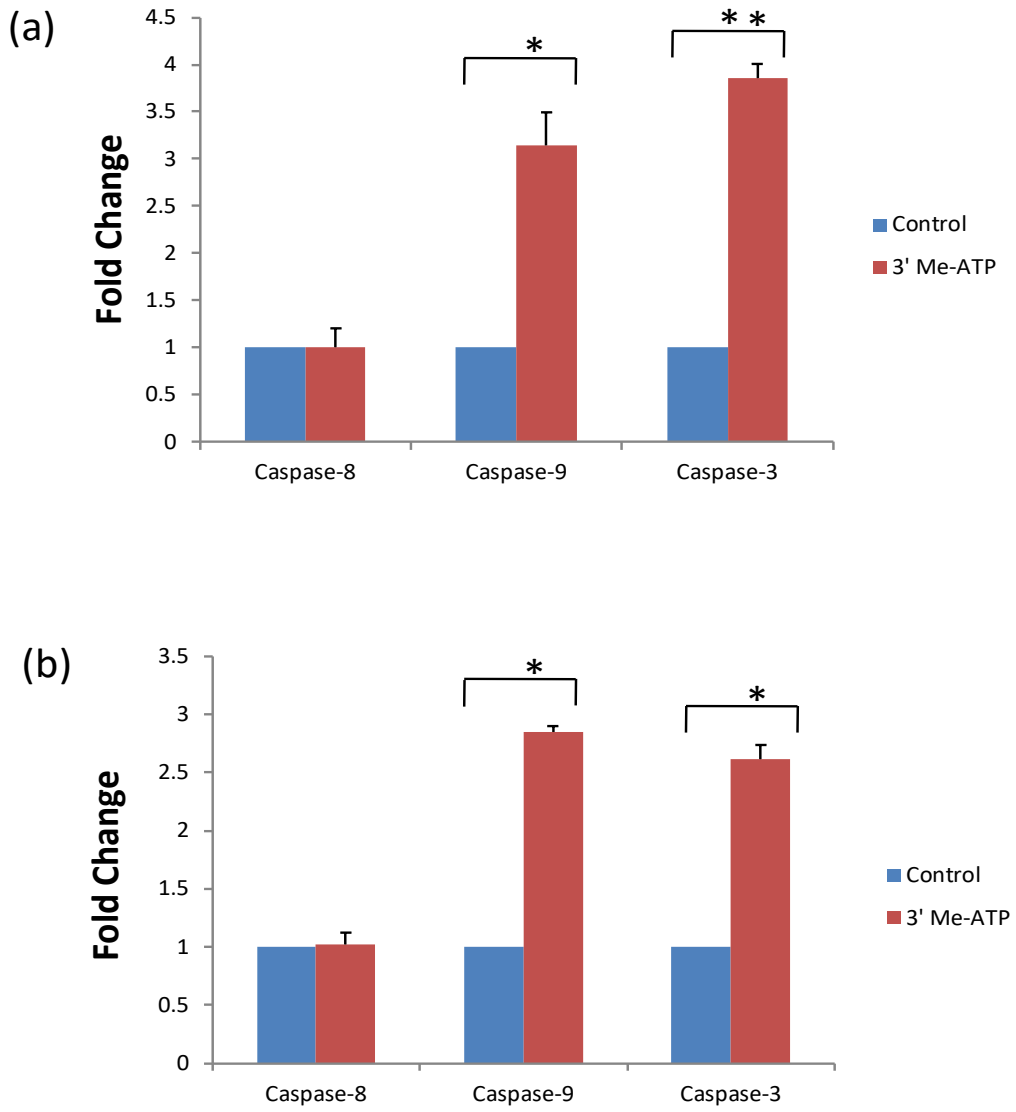

Figure 5. Caspase Activity Assay in (a) HeP2 and (b) SiHa Cells. *Significantly higher than control, $p<0.05$, **Significantly higher than control, $p<0.001$

The well-established intrinsic and extrinsic signaling pathways of apoptosis, along with regulatory factors, have been extensively depicted. Consequently, gaining insights into the apoptotic mechanisms and the factors influencing them is pivotal for the design of more potent, specific, and effective cancer therapies. Nucleoside or nucleotide analogs are highly successful as chemotherapeutic agents for for various types of cancer treatments [20-21]. Several such compounds, including gemcitabine and cytarabine, has a wide range of antitumor activity and are option in first-line treatments [6]. However, these materials do have limitations and the development of next generation compounds remains a topic of significant interest and necessity.

Malfunctions in the apoptotic pathways are significant contributors to the development of cancer, and there are numerous viable treatment approaches focused on manipulating apoptosis that could be applied in treating different cancer types. In the present study, we analyzed the apoptotic signaling mechanism induced by ATP analogs (2' Me-ATP and 3' Me-ATP) in SiHa (human cervical cancer cell line), H520 (Non-small cell lung cancer) and Hep2 (human laryngeal cancer cell line). Various studies from the past have concluded that ATP and its derivatives have a strong cytotoxic effect on various cancer cell types [12, 22-23]. We analysed induction of apoptosis by both the ATP analogs in all three above-mentioned cell lines. The IC_{50} value of 2' Me-ATP and 3' Me-ATP was found to be 3 mM and 2 mM respectively for HeP2 and SiHa cells. H520 cells were not as responsive for these doses of ATP analogs. Further, we had limited amount of these drugs, therefore we decided to proceed with SiHa and HeP2 cells only, for further experiments.

Our investigation focused on the impact of ATP analogs on the expression of pro- and anti-apoptotic members of the Bcl-2 family of proteins, given the crucial role of mitochondria in regulating cell death. Our findings revealed that in Hep2 cells, 3'-Me ATP led to a downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL, accompanied by an increase in the expression level

of the pro-apoptotic protein Bax. Additionally, treatment with 3'-Me ATP in Hep2 cells resulted in an elevation of p53, apoptosis-inducing factor (AIF), and cytochrome c in cytosolic extracts compared to the control, indicating the involvement of mitochondria in ATP analog-induced apoptosis.

The Bcl-2 family proteins play a significant role in apoptosis regulation, particularly through the intrinsic or mitochondrial pathway [24]. Numerous studies have confirmed the pro-apoptotic activity of ATP analogs by inducing pro-apoptotic genes, downregulating anti-apoptotic genes, and activating caspases [25-27]. Pro-apoptotic members of the Bcl-2 family, such as Bad and Bax, often mediate their effects through the mitochondria, either by interacting with Bcl-2 and Bcl-xL or through direct interactions with the mitochondrial membrane. These proteins regulate the intrinsic apoptotic pathway by either preventing or activating the release of apoptogenic factors, such as cytochrome c, to the cytosol from the mitochondrial interspace membrane [28-29].

Our study suggests that ATP analogs induce the intrinsic or mitochondrial apoptotic pathway, leading to the downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL and upregulation of the pro-apoptotic protein Bax. This process is followed by changes in mitochondrial membrane potential, subsequent release of cytochrome c and AIF, and activation of procaspase-9 to active caspase-9. Ultimately, this cascade leads to the activation of effector caspase-3, resulting in cell degradation or apoptosis.

In our experiments, 3'Me-ATP-induced caspase-9 activation was followed by the activation of downstream effector caspase-3, while caspase-8 showed only a limited, non-significant increase in Hep2 cells. This suggests that 3'Me-ATP induces apoptosis primarily via the mitochondrial pathway, followed by caspase-3 activation. Caspase-8 was not found to be a requirement for 3'Me-ATP-induced apoptosis in HeP2 as well as SiHa cells. The observed increase in p53 expression and cytochrome c levels after 3'Me-ATP treatments further supports the involvement of mitochondria in the apoptotic signaling pathway induced by 3'Me-ATP. The protein p53, known as a tumor suppressor, plays a pivotal role in hindering cell transformation and the advancement of tumors. Triggered by diverse stimuli, p53 manages cell-cycle arrest and apoptosis. In addition to its extensively studied role in controlling cell-death programs at the nuclear level, p53 also plays essential roles in the cytoplasm. It directly influences the apoptotic response at the mitochondrial level, contributing to its critical functions in preventing tumor development [30-32].

Consistent with our findings, ATP analogs have been demonstrated to induce apoptosis in various leukemic and ovarian carcinoma cell lines [33, 23-24]. The observed antiproliferative effects of these analogs seem to stem from their capacity to reduce intracellular levels of both purine and pyrimidine deoxynucleotides by inhibiting ribonucleotide reductase [34-35]. They selectively inhibit tumor cell growth and induce apoptosis in various human tumor cell lines [34]. In conclusion, our results indicate that 3'Me-ATP induces apoptosis through the intrinsic mitochondrial pathway in Hep2 cells. The downregulation of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and the upregulation of survival genes like p53, along with the activation of initiator and effector caspases-9 and -3 by 3'Me-ATP, suggest its potential utility as a therapeutic agent for cancer treatment.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

The work was approved by Institute ethical committee as a part of student thesis. There was no conflict of interest. Author is grateful to her mentor Prof. Neeta Singh and Late Prof. H. N. Jayaram for this work.

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