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Research Paper

Anti-tumor activity of BML proteases in Breast cancer, Prostate cancer and Cervical cancer by regulation of p53 gene, NF-κB and COX-2 expression through targeting MAPK Pathway and Intrinsic Pathway of Apoptosis

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Abstract

Chemo impediment impels the quest for moreover single targeted or brew of multi-targeted agents. BML proteases, potential agent in this regard, is a pharmacologically active compound, present in stems and fruits of *Ananas comosus*, endowed with anti-inflammatory, anti-invasive and anti-metastatic properties. BML protease is a complex or proteolytic enzymes. These proteolytic enzymes are paraphernalia that promise an impressive number of medical and therapeutic uses, particularly as anti-tumor agents (Swaroop G *et al*. 2013, p.80). BML proteases is a pharmacologically active compound, present in stems and immature fruits of pineapples (*Ananas comosus*), which has been shown to have anti-edematous, anti-inflammatory, anti-thrombotic and anti-metastatic properties (Swaroop G *et al*. 2013, p.80). In the present study anti-tumorigenic activity of BML was recorded in HeLa, MCF 7 and PC3 cell lines. Results showed that BML proteases application delayed the onset of tumorigenesis and reduced the cumulative number of tumors, tumor volume and the average number of tumor cells. To establish a cause and effect relationship, we targeted the proteins involved in the cell death pathway. BML proteases treatment resulted in up-regulation of p53 gene and subsequent activation of caspase 3 and caspase 9 with concomitant decrease in anti-apoptotic protein Bcl-2 in cancer cells targeting intrinsic pathway of apoptosis. BML treatment attenuated phosphorylation of extracellular signal regulated protein kinase (ERK1/2) and mitogen-activated protein kinase (MAPK). Taken together, we conclude that BML induces apoptosis-related proteins by blocking the MAPK kinase signaling in tumor cells, which may account for its anti-tumorigenic effects. Flow cytometry studies were carried out for the study of cell cycle progression.

Keywords: *Ananas comosus, BML Proteases, Cyclooxygenase-2 (COX-2), Epidermal Growth Factor Receptor (EGFR), HeLa Cells, MCF 7 Cells, Nuclear factor-κB(NF-κB), PC3 Cells, Receptor Tyrosine Kinases (RTK)*

1. Introduction

Carcinogenesis is a multistep process in which genetic and epigenetic events determine the transition from a normal to a malignant cellular state. The rate of the process of tumor progression is accelerated by mutagenic agents (tumor initiators) and by mitogenic agents (tumor promoters).

Reversal of aberrant epigenetic events, including those that modulate the transcriptional activity of genes associated with various signaling pathways, holds the prospect of influencing multiple stages of tumorigenesis.

This study has provided insight into the mechanism of carcinogen–DNA interactions and the nature of mutations in the critical target genes. The dietary components as chemopreventive agents have received much attention among the researchers. A greater understanding of the pivotal events associated with carcinogenesis will facilitate the use of dietary intervention as a key strategy to prevent cancer development. Experimental studies have revealed that the dietary components regulate the molecules in several cell signal transduction pathways including mitogen-activated protein kinase (MAPK), Akt and p53 pathways.

Dietary agents, among other mechanisms, activate cell death signals and induce apoptosis in precancerous or cancer cells, resulting in the inhibition of cancer development and progression.

Recently, BML proteases, an extract from pineapple stem (*Ananas comosus*) has been used clinically for a wide variety of maladies including edema, thrombophlebitis, sinusitis, inflammation, rheumatic arthritis and as adjuvant in cancer treatment (Swaroop G *et al*. 2013, p.80). Although poorly understood, the pleiotropic effects of BML proteases are considered to be due to the complex mixture of closely related cysteine proteinases, proteinase inhibitors, phosphatases, glucosidases, peroxidases and other undefined compounds. It has also been shown that BML may play a role in the differentiation of malignant cells. BML proteases also act as immunomodulator by raising the impaired immune-cytotoxicity of monocytes against tumor cells from patients. In addition, bromelain has shown both antiproliferative and antimetastatic effects in tumor models in vitro and in vivo. It has also been shown to possess anticancer properties in cancerous cells. In view of the anti-inflammatory, anti-proliferative and anti-metastatic activities of BML, we tested anti-tumorigenic potential of bromelain in MCF 7, PC3 and HeLa cell line models.

2. Materials and Methods

2.1. Preparation and standardization of Stem BML proteases extracts

Stem BML Proteases (Swaroop G *et al*. 2013, p.80), DMEM (Hi-Media, Bangalore), MCF 7 cell line, PC3 cell line, HeLa cell line, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (Sigma Aldrich, Bangalore), ELISA plate reader (Wallac Model 1420 Multilabel counter, michigan, USA).

2.2. Media preparation and Sterilization

DMEM (10.2g) (HI-MEDIA, Bangalore) was dissolved in 1000mL of autoclaved double distilled water. 1.2g of Sodium Bicarbonate was added while the media turns pink in color.
For the sterilization of the media the filter apparatus was autoclaved and transferred to laminar air flow cabinet. The upper chamber was removed, the membrane filter was placed and upper chamber was replaced and autoclaving was carried out for the second time. Filter apparatus was transferred to LAF cabinet and one of the lower chamber`s nozzle was attached to a vacuum pump. The media was then poured and pressure was applied to create suction to filter the media. The filtered media was collected in the lower chamber. 20% of serum was added according to requirement before use.

2.3. Trypsinization and Subculturing

Once the cells reach confluency stage sub culturing was carried out. All the spent media were removed and the cells were washed with saline water. 200µL of Trypsin-EDTA (0.25%) was added and incubated for 3-4 min for the cells to get detached and 1mL of 10% media was added and mixed thoroughly to stop Trypsin action. Cell suspension (0.6mL) like MCF 7, PC3 and HeLa was then transferred separately into a fresh culture dish, to this 1mL of fresh media was added. The cells were then incubated at 37ºC, with 5% of CO_2 supply and maintained with 60-70% humidity.

2.4. Cryopreservation

The cultures was removed from the dish and collected in a tube and centrifuged at 1500 rpm for 10min. The pellet was dissolved in 0.5mL of 10% media. Meanwhile freezing mixture (1.3mL of 20% media + 0.2mL DMSO) was prepared in ice bath. Freezing mixture (1.5mL) was added to the cell suspension in the ice bath. The cryo-vial was kept in the slow cooling device then the sample was transferred to long term storage device.

2.5. Thawing

It is the process of transferring cells from frozen state to normal state. The cryo-vial was removed from the cryo-barrels and was made to attain room temperature for some time to allow the liquid nitrogen present on the surface of vial to vaporize. Then the vial was incubated at 37ºC using water bath. Meanwhile 10mL of saline was taken in a 15mL centrifuge tube and the contents of vial were transferred into the centrifuge tube and centrifuged at 1500 rpm for 10min. Supernatant was discarded and 0.5-1.0mL of media was added to the pellet.

2.6. Trypan blue exclusion assay for cell viability

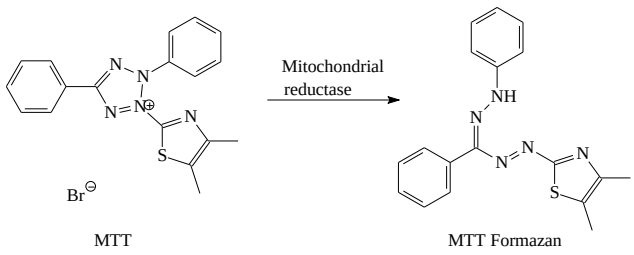
This rapid and simple test is used to determine the approximate number of viable cells present in the cell suspension. It is based on the principle that a viable or live cell, will have an intact cellular membrane and therefore will exclude the dye and have clear cytoplasm whereas a nonviable or dead cell do not have such integrity and so the cytoplasm takes up the blue color.

The cell suspension obtained after thawing was mixed properly and taken in seven fresh culture dishes. To all four culture plates 2mL of fresh media was added to the first dish 20mg of the ethanol extract of BML proteases was added to the second dish 20mg of ethanol extract of BML was added. To the third dish 20mg of methanol extract of BML was added. The fourth dish was taken as control. No extract was added. It contained untreated cells. All the plates were kept for 24 hrs incubation to test the effect of the sample on the cells. 10µL of MCF 7, PC3 and HeLa cell suspensions was mixed with 20µL of Trypan Blue dye in an eppendorf tube.
Meanwhile the surface of haemocytometer was cleaned using ethanol and 10µL of the prepared mixture was loaded such that the suspension goes in between the cover slip and the surface of haemocytometer. And the number of live cells and dead cells were counted inside L_1, L_2, L_3, L_4 chambers using a clicking counter and the percentage viability was calculated.

The percentage of cell viability was calculated by the formula:

$$\% \text{ of cell viability} = \frac{\text{Number of living cells} \times 100}{\text{Number of total cells (live + dead)}}$$

2.7. MTT Assay



Cells were diluted with RPMI 1640 complete medium to give final concentration of $1x10^4$ cells mL^{-1} and seeded in a 96-well microtiter plates in the volume of 200µL/well. The cultures were incubated at 37°C under 5% CO_2 supply humidified incubator for 24hrs. After incubation media was removed and 200µL of RPMI 1640 containing 0.5% FBS was added to each well and incubated for further 24 hrs. After incubation, media was removed and 100µL of stem BML protease extract was added to each well in triplicates to obtain the final concentrations of 10, 20, 30 or 40 μgmL^{-1} of BML or 50, 100, 200 or 400μgmL^{-1} of BML extract. Cells treated with final concentration of 0.1% DMSO in complete RPMI 1640 media containing 1% FBS were used as a vehicle control. After 48 hrs incubation, 20µL of 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent) was added to each well. Cultures were incubated in the dark at 37°C with a supply of 5% CO_2 incubator for 1hr. The absorbance (OD) of each well was measured at 490nm using ELISA plate reader (Wallac Model 1420 Multilabel counter, michigan, USA). The intensity of color developed in each well was corresponding to the cell number. Percentage of cell viability was calculated using a formula below. IC_{50} value was expressed as concentration of extract in $mgmL^{-1}$ that caused a 50% growth inhibition comparing with controls (Slater .T 1963, Alley M.C. 1988 & Van de Loosdrecht 1994).

$$\% \text{ of Cell Viability} = \frac{\text{OD (Test sample)-OD(Medium)}}{\text{OD (DMSO Control)-OD (Medium)}} \times 100$$

2.8. Flow cytometric analysis for cell cycle progression

The single cell suspension of HeLa Cells from experimental and control were prepared. It was performed in 6 well plate in which 50,000 cells per well (after 24hrs incubation) was incubated with drug at a concentration of its IC_{50} value and 10X' IC_{50} value. In flow cytometry, cells were fixed in its particular stage of cell cycle by ice cold 70% Ethanol. After addition of Ribonuclease A and

Propridium Iodide (PI), cells were analyzed in a flow cytometer. From flow cytometry it is easy to analyze the DNA content in cells (Darzynkiewicz . S 1992 & Carlo Riccardi 2006).

3. Results

3.1. Anti-tumor activity of BML proteases on Breast Cancer (MCF 7) Cell lines

Table 1: Inhibition Coefficient (IC_{50}) of Stem BML proteases on MCF 7 Cells

Drug: Stem BML proteases on MCF 7 Cells			
Dilution No.	Conc. (µg)	% of cell viability	IC_{50} (µg)
1	1.953	92.73	
2	3.906	75.17	
3	7.813	69.83	
4	15.625	56.22	
5	31.25	39.15	20
6	62.5	22.31	
7	125	15.06	
8	250	8.29	
9	500	6.30	

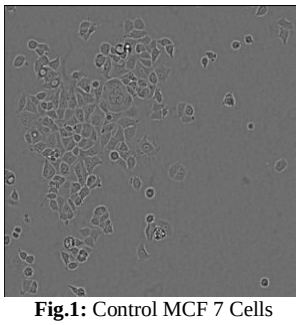


Fig.1: Control MCF 7 Cells

Fig.2: Tryphan Blue staining - Damaged MCF 7 cells

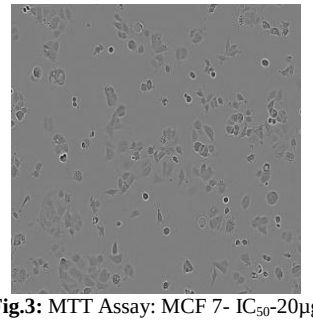


Fig.3: MTT Assay: MCF 7- IC_{50}-20µg

3.2. Anti-tumor activity of BML proteases on Prostate Cancer (PC3) Cell lines

Table 2: Inhibition Coefficient (IC_{50}) of Stem BML proteases on PC3 Cells

Drug: Stem BML proteases on PC3 Cells			
Dilution No.	Conc. (µg)	% of cell viability	IC_{50} (µg)
1	1.953	96.96	
2	3.906	83.72	
3	7.813	67.56	
4	15.625	45.19	
5	31.25	41.10	14
6	62.5	39.28	
7	125	35.08	
8	250	20.81	
9	500	4.83	

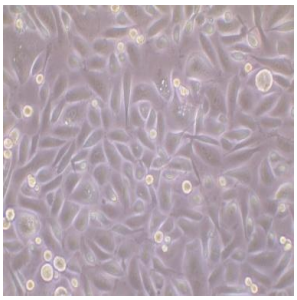


Fig.4: Control PC3 Cells

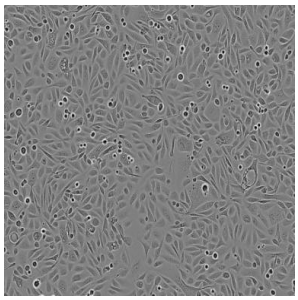


Fig.5: MTT Assay: PC3-IC_{50}-14µg

3.3. Anti-tumor activity of BML proteases on Cervical Cancer (HeLa) Cell lines

Table 3: Inhibition Coefficient (IC_{50}) of Stem BML proteases on PC3 Cells

Drug: Stem BML proteases on HeLa Cells			
Dilution No.	Conc. (µg)	% of cell viability	IC_{50}(µg)
1	1.953	98.43	
2	3.906	84.85	
3	7.813	86.59	
4	15.625	64.77	
5	31.25	50.42	35
6	62.5	46.34	
7	125	27.76	
8	250	14.94	
9	500	12.24	

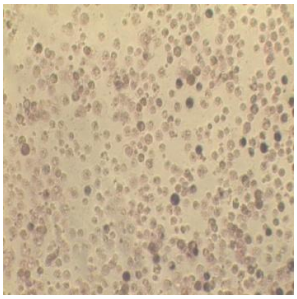


Fig.6: Control HeLa Cells

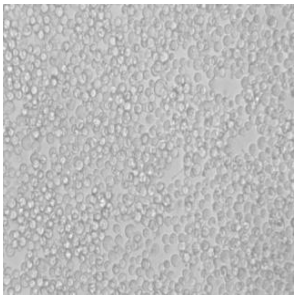


Fig.7: MTT Assay-HeLa-12 hrs incubation- 35µg

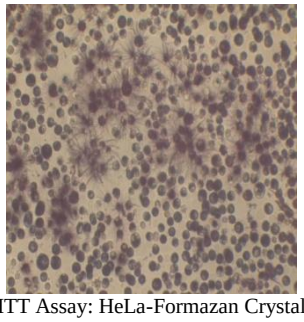


Fig.8: MTT Assay: HeLa-Formazan Crystals- 35µg

3.4. Flow Cytometry Analysis

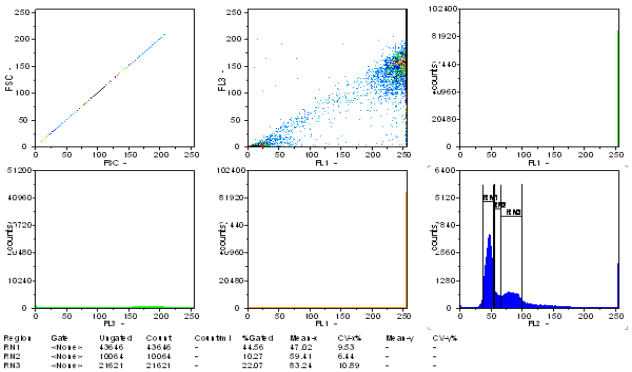


Fig.9: Flow cytogram of Control (only with cells and media). X-axis represents specified parameters and Y-axis represents number of cells * 10. Analysis of three phases (RN1, RN2, RN3) and its cell number

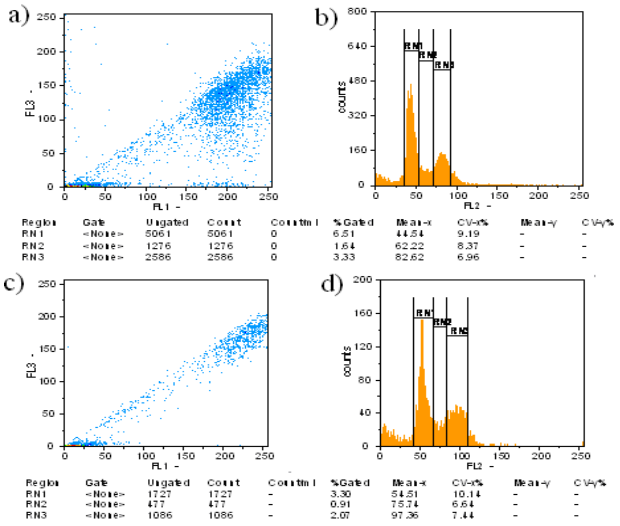


Fig.10: Cell cycle of Cervical cancer cells affected by dosage concentration of BML (a) and (c) The number of cells in each phase as dots with drug concentration of IC_{50} and 10X' IC_{50} value respectively. (b) and (d) The three phases of cell cycle affected by drug concentration of IC_{50} and 10X' IC_{50} value as RN1, RN2 and RN3 regions (G_1, S and G_2/M phases).

Table 4: Total DNA content in the Cervical Cancer Apoptotic cells

Drug Name	Dosage	RN1 (%) & Cell count	RN2 (%) & Cell count	RN3 (%)& Cell count
Control	None	57.94% (43646)	13.36% (10064)	28.70% (21621)
BML	IC_{50}	56.72% (5061)	14.30% (1276)	28.98% (2586)
	10X'IC_{50}	52.49% (1727)	14.49% (477)	33.01% (1086)

3.5. Predicted Anti-tumor activity of BML proteases on Mitogen Activated Protein Kinase (MAPK) Pathway

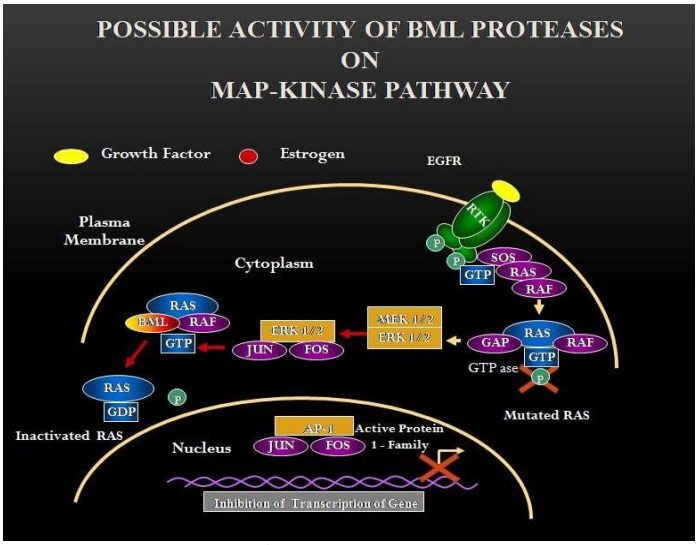


Fig.11: Activity of BML proteases on MAPK Pathway (Lauren Pecorino 2008)

3.6. Predicted Anti-tumor activity of BML proteases on Intrinsic Pathway of Apoptosis by up-regulation of p53 gene

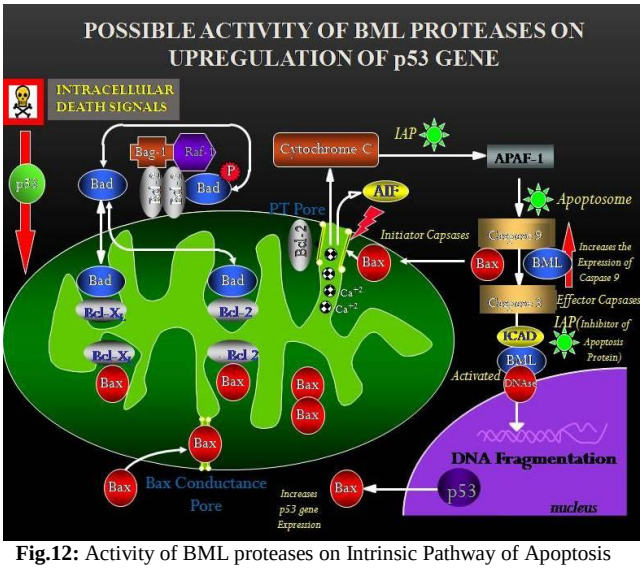


Fig.12: Activity of BML proteases on Intrinsic Pathway of Apoptosis (Lauren Pecorino 2008)

4. Discussion

Anti-tumor activity of BML proteases has given a better inhibition of MCF 7, PC3 and HeLa cells leading to apoptosis. The determination of cell growth is done by counting viable cells after staining with a vital dye. Trypan blue staining is a simple way to evaluate cell membrane integrity but the method is not sensitive and cannot be adopted for high throughput screening. Measuring the uptake of radioactive substances, usually tritium labeled thymidine, is accurate but it is also laborious involving handling of radioactive isotopes. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide a tetrazole is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active and therefore conversion can be directly related to the number of viable cells. When the amount of purple formazan produced by cells treated with MTT reagent is compared with the amount of formazan produced by untreated control cells. The effectiveness of the reagent in causing death can be deduced through the production of dose response curve.

Mitochondrial dehydrogenases of the viable cells cleave the tetrazolium ring yielding purple MTT formazan crystals (Fig.8) which are soluble in aqueous solutions. An increase in cell number results in an increase in the amount of MTT formazan crystals formed and an increase in absorbance

The Inhibition Co-efficient (IC_{50}) of BML proteases activity in MCF 7 cells was found to be 20µg, PC3 cells was found to be 14µg and the inhibition co-efficient in HeLa cells was found to be 35µg respectively.

The percentage of cells entering in each phase of cell cycle by drug activity of BML is studied from flow cytometry graphs and results. In most cases number apoptotic cells by drug action is high and DNA synthesis is reduced, due to high number of apoptotic cells. The apoptosis of cells happened in which phase of cell cycle was clearly shown in graph with FL2 in X-axis. For the drug BML apoptotic cells decreased DNA content based on drug concentration. At IC_{50} concentration apoptotic cells in S phase is 14.30% and at 10X'IC_{50} value, it is cells is 14.49%. These depicts that BML is effective against cervical cancer cells.

5. Conclusion

MAPK is known to regulate NF-κB activation by multiple mechanisms. Accumulating evidence indicates that NF-κB activation is modulated by ERK1/2 and MAPK. ERK1/2 and MAPK activation in cancerous cells, we examined whether BML proteases could inactivate the aforesaid MAPK thereby inactivating NF-κB and further suppressing COX-2 induction. Since NF-κB is known to regulate the COX-2 induction, BML treatment on the MCF 7, PC3 and HeLa cells significantly increased the phosphorylation of IκBα resulting in the activation and nuclear translocation of NF-κB. BML down regulated activation of both MAPK and ERK1/2 through inhibition of their phosphorylation.

BML treatment on MCF 7, PC3 and HeLa cells up regulated p53 expression. The expression of Bax and Bcl-2 has to reported to play a crucial role in apoptotic response mediated by agents. The effect of BML treatment resulted in up-regulation of Bax with contaminant decrease in the over expression of Bcl-2 in cancerous cells. Caspases receive the upsteam death signals and are the final executioners of apoptosis. Thus we showed that BML treatment resulted in activation of both caspase 9 and caspase 3 (Neetu Kalra et al. 2008 p.30)

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