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Changes in cell membrane morphology of colon cancer cells (HCT116) after treatment with azurin derived hexapeptide 4 by AFM and STEM

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ABSTRACT: A6-amino acid peptide fragment of the azurin molecule was observed to act on HCT116 cancerous cells and cell death was brought by apoptosis *in vitro*. Integrity of the cell membrane is an indispensable prerequisite for preserving the biological characteristics of a cell. In this study, changes in the morphology and ultrastructure of HCT116 (human colon carcinoma) was studied by atomic force microscopy (AFM) and Scanning Transmission Electron Microscope (STEM) before and after treatment with the azurin derived hexapeptide. In response to hexapeptide addition, the microstructure of the cell membrane of HCT116 cell displayed changes. With increase in reaction time, the degree of morphological changes on the surface of cell membrane increased. These changes included increase in the fluctuation of the surface components of the cell membrane, and roughness of cell. Cell viability was done by MTT assay. Analysis of the cell membrane root-mean-square (RMS) roughness showed that under the action of hexa peptide, RMS values for the cell membranes of HCT 116 cell type increased with time thus positively correlating to the reaction time. This work is of high significance for the visual diagnosis of early stage of apoptosis in colon cancer cells in response to azurin derived hexapeptide, The use of AFM and STEM can be a rapid and sensitive visual method for studying the interaction between cells and drug.

KEYWORDS: Azurin derived hexa peptide, HCT116, human colon carcinoma, Atomic force microscope, STEM, interaction.

I. INTRODUCTION

The cancer cell is distinguished by its acceleration of the cell cycle; genomic alterations; invasive growth; increased cell mobility, chemotaxis, changes in the cellular surface, secretion of lytic factors, etc. Morphological analysis of cancer cells is significant in studying the changes in cellular organization and the physiological state of the cells, and thus can be used as a qualitative and quantitative measure of effect of anticancer drugs. The cell membrane forms a barrier between the cell and the external environment and acts as an exchange border between the interior and exterior of the cell [6] where it functions in signal transduction, cellular transport, energy conversion, cell-surface recognition, cell survival, and differentiation [9]. Changes in cell membrane structure can therefore be construed directly as influence on the normal functions of cells. Mechanistic studies of human cancer are largely dependent on *ex vivo* culture of established cell lines.

Cell morphology is usually studied using Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and optical microscopy [2] which provides information regarding structural characteristics of cells. However,

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with the discovery of atomic force microscope (AFM) in 1986 AFM has become a novel and an important tool for visualizing cellular ultrastructure, such as the nuclear pore ion channels present in the membranes and cytoskeletal fibers [4]. AFM has advantages such as suitability for conductive and non-conductive samples and other special samples, where none of which require a metal coating. AFM is frequently used in the study of molecular and cell biology and in the medical field [36] on cancer cell morphology [35], cell elasticity [37] or the relationship of these to cell function [24]. The microscopic studies used to study cancer cells do not provide information on the morphological structures at the nanoscale level, the interactions between cancer cells and drugs and they fail to distinguish the micro-changes taking place on the surface of the cell membrane in response to antiproliferative drugs. Also exploitation of AFM to study real-time changes on the ultrastructure of cancer cell membranes in the context of anti-cancer drugs is at its infancy. Similarly, Scanning Transmission Electron Microscopy (STEM) combines the principles of transmission electron microscopy and scanning electron microscopy and can be performed on either type of instrument. The motivation for STEM imaging of biological samples is particularly to make use of dark-field microscopy, where the STEM is more efficient than a conventional TEM, allowing high contrast imaging of biological samples without requiring staining. The method has been widely used to solve a number of structural problems in molecular biology (Wall and Simon 2001).

Hence, the study was focussed on investigating the real-time changes in the ultrastructure of the cell membrane following azurin derived hexapeptide interactions on colon cancer (HCT116) cells as visualized using AFM and STEM. Experiments using optical microscopy and cell viability assays were also performed for comparison purposes.

II. MATERIALS AND METHODS

Peptides: All azurin-derived hexa peptides used in this study were chosen at the Met regions on the azurin sequence. Four hexapeptides were synthesized on a semi automated solid phase peptide synthesizer using standard Fmoc-chemistry. 3-(4,5-Dimethylthiazol-2-diphenyl-tetrazolium) bromide (MTT) was procured from Sigma-Aldrich Chemical Company. All solvents and other chemicals were purchased from local commercial suppliers and were of analytical reagent grade, unless otherwise stated. All solutions were prepared using sterile ultra-purified Milli-Q water. Cell culture plates were purchased from NUNC, USA.

Cell Culture and Cell lines: Human colon carcinoma cell line (HCT116), was obtained from NCCS, Pune, India. Cells were cultured in McCoy's 5A (Sigma-Aldrich, Bangalore, India) medium containing inorganic salts, essential amino acids, vitamins, D-glucose, and L-glutamine supplemented with 10 % heat-inactivated fetal bovine serum (Sigma Aldrich) 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂ to maintain cells in the exponential growth phase, they were passaged at a ratio of 1:3 once in every 2 days. Before use, cells were harvested through trypsinization with 0.25% trypsin for 3min at 37°C. Trypsinization was stopped by the addition of fresh supplemented McCoy's 5A. The numbers of cells were counted with a hemacytometer.

Hexapeptides: From the azurin protein sequence all the four hexapeptide was chosen at the Met regions on the sequence. Four hexapeptides were synthesized on a semi-automated solid phase peptide synthesizer as follows: The peptides were synthesized on a semi-automated solid phase peptide synthesizer (CS 136, C S Bio Co., California, USA) using standard Fmoc-chemistry, on Rink Amide AM resin (100–200 mesh size). As the peptides were amidated at the C-terminus., equimolar quantities of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and Hydroxybenzotriazole (HOBt) were used as coupling agents along with 20% diisopropylethylamine (DIEA) in dimethylformamide (DMF). 20% of piperidine in DMF was used for cleaving the Fmoc-group. Post synthesis, the peptides were cleaved off the resin using a cleavage cocktail containing 9 ml trifluoroacetic acid, 0.5 ml water, 250 µl ethanedithiol, and 125 µl thioanisole. The cleaved peptides were precipitated using cold diethyl ether after removing the trifluoroacetic acid from the cocktail *in vacuo*. The precipitated peptides were filtered, dried and stored in a desiccator.

Cell proliferation Assay: HCT116 cells were seeded into 96-well plates in triplicate at a starting density of 2×10^4 cells/well. After 48 h, cells were treated with of hexa peptides at the final concentration of 0 (as control) and 500µM/L

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separately. The cells were harvested at different intervals of time from 0, 15min, 30min, 45min, 60min, 12h, 24h and 48h. The proliferation of the cells was measured by the MTT method. The cells were treated with 5 mg/mL thiazolyl blue tetrazolium bromide (MTT) for 4 h at 37°C followed by addition of dimethyl sulfoxide (DMSO) solution (spectra grade, Sigma Aldrich, Bangalore, India) to the cells. Cell viability was evaluated in comparison with control culture (assumed to be 100% viable), which measures cell proliferation based on the ability of live cells to convert MTT into dark blue formazan crystals (OD at 590 nm) using a multi-well reader (Varioskan Flash multimode Plate reader, Thermo Scientific).

Preparation of glass coverslips: Glass cover slips were cleaned for 5 minutes in acetone, dried under a stream of nitrogen and then sterilized at 121°C for 20 min. The sterile coverslips were then UV irradiated for 30 minutes and then coated with poly-L-lysine (Sigma Aldrich, 0.01 %) for 30 min. Extra poly-L-lysine was removed and the coated coverslip was allowed to dry in laminar hood under clean air stream.

Growth of HCT 166 cell lines on coverslips: Clean sterile poly-L-lysine coated glass cover slips were placed in sterile 6 well plates and inoculated with 1×10^6 HCT116 cell lines. The plates were incubated in a humidified atmosphere of 5% CO₂ at 37°C for 48h (till it reached confluence), media was removed, cells were washed with sterile PBS and fresh supplemented culture medium containing hexapeptides at required concentrations was added. The plate was continuously cultured for a chosen period of time. Reaction times for hexa peptide treatments were 0h, 6h, 24h, and 48h. Blank controls were run concomitantly during each experiment.

For AFM imaging, attached cover slips were taken out and washed three times with phosphate-buffered saline (PBS, pH 7.4) fixed with 1% glutaraldehyde solution in PBS for 5min, and then again washed 3 times with PBS. All steps were performed at room temperature, and in a laminar flow clean hood to maintain sterility of all reagents. Caution was taken in handling all human biological material.

AFM Imaging: The cell AFM imaging was conducted in the atmospheric environment using non contact mode. A 100-μm AFM scanner (APE Research, Italy) and Si₃N₄ tip (MikroMasch Co., Russia) silicon tip were used for cell scanning in this study. The tip cantilever length was 3.4μm, width was 1.6μm, thickness was 0.4μm, and force constant was 5.5–22.5 N/m. Image acquisition was carried out using APE Research system programme dedicated AFM SPM Software was used. The scanning speed was half of the scanning area in nanometers / sec. At least 10cells were randomly selected in each attached culture slide for this study, and at least three independent imaging scans for each cell were carried out. Untreated cells were used maintained as control.

STEM Analysis: The STEM imaging was done using ZEISS Model EVO/LS15 instrument (Italy). The samples prepared on cover slips were mounted on aluminium stub which was mounted on a carbon tape. Electron beam imaging was done to observe the treated cells. The image acquisition was carried out using Smart SEM software (Italy).

Statistical analysis The root-mean-square (RMS) roughness data acquisition and analysis of the surface of cell membranes of the three cells were carried out with software. Data are presented as mean ± S.D for the measured root-mean-square roughness for each experiment. Statistical analysis was performed by analysis of variance (ANOVA). Differences among means were tested using Duncan's multiple range tests. A p-value of 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

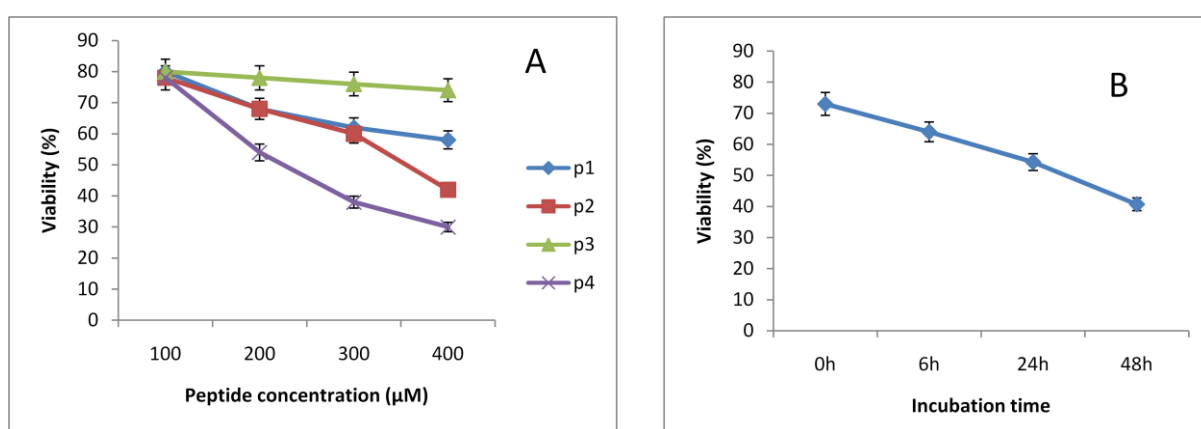
Bioactive peptides derived either from natural resources or synthesized by rational design, have been established to be prospective therapeutic agents against numerous human diseases, including cancer. In *in vitro* studies, transformations in the external morphology of tumor cells treated with anti cancer drugs are generally used as a basis for judgment of drug effects. Hence an attempt was made to observe the changes in the surface morphology of the cells after exposing the cells to azurin derived hexapeptides.

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Cell viability Assay: Prior to the AFM studies, the effects of the four Azurin derived hexapeptides were assessed on viability of HCT116 cells by MTT assay (Fig1). The decrease in viability was concentration and time dependent. At the same concentrations of all peptides no effect on proliferation of cells was observed in the cell line following exposure for shorter time periods. Thus, the toxicity of the azurin derived hexa peptides under the experimental conditions used here were minimal in all in the earlier periods of incubation. Of the four hexapeptides used, peptide 4 showed maximum cell death and this was chosen for AFM and STEM imaging.



giF. .A .1Cell viability assay

Optical microscopy of cell morphology: Of the Azurin derived hexapeptides, hexa peptide 4 was found to be the best in bringing effect on growth and proliferation of HCT116 cells. As shown in (Fig. 2) the morphology of HCT116 cells did not change significantly following exposure to azurin derived hexa peptide 4 over the experimental period used in this study. When observed under visible light, outlines of tumor cells were clear with increase in incubation time when peptide was used at 400 μM/L concentration. Thus, no significant optically observable damage to cell integrity as seen in response to the hexa peptide 4 used (Fig 2).

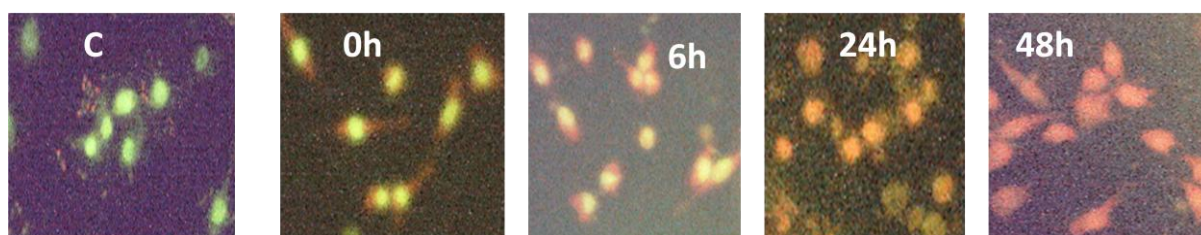


Fig .2 Optical microscopy of cell morphology

Changes in Microscopic Ultra structure following Azurin derived Hexapeptide 4 treatment: Because the surfaces of the tumor cells used in this study are relatively soft, the non contact mode was used for cell scanning, which allowed a large number of AFM images to be obtained following hexapeptide 4 treatment. Under different reaction times, a variety of changes in surface morphology and ultrastructure were seen with HCT116 cells. As seen in Fig. 3A HCT116 cells under normal culture conditions (untreated control) had a regular shape and the cell surface was relatively smooth and intact. The cell membrane ultrastructural components were uniformly distributed. STEM image also showed smooth surface in untreated control cells (Fig. 3, B). Following treatment with 400μM/L hexapeptide 4, the surface morphology began to change gradually with increase in reaction time (Fig.3) and the membrane surface root-mean-square roughness increased with time of incubation (Fig4) and the particles became enlarged and heterogeneous (Fig

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3). The STEM image showed that in the early hours of peptide addition slight perturbations were seen on the cell surface. With increase in time of incubation, by 6h the cell had become slightly elongated and surface is slightly irregular (Fig 3B). At 24h the surface irregularity increased further. By 48h the cell surface was very irregular. These results are well supported by the RMS data, where the roughness increased with time (Fig 4). These changes could be due to the depolymerization of microtubules induced by hexapeptide 4, which would have resulted in decreased cell height, increased membrane roughness, and appearance of various sized pores [12]. Since the HCT116 cells showed decreased viability over incubation period (0–48h) of hexapeptide 4 treatment, it was speculated that the changes observed on the cell membrane ultra structure may be an injury response of tumor cells to hexapeptide 4.

The onset and progression of some human disease states can be strongly associated with the mechanical properties of living cells [28] and any deviations in the mechanical properties may affect its physiological functions [28,3]. As such, single cell mechanics [33] can play a pivotal role in the study of human diseases and further help to understand how diseased cells differ from healthy ones

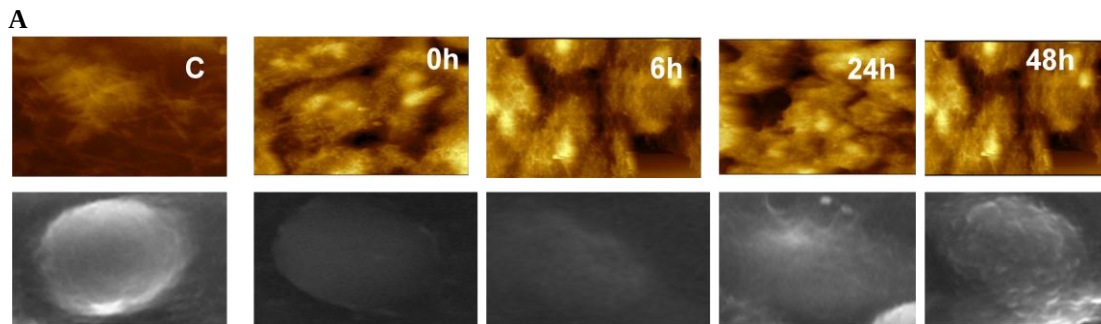
Cancer has long been one of the leading causes of death worldwide and is presently responsible for about 25% of all deaths [12]. It is estimated that there will be about 15 million new cases of cancer annually by 2020 [28]. Since it is known that diseases like cancer, can induce mechanical property changes in cells, this may potentially serve as a useful biomarker in the early detection of cancer [28] and for anti-cancer drug efficacy test. Basically, AFM is a mechanically sensitive surface probe, and local mechanical property differences on the sample can lead to differential surface deformation and contribute to image contrast. The potential of AFM in live cell imaging under near physiological conditions was illustrated since the early 1990s [10; 22]. Since then, high resolution AFM images of various types of living cells have been acquired [18]. Moreover, investigating the mechanical properties of cancer cells may help in better understanding of the physical mechanisms responsible for cancer metastasis. This can potentially lead to the development of different and new strategies for cancer prevention and diagnosis. With the recent advances in biomechanics and nanotechnology, it has now become possible to probe mechanical influences acting on biological structures not only as small as cells but also molecules using biophysical tools and techniques such as AFM [17, 21; 26]. AFM has been established as a versatile tool for imaging and measuring the elastic properties of living cells [5; 22]. In addition, AFM has been applied to investigate the mechanical properties of *ex vivo* cancer cells obtained from patients [32].

In vitro studies by different techniques have shown that some cancerous cells are softer than their normal counterparts [17; 11; 19; 25; 31]. Lincoln et al., (2004) were the first to investigate the deformability of non-malignant and malignant human breast epithelial cells using the optical stretcher and they found that malignant cells can stretch about five times more than their non-malignant counterparts [11; 19]. Lekka et al., (2001) were the first to compare the elastic properties of normal human bladder epithelial cell lines (Hu609 and HCV29) and cancerous ones (Hu456, T24, BC3726) [17]. The authors used the atomic force microscope (AFM) to study the elasticity of normal human bladder epithelial cells (Hu609 and HCV29) and cancerous ones (Hu456, T24, and BC3726) by performing AFM indentation on these cells [21]. AFM imaging and confocal fluorescence imaging of benign (MCF-10A) and cancerous (MCF-7) human breast epithelial cells showed a significant difference in the organization of their sub-membrane actin structures which directly contribute to their difference in cell elasticity [27]. Using AFM [15] observed morphological features on A549 cells. Recently, human bladder and breast cancer cells were extensively studied with AFM [27; 18]. Other types of cancer cells like lung cancer cells, malignant transformed fibroblasts were also investigated with AFM [40; 26]. The effect of chitosan on the stiffness and glycolytic activity was tested on these human bladder cells. Cancerous cells became stiffer due to addition of chitosan, whilst healthy ones were not affected. Chen and Cai (2004) investigated the interaction between human breast carcinoma cells and extracellular matrix with AFM [13] compared morphology and ultrastructure of HeLa (human cervical carcinoma), HepG2 (human hepatocellular liver carcinoma), and C6 (rat glioma) cell membranes by atomic force microscopy (AFM) both before and after treatment with the anti-cancer drugs, colchicine or cytarabine. The changes observed included, increases in the fluctuation of the surface components of the cell membrane, increase in shrinkage, or even the appearance of pores.

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B

Fig. 4. Changes in morphological ultrastructure following azurin derived hexa peptide treatment.

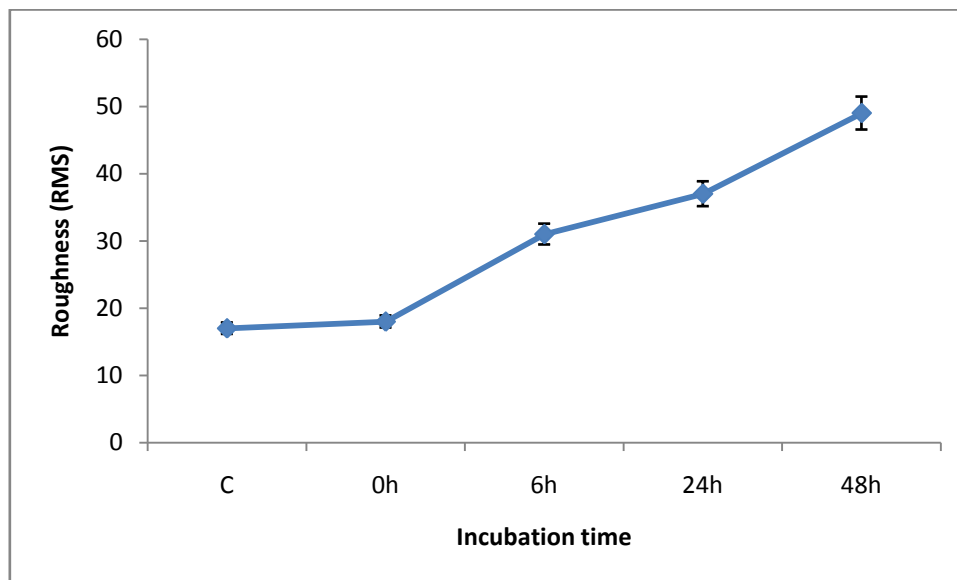


Fig. 4. Changes in morphological ultrastructure following azurin derived hexa peptide treatment.

IV. CONCLUSION

In summary, colon cancer (HCT116) tumour cell lines exposed to azurin derived hexapeptide showed apoptosis as determined by fluorescence microscopy and MTT assays. AFM and STEM scanning analysis showed that the ultrastructure of the cell membrane had been substantially changed, with membrane roughness showing a particularly evident increase. This suggested that the degree of damage to a tumour cell membrane has a certain positive correlation with azurin derived hexapeptide exposure time with longer reaction times, a greater of the degree of cell damage will occur. These findings have significance for the exploration of real-time changes during drug interactions with receptors and this will aid in establishing role of the membrane ultrastructure on the surface of cancer cells in the action of drugs. At the same time, the results presented here also provide a new research emphasis and frame work for the establishment of new experimental methods that incorporate AFM for visual studies of toxicity and for construction of visual trace drug screening models. AFM and STEM can be used as an indenter and also a powerful high resolution imaging tools. The combination of AFM (both as indenter and high resolution imaging tool), STEM and fluorescence microscopy has proven to be very useful in studying the mechanical property and corresponding structures. Changes in the mechanical

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property brought about by the malignant transformation process may be attributed to the reduction in the amount of organized actin filaments or stress fibers.

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