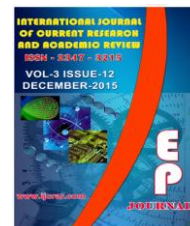




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Hexapeptide Fragment of Azurin Induces Apoptosis in Human Colon Carcinoma (Hct 116) Cell Line

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A B S T R A C T

Bioactive peptides, synthetic or natural have been studied as potential remedial agents against many human ailments, including cancer. However, the mode of action of these remedial peptides against cancer have not been clearly elucidated. In this paper, we discuss the effect of azurin derived hexapeptides on *in vitro* proliferation of human cancer cell lines (human colon carcinoma, HCT 116). The antiproliferative activities were evaluated using the MTT cell growth assay and apoptosis was confirmed by microscopic and FACS analysis. The hexapeptides studied exerted significant, dose and time related apoptotic effects. There was no elevation of p53 levels in treated cells. The penetration of peptide 4 into HCT116 was completely blocked by Nystatin and Filipin, inhibitors of caveolae-mediated endocytosis.

Introduction

Azurin (AZ) is a 128-amino-acid (aa) secondary metabolite secreted by the opportunistic pathogen *Pseudomonas aeruginosa*. It is also produced in plants (Fuchs SM and Raines RT, 2004). The presence of the copper ion in azurin molecule provides this protein few

beneficial effects, including a strong blue color, a high reduction potential and a small parallel hyperfine coupling in the electron spin resonance spectrum (Adman ET, 1991). Azurin has been shown to induce apoptosis in tumour cells implying it to be a potential anticancer agent.

In vitro studies have indicated a direct physical interaction between azurin and p53 (Punj V *et al.*, 2003). In J774 cells, azurin has been observed to induce apoptosis by enhancing the intracellular level of p53 by the complex formation and brought out apoptosis by caspase-mediated mitochondrial pathways (Yamada T *et al.*, 2002). The region in azurin molecule interacting with p53 contained amino acids Met44 and Met64, which were critical for the formation of a p53 complex (Melnick A, 2007).

Peptides have been exploited for a variety of purposes such as drugs, including cancer (Jyothi Thundimadathil, 2012). Cell penetrating activity of short amphipathic and cationic peptides and peptide derivatives, usually containing multiple lysine and arginine residues have been studied (Fischer PM, 2007).

Peptides also have been used as potential transport agents for a variety of drugs including cytotoxic drugs (Gusarova GA *et al.*, 2007; Melnick A *et al.*, 2007), antisense oligonucleotides (Astria-Fisher *et al.*, 2002), in gene therapy (El-Andaloussi S *et al.*, 2006; Cashman SM *et al.*, 2002), and as decoy peptides (Fisher L, 2004).

In the present study, we choose methionine (Met) containing regions in the 148 aa containing azurin molecule and studied hexapeptides from aa 74-79, 82-87, 126-131, 139-144 on human colon carcinoma (HCT116) cell lines.

Here, we show that the hexapeptides can effectively inhibit cancer cell proliferation, induce cancer cell apoptosis. The findings in the present study give the proof of concept for using azurin derived hexapeptide as a potential therapeutic agent for the treatment of human colon carcinoma.

Materials and Methods

Cell Culture and Cell Lines

Human colon carcinoma cell line (HCT116), and normal cell line NIH 3T3 were obtained from NCCS, Pune, India. Cells were cultured in McCoy's (Sigma-Aldrich) 5A 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% carbondioxide.

Peptide Synthesis

All azurin-derived hexapeptides were chosen at the Met regions on the sequence. The hexapeptides with azurin sequence are given in Fig. 1. 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-tetramethyluronium hexafluorophosphate (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.

Purification and Characterization of the Peptides

The crude peptides obtained after cleavage from the resin were purified by gradient HPLC using water, acetonitrile, and 0.1% TFA as the mobile phase on a C₁₈ column (Varian pursuit XRs C₁₈ column, 250 mm length and 21.2 mm width with particle size 10 µm), attached to a LC-8A, Shimadzu HPLC system (Shimadzu Asia Pacific Pvt. Ltd., Singapore). ESI mass spectroscopy was used to pool the HPLC fractions and characterization of the pure peptides (Q-TOF Ultima mass spectrometer with TOF analyser of Waters Corporation, Micromass UK limited, UK).

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 various concentrations of hexapeptides at the final concentrations of 0 (as control), 100, 200, 300, 400 and 500 µM/l separately. The proliferation of the cells was measured after 48h 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). Similarly, experiments were conducted using normal cell lines NIH 3T3.

Morphological Observation of Cells Treated with Hexapeptide

The morphological changes of the HCT116 cells treated with hexapeptides were observed with fluorescence and confocal microscope. For fluorescence microscopy, the cells were suspended in a 96-well plate (Corning Sigma-Aldrich) at a density of 2×10^4 cells per well. After 48 h of growth, they were treated with various concentrations of hexapeptides separately, for 48 h.

Then the medium was removed, the cells were washed with sterile PBS and then stained with a mixture of ethidium bromide: acridine orange at 0.9: 1 ratio. The cells were observed under fluorescence microscope by 30 min of staining. For confocal microscopy in poly-L-lysine coated glass coverslips, cells were grown to confluence. These cells were treated with hexapeptide 4 at 400 µM/l. The cover glass nearly full of the cell on its surface (by 48h) was taken for staining with the mixture of ethidium bromide: acridine orange at 0.9: 1 ratio. The method was used to observe the apoptosis of hexapeptide treated HCT116 cells.

Detection of Apoptosis in Cultured Cells by FACS

Apoptosis of cells were detected using FITC-conjugated Annexin-V and propidium iodide (PI) (Sigma-Aldrich). The cell growth and peptide treatment are as given under 2.5 peptide treated cells were washed twice with cold PBS and resuspended in Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl and 5 mM CaCl₂) at a concentration of

1×10^6 cells/ml. Then a single suspension of 1×10^6 HCT116 cells was prepared in a 5 ml culture tube, in which 5 μ L Annexin-V-FITC (10 μ g/ml and 10 μ l) propidium iodide (10 μ g/ml) was added. Then the tube was gently vortexed and incubated for 15 min at room temperature in the dark. Binding buffer (400 μ l) was then added to each tube and the cells were analyzed by flow cytometry (Beckman coulter cell lab quanta SC).

DNA Fragmentation Assay by Agarose Gel Electrophoresis

DNA fragments in the cell lines were assayed by agarose gel electrophoresis, according to the method described by (Sambrook and Russell 2001). DNA in the cell lines was extracted using Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma), and subjected to electrophoresis in 1 % agarose gel. The electrophoresed gel was stained with 0.5 μ g/ml ethidium bromide. The electrophoretic bands in the gel were visualized and photographed under transmitted ultraviolet light and photographed using Cleaver Scientific Ltd Gel Doc system.

Immuno Analysis of Apoptotic Pathway Proteins

The cell lysates were isolated from the hexapeptide 4 treated HCT116 cells. The lysates were immobilized onto ELISA wells. Then after blocking the wells with 1% (w/v) BSA, wells were incubated with primary, rabbit monoclonal anti p53, anti CDK antibodies, according to the manufacturer's instructions (Sigma-Aldrich) and then incubated with alkaline phosphatase conjugated secondary antibody (goat anti-rabbit IgG). The proteins were detected with colour developed using *p*-NPP as a substrate.

Entry Inhibitors

HCT116 cells (1×10^5 per 200 μ L) maintained in serum-free McCoy's 5A at 37°C, were pretreated with the following inhibitors (Sigma-Aldrich) Chlorpromazine (CPZ), Nystatin, Filipin, Taxol, Sodium azide, Cytochalasin D, Benzyl-2-acetamide, Staurosporine, Wortmannin, Monensin, Nocodazole, Tunicamycin, and Neuraminidase for 5 h. Hexapeptide 4 (400 μ M/L) was then added to the cells, incubated for 1 h, and the cells were then washed, fixed and prepared for flow cytometric analysis as given in Fig. 2.

Hemolysis Assay

Mammalian whole blood samples (2–3 ml) were centrifuged for 10 min at 1,000 g, the pellets were washed once with PBS, once with HKR buffer (pH 7.4) (Sharikov Y et al., 2008) resuspended in HKR buffer to 4% erythrocytes, and 50 μ l was transferred to a 1.5 ml tube with 950 μ l of peptide 4 or 0.1% Triton X-100 in HRK buffer to disrupt the RBC membrane. After 30 min at 37°C, tubes were centrifuged for 2 min at 1,000 g, 150 μ l of supernatants were transferred to a 96-well plate, and absorbance was recorded at 540 nm.

Cell Membrane Toxicity Assays/Lactate Dehydrogenase Leakage Assay

The lactate dehydrogenase (LDH) leakage assay was performed according to the manufacturer's instructions (AGAPE kit) with 100 μ l of HCT116 cells (5×10^3). Cells without peptides were used as a negative control. Experiments were carried out in triplicate.

Tunel Assay

The TUNEL assay was used to detect apoptotic cells. This assay utilizes terminal-deoxynucleotide-transferase (TdT) containing FITC labelled dUTP on the 3'-OH ends of fragmented DNA (Gavrieli *et al.*, 1992) (TdT-mediated dUTP Nick-End Labeling assay or TUNEL). The Kits instructions were followed (Life Technologies). Briefly, cells were grown to about $50 \pm 75\%$ confluency on coverslips in culture media. After 24 h of zero or 400 μM peptide 4 exposure (48 h) the coverslips were removed from the media, washed twice in PBS and the cells were fixed for 25 min (4°C) in freshly prepared 1% paraformaldehyde (HiMedia, Mumbai, India) in PBS.

The cells were permeabilized in PBS plus 0.2% Triton X-100 for 5 min on ice and washed with PBS. The coverslips were placed at room temperature and incubated for 5 min in the equilibration buffer supplied by the manufacturer. After incubation this solution was removed and a solution containing equilibration buffer (90 μl), nucleotide mix (10 μl) and TdT enzyme solution (2 μl) was applied and this was incubated in the dark at 37°C for 60 min.

The reaction was terminated by washing with 2 X SSC followed by a wash in PBS. The coverslips were then incubated with primary antibody (Anti-BrdU mouse monoclonal antibody PRB-1, Alexa Fluor 488 conjugate) for 30 min at room temperature away from sunlight. Then 0.5 μl of the propidium iodide/RNase A staining buffer was added. The cells were incubated for an additional 30 min at room temperature protecting from light during the incubation. The coverslips were then mounted on glass slides for microscopy.

Statistical Analysis

All measured data were presented as mean \pm 3 SD. The differences among groups were analyzed using the one-way ANOV.

Results and Discussion

Peptide Synthesis

All azurin-derived hexapeptides were chosen at the Met regions on the azurin protein sequence. They corresponded to Ala⁷⁴-Val⁷⁹, Asp⁸²-Gly⁸⁷, Cys¹²⁴-Phe¹²⁹, Ala¹³⁷-Thr¹⁴² regions of Azurin protein molecule. They were synthesized and purified to homogeneity (Fig. 1).

Hexapeptide Treatment Induced Cell Proliferation Inhibition and Apoptosis of HCT116 Cells *In vitro*

Peptides (p28) derived from azurin protein have been shown to induce apoptosis of UISO-Mel-2 cells cancer cells. They have observed that the methionine of peptides had a major role in inducing apoptosis. This intrigued us to investigate the possible effect of methionine containing azurin hexapeptides as anti-proliferative and apoptotic peptides. We examined the effect of four hexapeptides on the proliferation of HCT116 cells. To our surprise, all hexapeptides could effectively suppress the HCT116 cells growth even at a low dosage of 50 μM concentration (Table 1).

The cell proliferation inhibition was concentration-dependent. Of the four hexapeptides studied, peptide 3 and 4 showed maximum inhibition of cell proliferation. Peptide 4 was chosen for further studies.

The treated cells also showed a deeply stained in the nuclear portion, which is the

typical characteristic of apoptotic cells (data not shown). To validate this observation, we performed the apoptosis assay with Annexin V-FITC and PI double staining method. Hexapeptide treatment clearly induced HCT116 cells to apoptosis after 48 h peptide exposure (Fig. 2, Table 2).

Peptide 3 and peptide 4 showed 97 and 98% apoptosis and peptide 1 did not show apoptosis. Peptide 1 induced only inhibition of cell growth. Inhibition of HCT116 cell proliferation, measured directly, suggested that the inhibitory effect does not reside at the level of cell cycle with apoptosis (Table 2) suggesting that only cytotoxic activity of hexapeptide 4 was responsible for bringing out apoptosis. The hexapeptide 4 did not exhibit the cytostatic activity (Table 2).

Confocal analyses initially suggested that hexapeptide 4 (Fig. 3) apoptosed human cancer cells with good efficiency but did not apoptose normal cell lines to the same degree (Fig. 4). The peptide 4 was efficiently taken up by the HCT cell lines. These observations were confirmed further by a sensitive fluorescence-activated cell sorting (FACS) analyses (Fig. 2) where peptide 3 and peptide 4 brought 98% apoptosis followed by peptide 2.

Cell Penetration is not a Result of Membrane Disruption

Cell penetration by hexapeptide 4 does not result in membrane disruption. An LDH leakage assay (Fig. 5) suggested that peptide did not enter cells by altering the integrity of the plasma membrane (Saar K et al., 2005). The absence of membrane disruption was also confirmed by determining the hemolytic activity of hexapeptide 4 on mammalian erythrocytes where no cell lysis was observed (Fig. 5).

Confocal analyses initially suggested that hexapeptide (Fig. 3) penetrated HCT116 cells with good efficiency but did not penetrate normal cell lines to the same degree.

DNA fragment assay also demonstrated the remarkable DNA degradation in hexapeptide-treated HCT 116 cells (Fig.6). The hexapeptide 4 treated cells were TUNEL positive (Fig. 6).

In the peptide 4 treated HCT 116 cells there was no increase in the p53 levels (Fig. 7) . However, there was a decrease in Cdk levels in hexapeptide 4 treated cells (Fig. 7).

Hexapeptide 4 Penetration into HCT 116 Cells

The penetration of hexapeptide 4 (Fig. 7) into HCT116 cells in the presence or absence of a specific inhibitor of O-linked glycosylation, neuraminidase, which cleaves sialic acid residues, was not inhibited. Tunicamycin, an inhibitor of N-linked glycosylation, also did not reduce the penetration of peptide 4 across the cell membrane. Sodium azide, an inhibitor of energy-dependent transport mechanisms, i.e., ATP reduced the penetration of peptide 4, suggesting non-endocytotic pathways might also be involved in the penetration of these peptides (Fig. 7). Stabilization of microtubules with Taxol had no effect on penetration, but disruption of actin filaments and macropinocytosis with Cytochalasin D produced 41% inhibition of peptide 4 entry. Inhibition of the cell cycle with staurosporine reduced penetration, suggesting that penetration could be cell cycle specific. The reduced penetration of peptide 4 by staurosporine into the cancer cell plasma membrane also suggested that a Src kinase/ tyrosine kinase-dependent pathway might be involved in the entry

(Pelkmans L et al., 2002) . Nocodazole, which disrupts caveolae transport and inhibitors of cholesterol mobilization and, hence, caveolae-mediated endocytosis, inhibited penetration by 20%. Wortmannin, an inhibitor of early endosome formation, inhibited the intracellular accumulation of peptide 4 by 23% suggesting that a caveolae to early endosome pathway might be involved in the intracellular trafficking of peptide 4. The lesser involvement of early endosome in the intracellular trafficking of peptide 4 also suggested that clathrin-mediated endocytosis might be involved in internalization of these peptides (Tran D et al., 1987; Parton RG and Richards AA 2003). Monensin, which inhibits late endosome/lysosome, influenced the intracellular accumulation of peptide 4 by 72%. The penetration of peptide 4 into HCT116 was also inhibited by Nocodazole (20%), Monensin (72%), Tunicamycin (22%) and Sodium azide (71%). Nystatin and Filipin inhibitors of caveolae-mediated endocytosis completely blocked the entry of peptide 4.

Peptide remedy is a promising field for the development of anticancer agents, mainly due to the fact that these peptides can easily be obtained either from natural resources or rational design based on the target protein structure. Numerous studies have indicated that a number of bioactive peptides inhibited tumor cell growth in preclinical trails (Su L et al., 2010; Kannan A et al., 1920; Li D et al., 2011).

An advantageous property of these therapeutic peptides is that they usually have no or limited toxicity (Almansour NM et al., 2012). Goat spleen extracted anticancer bioactive peptide (ACBP) radically inhibited human gastric tumour growth in a xenograft model with no obvious cytotoxicity to host (Su L et al., 2010). Anticancer properties of some bioactive peptides could be accredited

to their capabilities to induce apoptosis and cell cycle arrest (Su L et al., 2010; Cheok CF et al., 2012; Li D et al., 2011) . Some studies have revealed peptides can impair a specific signaling pathway and subsequently inhibit the tumor growth or metastasis. SAHM1, the hydrocarbon stapled peptide, foiled assembly of the active transcriptional complex of Notch, and thus inhibited cell proliferation *in vitro* and tumorigenesis in a mouse model of NOTCH1-driven T-cell acute leukemia and lymphoma (Moellering RE et al., 2009).

Azurin, a cupredoxin redox protein is observed in few bacteria and green plants. Azurin preferentially enters cancer cells (Almansour NM et al., 2012; Su L et al., 2010; Kannan A et al., 1920; Li D et al., 2011) and induces a p53-mediated apoptosis in murine J774, human breast cancer, melanoma (Almansour NM et al., 2012; Su L et al., 2010; Li D et al., 2011) and osteosarcoma cells but not in p53-negative osteosarcoma and normal liver cells. Azurin has been shown to bind to the NH₂-terminal domain of p53 [24] and DNA-binding domain (DBD), which include amino acids 1 to 292, but not the COOH-terminal region of p53 (Li D et al., 2011; Leslie H, Kondejewski et al., 2002) . Amino acids 50 to 77 of azurin (p28), which encompass Met 64, essentially act as a protein transport domain for azurin (Kanwar JR et al., 2009) . It was suggested that the COOH-terminal 10 to 12 amino acids of p28 were responsible for its antiproliferative activity (Urista CM et al., 2011) . p28 has been shown to act by increasing intracellular levels of p53, increasing the cyclin-dependent kinase (CDK) inhibitors p21 and p27, and inhibiting cell cycle at G2-M in a time-dependent manner (Brad N et al., 2009) . It has been observed that Met of p28 played a key role in inducing apoptosis (Brad N et al., 2009).

These reports made us choose hexapeptides from methionine containing regions of azurin. Of the four hexapeptides chosen, peptides 3 and 4 showed maximum apoptotic activity which was concentration dependent. Tylor et al., (2009) observed entry of 5 μ M/L p28 or p18 into UISO-Mel-2 cells after 30 min in the presence of a 200-fold surplus of unlabeled peptide indicating that entry was saturable process. In general, cationic peptides initially interact with the cell membranes of prokaryotic and eukaryotic species (Leslie H et al., 2002) by binding to negatively charged surface glycoproteins, facilitating efficient entry into a broad range of normal and malignant cell lines (Fuchs SM and Raines RT 2004) . Azurin and the two peptides derived from it (p28 and p18) possessed the selective property of preferentially entering cancer cells and inhibiting their proliferation through cytostatic and cytotoxic mechanisms (Brad N et al., 2009) . p28, residues 54 to 67 of azurin has been shown to possess predominantly α -helical as well as a partial β -sheet structure (Garnier J et al., 1978) and described as the minimal sequence required for cancer cell entry and cell cycle inhibitory activity. However, in our studies we observed Met containing hexapeptides of azurin bring about apoptosis of HCT116 cell. Hexapeptide PGPIP has been shown to bring apoptosis of SKOV3 cells and female nude mice (Wang W et al., 2013).

The penetration of peptide 4 into HCT116 was blocked by Nocodazole (20%), Monensin(72%), Tunicamycin(22%), Sodium azide (71%), and by CPZ(95%). Nystatin and Filipin inhibitors of caveolae-mediated endocytosis completely blocked the entry of peptide 4. However, an energy-dependent endocytotic or porerelated process did not seem to be the only entry mechanism available to p28 and p18

peptides. Sodium azide, which inhibited the entry of cationic peptides (36), did not harm the entry of either p18 or p28 into UISO-Mel-2 cells or fibroblasts signifying that both peptides may also directly penetrate the cell membrane. However, the non-endocytotic mechanism described for penetration and related peptides needed a more basically charged peptide (than either p18 or p28). Clathrin-mediated endocytosis, which triggers cellular penetration of a wide variety of cationic peptides (Richard JP et al., 2003; Potocky TB et al., 2003), was also not a entry route of p18 and p28 as CPZ had no effect on penetration into cancer cell membrane. Azurin-derived peptides also do not follow the proposed modes of cellular penetration like other cationic CPPs, i.e., macropinocytosis, distribution to late endosomes or lysosomes along actin filaments or microtubules, and penetration at exact cell cycle stages, because inhibitors of each of these pathways were alone not effective with p18, p28, and possibly azurin appeared to penetrate the plasma membrane and reach late endosomes, lysosomes, and the Golgi associated with caveolae in a dynamin-independent clathrin-independent carrier-mediated manner (Kirkham M et al., 2005). The non inhibition of penetration by nocodazole and relatively reduced inhibition by cytochalasin-D, which disrupts actin filaments, supported the idea of caveolae-mediated entry of hexapeptide 4. The lack of effect of staurosporine suggested that dynamin does not play a large role in the penetration of hexapeptide (Kirkham M et al., 2005) . This mode of entry has been described for integral cell-surface components and a broad range of pathogens or their products that also use caveolae to avoid classic endocytic pathways. Diminution of cholesterol from the plasma membrane with Nystatin to disrupt lipid rafts, plasma membrane domains that provide fluid platforms to separate

membrane components and compartmentalize membranes (Fischer PM 2007) significantly inhibited the penetration of hexapeptide (100%) into HCT 116 cells

and suggested a significant percentage of hexapeptide 4 penetrated the plasma membrane *via* caveolae.

Table.1 Viability of HCT 116 Cells after Hexa Peptide Treatment

Concentration of peptide (µM)	Viability (%)			
	Peptide 1	Peptide 2	Peptide 3	Peptide 4
0 (Control)	100	100	100	100
100	75	85	80	80
200	65	70	75	64
300	59	60	73	25
400	50	40	65	20

Table.2 Flow Cytometric Analysis Hexapeptide Treated HCT116 Cell Line

	Apoptosis (%)
Control	0
Peptide 1	0
Peptide 2	87.54
Peptide 3	97.82
Peptide 4	97.95

Fig.1 Pseudomonas Aeruginosa, Azurin Protein Sequence Showing four Hexapeptide Chosen for this Study

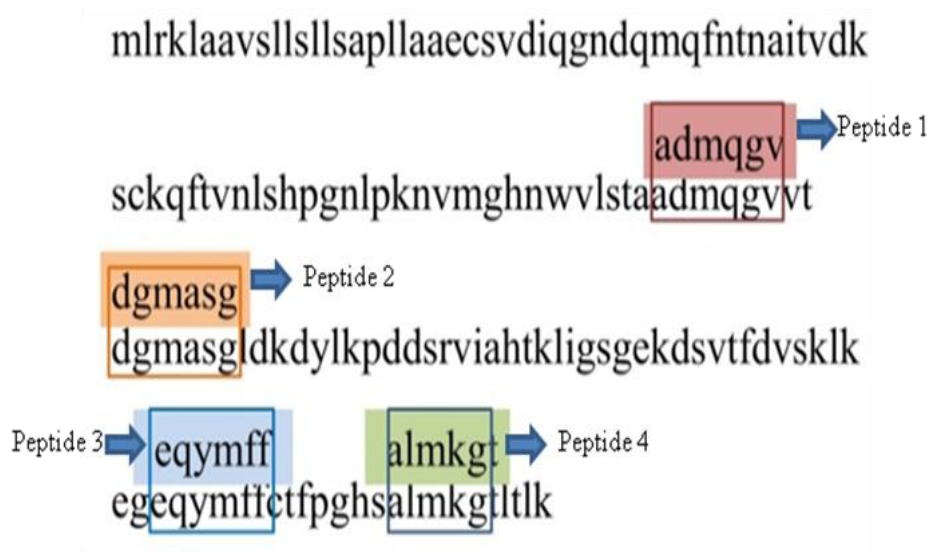


Fig.2 Flow Cytometric Analysis of Hexa Peptide Treated HCT 116 Cells

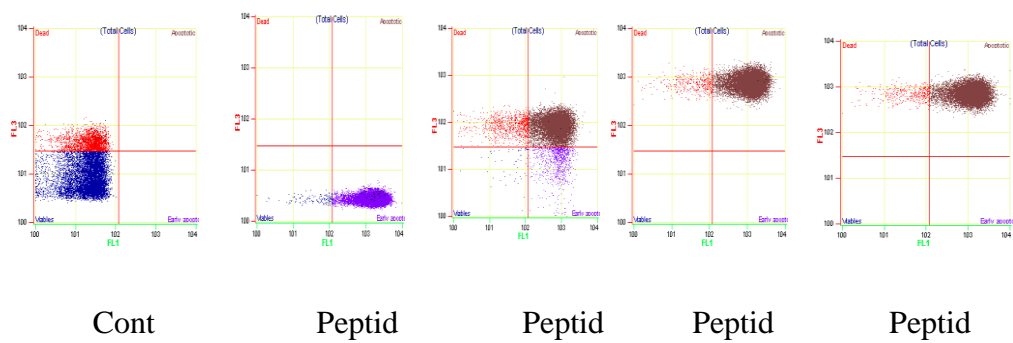


Fig.3 Confocal Microscopic Picture of Hexapeptide Treated HCT116 Cells

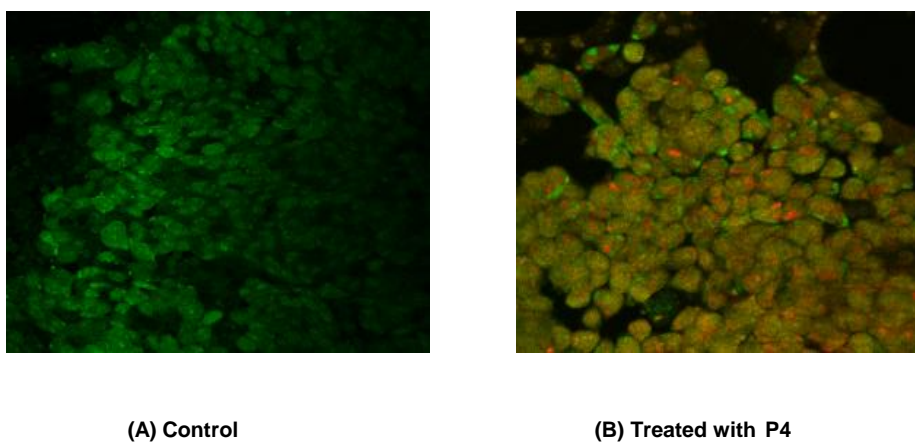


Fig.4 MTT Assay of NIHT3T Cell Line

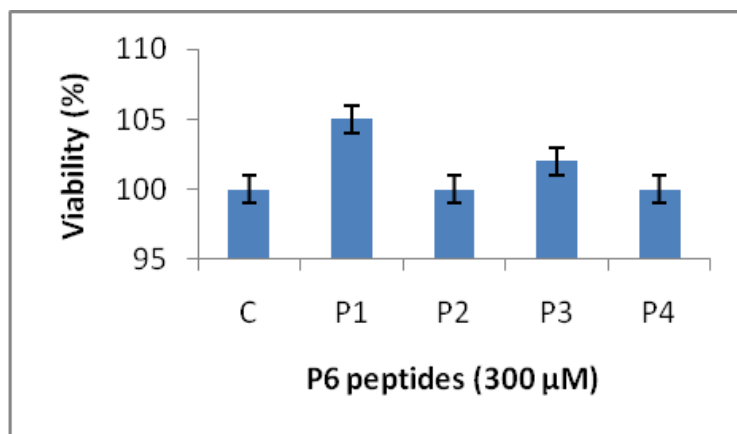


Fig.5 (A) DNA Fragmentation and (B) TUNEL Assay

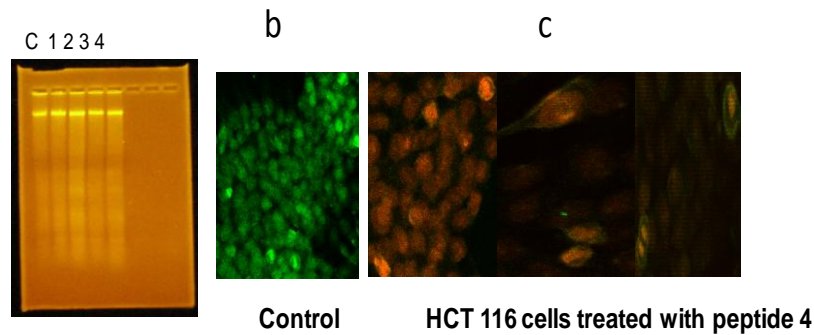
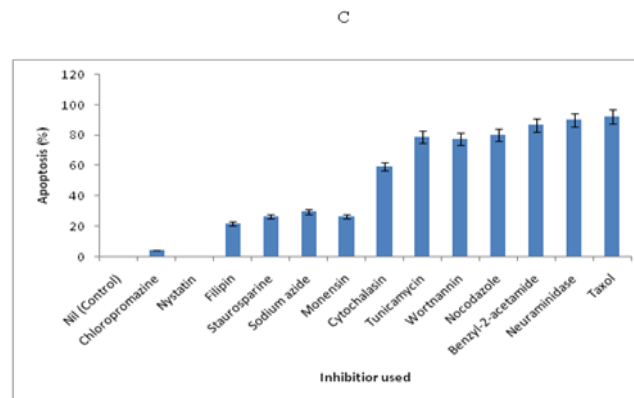


Fig.6 Levels of p53 and CDK in Hexapeptide 4 Treated HCT 116 Cells



Fig.7 Effect of Entry Inhibitors



Caveolae are a 50 to 100 nm N-shaped subset of lipid raft invaginations of the plasma membrane identified by the existence of caveolin-specific proteins (caveolin-1, caveolin-2, or caveolin-3) that function as regulators of signal transduction (Scherer PE et al., 1997). Cell penetration of hexapeptide 4 *via* caveolae also fits well with our observation that inhibitors of N-

glycosylation (Tunicamycin) reduced cell entry of peptide 4 by 22% in HCT116 cells.

Hexapeptide 4 all bind to cancer cells with high affinity and high capacity relative to other potential anticancer peptides (Hohla F et al., 2007) , indicating that this protein/receptor complex localizes in caveolae and is internalized, ultimately

moving (*via* caveosomes) to the Golgi, endoplasmic reticulum, and nucleus. A clathrin and caveolin independent pathway can exist as a constitutive internalization mechanism, such as for the interleukin 2 receptor (Lamaze C et al., 2001) and for certain glycosyl-phosphatidylinositol-anchored proteins (Sabharanjak S et al., 2002). Clathrin- and caveolin-independent endocytosis has also been used by pathogens to enter cells, either completely, as for the murine polyomavirus (Ewers H et al., 2005), or in combination with a conventional pathway, as is the case for the influenza virus (Sieczkarski SB and Whittaker GR 2010). However, an additional investigation into the preferential penetration of these peptides into cancer cells is required.

Conclusion

In conclusion bioactive peptides, synthetic or natural have been studied as potential remedial agents against many human ailments, including cancer. However, the mode of action of these remedial peptides against cancer have not been clearly elucidated. In this paper we discuss the effect of azurin derived hexa peptides on *in vitro* proliferation of human cancer cell lines (human colon carcinoma, HCT 116).

The antiproliferative activities were evaluated using the MTT cell growth assay and apoptosis was confirmed by microscopic FACS analysis. The hexa peptides studied exerted significant, dose and time related apoptotic effects. There was no elevation of p53 levels in treated cells. The penetration of peptide 4 into HCT116 was blocked by nocodazole (20%), monensin(72%), tunicamycin(22%), sodium azide (71%), and by CPZ(95%). Nystatin and Filipin inhibitors of caveolae-mediated endocytosis completely blocked the entry of peptide 4.

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