CHAPTER –3

REVIEW OF LITERATUR
**P. aeruginosa**: Among bacteria inducing apoptosis in host, *P. aeruginosa* infects compromised/uncompromised hosts, and occurs naturally almost everywhere in lakes, streams, soil and even our drinking water. *P. aeruginosa*, a prevalent opportunistic human pathogen, is a gram-negative bacterium found in nosocomial infections (Filon et al., 2002; Sadikot et al., 2005). Cystic Fibrosis patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population (Greenberg, 2000; Patricia and Jay 2007). *P. aeruginosa* is often resistant to antibiotics and causes serious respiratory infections in people who are particularly susceptible, such as patients with cystic fibrosis or severe burns. *P. aeruginosa* protects itself from destruction by killing macrophages, the immune system’s first line of attack against a foreign body.

It was reported that *P. aeruginosa* secretion protein azurin shrink human melanoma tumours in immune deficient mice by 60%, suggesting that azurin is an entirely new source of anticancer agents. Furthermore, it was found that redox protein, azurin from *P. aeruginosa* triggered apoptosis in cancer cells and associated with tumour-suppression protein (Yang et al., 2005; Chaudhari et al; 2007). The induction of apoptosis in bacterial infection results from a complex interaction of bacterial proteins with mammalian proteins finally mediating apoptosis. Bacterial proteins are able to activate pro-apoptotic proteins eg. caspase.

**Azurin Protein Molecule**:

For the first time in 1956, the *Pseudomonas aeruginosa*, a Gram-negative, aerobic, rod shaped bacterium with unipolar motility was reported to contain a blue protein (Verhoeven and Takeda 1956, Sutherland and Wilkinson 1 in 1963). The blue protein was purified in 1958
(Horio 1958). It was then revealed to be a virulence factor, which gives bacteria the ability to escape the host defence system. Similar proteins are broadly spread in the genera *Pseudomonas*, *Bordetella* and *Alcaligenes*. The same blue protein was discovered in strains of *Bordetella* in 1963 (Sutherland and Wilkinson 1963). They proposed the name ‘azurin’ Az for this class of proteins due to purplish shade of blue of the copper ion present in their structure. Az is located in the periplasmic space of the bacterium. The sequence of Az extracted from *Pseudomonas fluorescens* was determined in 1967 (ambler and Brown 1967) and it was revealed to contain single peptide chain with 128 amino acids. It functions as an electron carrier (Hotink Carla and Canters 1992) and eradicates the host defense system by encouraging apoptosis in phagocytic cells (macrophages) (Jain and Forbes 2001). Az is now known as a member of copper-containing proteins called cupredoxins or blue-copper proteins, due to their striking blue color with $\lambda_{\text{max}} = \text{ca.} 600 \text{ nm}$ (nanometer).

**Azurin protein molecular structure:**

Az molecule is a small 128 amino acids copper protein, demonstrating a rather large stability (Fuentes et al 2004). Az is the simplest of all the copper proteins so far discovered containing only one copper atom/molecule. It has a low molecular weight (14 kDa) and contains no carbohydrate unlike ceruloplasmin, which is another copper containing protein and laccase, a copper containing oxidase copper-protein is small (10-14 kDa) water soluble proteins which contain at least one copper ion bound to a site called the type-1 copper site. The site confers their unique spectroscopic properties. Copper sites are classified as types 1, 2 or 3 due to their optical and electron paramagnetic resonance (EPR) spectroscopic feature (Rosenzweig and Sazinsky 2006). Type – 2 copper site is spectroscopically similar to the aqueous copper (II) ion and has square planar coordination geometries, proteins containing a type-2 copper site are frequently involved in substrate binding, like superoxide dismutase, which is a superoxide scavenger (Kolczak et al., 1999).

Some blue-copper proteins have combination of four or more copper ions per molecule, of which one or more are bound to the type I site. Their function is to shuttle electrons and to catalyze dioxygen reduction to water (Farver and Pecht 1991).
The copper content of Az accords to one copper atom per 16,000 molecular weight (Antonini et al. 1970). Az is composed of one α helix and two β sheets, which create a β-barrel motif (Leckner et al., 1997, Fig: 2)

![Fig 1. Azurin showing α- and 2 β-sheets arranged in Greek -key folding motif.](image)

In 1998 it was proved (Baker 1988) that the copper ion in Az is coordinated by a Sγ- atom (sulfur) of cysteine and Nδ-atoms (nitrogen) of two histidines. It was also confirmed that the copper coordination is best described as distorted trigonal planar, with strong in-plane bonds to His46 Nδ-atom, his 117 Nδ-atom, His 117 Nδ-atom and Cys 112 Sγ- atom, and much weaker axial interactions with Met 121 Sγ- atom and Gly 45 C=O. The Sγ- atom of a methionine holds the axial position (fourth ligand), resulting in a trigonal pyramidal geometry around the copper as it is shown in Fig: 3.

Differently stated, there are three potential binding sites in Az 9planar trigonal ligands), two imidazole nitrogen groups of histidines (His 117, His 46) and a thiolate group of cysteine (Cys 112) (Messerschmidt 1998), which are coordinated somewhat equatorially 2.0-2.2 nm from the copper. Conformation of the copper ligands of Az is depicted in Fig: 1.
Alongside, there are two axial ligands, methionine 121 and glycine 45 occupying the more distant axial positions at 0.30-0.31nm (antholine et alk 1993), thus, giving the Cu site in Az a trigonal planar or trigonal bipyramidal (3+1+1) form as shown in Fig : 1.

This conformation of the copper ligands of Az is retained by the rigid protein matrix, which has become known as the cupredoxin fold (Adman 1991). In this fold, a Greek-key folding motif, referring to super secondary structure in a protein, is formed by eight β-strands arranged in two β-sheets opposing each other in a β-sandwich.

In 1992 it was revealed (Lowery and Solomon 1992) that the copper site of Az is distinct from other type-1 copper sites since the back bone carbonyl oxygen (A-Co-B) of a glycine residue (H2N-CH2-CO-OH) has a non-negligible electrostatic interaction with the copper ion. Copper sites of all blue-copper proteins have an axial ligand, which is either methionine [O-CO-CH(NH3)-CH2-CH2-S-CH3] or a glutamine [CO(NH2)-CH2-CH2-CH(NH2)-COOH]. However, in Az, opposite to the methionine ligand, there is a carbonyl oxygen (O=C) at a distance of ~0.21 nm the copper ion. Additionally, the distance between the copper ion and the axial methionine ligand (0.318) is relatively longer comparing to other blue-copper proteins (0.28-0.29) (walter et al., 1996).

Az also unique in that it contain a disulfide bond [=C-S-SC=] between Cys3 and Cys26, which connects the first two N-terminal β-strands in the structure. Az has a hydrophobic patch exposed to the surface. It was shown that the hydrophobic patch exposed to the surface. It was shown that the hydrophobic patch of Az was the interaction site with the redox partners, Cytochrome (cyt) c-551 and nitrite reductase. When two methionine residues (Met-44 and Met-64) in this hydrophobic patch were replaced by two polar aminoacids (lysine and glutamic acid) to reduce the hydrophobicity of the patch, an electric dipole was created in the hydrophobic patch, thus greatly reducing the electron transfer property of Az (Goto et al., 2003). The consequential Az-mutant could still enter the cells, but with severely decreased cytotoxic property. Therefore, it seems the Az hydrophobic patch has a key role in apoptosis induction(Yamada et al., 2004).
Azurin contains a single tryptophan residue at position 48 masked in the hydrophobic core of the protein (Hansen et al., 1990). The core is highly shielded from solvent and surrounded by hydrophobic residues, causing it to display fluorescence with the smallest Stokes shift known for the tryptophan residue in any protein (shift of the absorption and fluorescence to 291 and 309 nm respectively) (Leckner 2001).

There is an unusual tryptophan environment in this protein, which is characterized by the absence of any hydrogen bonding or other polar interaction of tryptophan with its environment (Gilardi et al., 1994). Some modifications in this structure have been examined. For example, a remarkable increase in tryptophan fluorescence quantum yield was observed by removing the copper ion from the Az structure (Burstein et al., 1997).

Az holds two potential redox centers: the T1 blue-copper ion coordinated directly to amino acid residues, and a disulfide bridge (R=S=S-R) present at the opposite end of the molecule, separated by a direct distance of 2.65 nm (Farver et al., 1999). Intramolecular electron transfer between these sites was investigated in a large number of wild-type and single site-directed Az-mutants. Also the effect of specific changes in the protein structure on electronic couplings, reorganization energies, and the nature of the medium separating donor and acceptor were examined.

Az has a strong charge-transfer absorption with the maximum absorbance at around 625 nm due to the bond maximum absorbance at around 625 nm due to the bond between Cu and Cys-112 (Leckner 2001). The absorption band probes the oxidation state of the copper ion and other alterations around it (Fuenetes et al., 2004). The formation of active Az dramatically increases if the copper is introduced before polypeptide folding comparing to folded protein (Pozdnyakova and Wittung-Stafshede 2001).

An Az variant has been engineered (called purple CuA Az) (Hay et al., 1996) where the blue-copper site is replaced by the purple cuA center. This binuclear purple CuA center is a more efficient electron transfer agent than the blue single copper center; because reactivity of the former involves lower reorganization energy (Farver et al., 1999).
Az is a very stable protein. Its oxidized form melts at a temperature of approximately 80 degrees C and its chemical denaturation energy stability has been measured to be 52 kJ/mol. The presence of a disulfide bond is one reason for Az high stability. Other stabilizing factors are the metal, and the tyrosine corner. Removal of the metal significantly destabilizes the protein. (Bonander et al., 2000).

**Az Electron transfer:**

Az functions in the electron transport cycle during respiration in microbes. It transports an electron between cyt c-551 and cyt oxidase in their respiration process (Kakutani et al. 1981). Az and cyt c-551, two globular metallo proteins, are generally regarded as the physiological electron donors for Pseudomonas nitrite reductase (or Pseudomonas oxidase) which is known to react with both O2 and NO2 (Silverstrini et al., 1982).

Pseudomonas cyt oxidase is a bi functional enzyme, consisting of two identical sub units each containing one heme c and one heme d1 moiety and capable of using either inorganic nitrite or molecular oxygen as the ultimate electron acceptor. The Pseudomonas cyt oxidase can accomplish two functions, the four-electron reduction of nitrite (O2) to water and the single-electron reduction of nitrite (NO2-) to nitric oxide. It carries out these functions by accepting electrons from either of two protein substrates, the Pseudomonas cyt c-551 or the copper protein Az (Parr et al., 1977).

In 1970, stopped-flow kinetic studies on the reaction between Az and cyt c-551 suggested that electron transfer occurs within a complex, or complexes, which form between these two proteins. The kinetics of electron transfer between the Az and cyt c-551 from Pseudomonas was studied using rapid mixing methods. It was shown that the reaction in both directions is fast; but depended on reagent concentrations (Antonini et al., 1970).
In 1975 the kinetics of electron transfer between Pseudomonas cyt c oxidase and the Pseudomonas Az was reported (Brunori et al., 1975). It was shown that electron transfer between Az and Pseudomonas cyt oxidase is rate limited in two directions, suggesting the formation of a molecular complex within which electron transfer takes place. Besides, a slower process which was attributed to internal electron transfer between the heme c and heme d moieties of the Pseudomonas cyt c oxidase was observed. It was also shown that reduced Az existed in two stable forms, of which, only one was capable of exchanging electrons with the Pseudomonas cyt oxidase. Also, it was noticed that the internal electron transfer with in the molecular complex of Az and Pseudomonas cyt oxidase because rate limiting at high reagent concentrations.

Using the stopped flow technique, oxidized Az was mixed with reduced cyt c. The experiment resulted in monophasic progress curves, which corresponded spectrally to the production of oxidized cyt c. The rate of the process was shown to be linearly dependent on the Az concentration (Wilson et al., 1975).

Investigations on the electron transfer between Pseudomonas cyt c-551 and Az have resulted in the formulation of a kinetic model requiring that the reduced Az molecule should exist in two forms, but just one of them is capable of electron transfer. Similarly, temperature-jump studies on the Az-Pseudomonas cyt oxidase reaction (Brunori et al., 1975; Parr et al., 1977) have reinforced this hypothesis and also indicate that electron transfer occurs within a molecular complex of the two proteins (Parr et al., 1977).

In 1982, the electron exchange between cyt c-551 and chromium-labeled Az by temperature-jump chemical relaxation measurements was reported (Farver et al., 1982) which demonstrated that the same reaction mechanism occurs as the one that takes place between native Az and cyt c-551. But Az physiological cycle has another aspect that deals with electron transfer to cyt c oxidase. After several investigations, it was recommended that functional sites for electron transfer to cyt c-551 and cyt oxidase are different. The site that is involved in electron exchange with cyt c-551 engages His-35 and the other one which transfers electron to
cyt oxidase, possibly involves His-117 in the hydrophobic northern end of the protein (Farver et al., 1991).

The copper coordination both in reduced and oxidized forms is close to trigonal, that is typical for Cu (I) but quite unusual for Cu (II) complexes (Gray et al., 200). These observations support a suggestion that the rigid protein matrix serves as a rack to compel the copper its coordination geometry, leading to minimal reorganization energy related to the electron transfer (Malmstrom et al., 1994) So, because of high structural similarity of reduced Az to oxidized protein state, the reorganization energy for reduction or oxidation is low. Low reorganization energies promote electron transfer under biological conditions, at low driving forces (Marcus and Sutin 1985).

**Functions**: it has been reported to induce and trigger apoptosis in several human cancer cells selectively. These findings in vitro have been confirmed in nude mice bearing tumour xenograft in vivo.

**Anticancer Action**:

1. **Azurin entry into Mammalian cells**: Az entry into mammalian cells has been the subject of several studies. It is established that the ability of Az to enter cells varies depending on the cell types. Az is able to enter both J774 (a murine macrophages cell line established from a tumour that arose in a female BALB/c mouse) and human cancer cells. While Az in Az-treated mouse, induced apoptosis in vivo in the tumour cells in nude mice (a genetic mutant that has a deteriorated or removed thymus gland, resulting in an inhibited immune system), it was much less cytotoxic towards normal tissues (Yamada et al., 2005)

   Interestingly, the cytotoxic activity of Az towards normal breast MCF -10F cells (non-tumourigenic epithelial cell line) was different from MCF-7 cells (Human breast adeno carcinoma cell line) (Punj et al., 2004). Thus, a group of investigators compared Az entry into such cells (Yamada et al., 2005), in order to find a way to explain this difference. Az showed more internalization in human breast cancer MCF-7 cells comparing to normal
mammary epithelial MCF-10F or MCF 10A1 cells. It was also important to determine if a cupredoxin such as Az can enter normal peritoneal macrophages or mast cells. It should be noted that J774 cells are not normal macrophages. They actually are ascites forms of murine reticulum cell sarcoma. Interestingly, Az has shown preferential internalization in J774 than the corresponding normal cells like primary peritoneal macrophages and mast cells. This might explain the low level of apoptosis during in vitro treatment of the latter cells.

To see if the cellular entry is the major constraint in the ability of Az to induce apoptosis in normal cells, Az was microinjected in fibroblasts and MCF-10F cells and apoptosis induction was determined. Significant nuclear DNA fragmentation and condensation were observed after five hours but not during the 30 min incubation with Az, showing that Az is able to induce apoptosis once inside the normal cells (Yamada et al., 2005). This will suggest that with nanotechnology targeting to deliver Az into cancerous cells, we may develop an effective strategy for cancer treatment.

Lately, it has been shown that amino acids 50 to 67 of Az (p18) are responsible for selective entry of Az into human cancer cells (Taylor et al., 2009). After internalization, the peptides 50 to 77 (p28) are revealed to induce a cytostatic mechanism to inhibit cancer cell proliferation. Accordingly, these peptides are considered essential in the nanotechnology trials intending to eradicate malignant cells using Az. Interestingly, it is reported that Az penetration into cancer cells does not lead to membrane disruption.

2. Mechanism of azurin cytotoxicity: The story began in the year 2000, when it was reported (Zaborina et al., 2000) that cyt c-551 and Az from \textit{P.aeruginosa} induced apoptosis in macrophage cells. It was also demonstrated that a kind of unknown cytotoxic factor triggered the proteolytic conversion of procaspase-3 to active caspase-3 in an ATP-independent manner. Amusingly, two redox proteins, cyt c-551 and Az, were identified in the cytotoxic preparation.

Identifying the mechanism underlying Az cytotoxicity is a fundamental concern before we can utilize it as an apoptosis inducing factor. It may also explain its unequal behavior.
facing different cell types. Therefore, in recent years, Az mode of cytotoxicity has been studied in association with some apoptosis regulating genes like caspases, bax and P53. It is demonstrated (Yamda et al., 2002) that Az forms a complex with the tumour-suppressor protein p53, generates reactive oxygen species (ROS), and induces apoptosis in macrophages. To see if treatment with Az-cyt c-551 might change the intracellular level of p53, macrophages were treated with Az and cyt c-551 for 0, 3, 6 and 12 hours and the level of p53 was determined in the extracts of the treated macrophages. The level of p53 was significantly increased when the macrophages were treated with the redox proteins for 12 hrs. To see the subcellular localization of both Az-cyt c 551, the macrophage cell extract was fractionated to obtain cytosolic, mitochondrial and nuclear fractions. The level of p53 and Az were then determined in such fractions (for the details of the process see (Yamada et al., 2002)). P53 level rose steadily in the cytosol and in the nuclear fractions during 1 hr-period, but little p53 was observed in the mitochondria.

On the contrary, the Bax (an apoptosis promoter protein) level increased significantly in the mitochondria, particularly during 6 to 12 hours after treating macrophages with Az and cyt c-551. A steady increase of cytochrome c in the cytosol was observed during the 12-hour period, suggesting a possible cyt c release from the mitochondria to the cytosol. Overall, Az-cyt c-551 treatment of the macrophages resulted in accumulation of p53 in the cytosolic and nuclear fractions, but the Bax level increased mostly in the cytosolic and mitochondrial fractions. Az was found to be located within the macrophage cells in the cytosol and the nuclear fractions. No Az was found in Mitochondria.

To confirm that macrophage cell death, triggered by Az and cyt c-551, is due to induction of apoptosis, investigators (Yamada et al., 2002) incubated macrophages overnight either with phosphate buffered saline (counted as untreated samples) or with a mixture of Az and cyt c-551 (treated samples) and then measured caspase 3 and 9 (two proteins of caspase family of proteins which act as central mediators of apoptosis, ) activities. The results proved that macrophages treatment with Az-cyt c-551 resulted in
significant activation of caspase 9 and 3. Activation of these two proteins indicates extensive apoptosis in such cells.

The localization of p53 to mitochondria is postulated to be evocative of the proapoptotic protein Bax. In apoptotic cells, the cytosolic Bax undertakes a conformational change leading it to be relocalized in the mitochondria (Gross et al., 1998). In addition, the BH3 (borane molecule)-domain-containing protein Bid (an apoptosis promoter protein), during staurosporine-induced apoptosis in HeLa cells, translocates from the cytosol to the mitochondria, leading to a change in the conformation of Bax and resulting in the release of cyt c from mitochondria (Desagher et al., 1999). Using melanoma cell lines, it was shown (Yamada et al., 2002) that the Bax level was low in the cytosol but increased steadily in the mitochondria up to 12 h after treatment of UISO-Mel-2 cells (p53 negative melanoma cell line), where Az showed very little cytotoxicity, very little enhancement of Bax in mitochondria or release of cyt c from mitochondria was observed, suggesting a role of p53 in such a process. Mainly, Az was found in the cytosol, but it was also found in the nuclear fraction. Az was also localized in the mitochondria, but not during the earlier period. In the p53-null UISO-Mel-6 cells, Az was located in the cytosol and in mitochondria, but not in the nucleus, suggesting that p53 may play a role in the nuclear transport of Az was verified. Further observations suggested that Az forms complex with p53 and this complex formation is specific. In addition, it was found that wild-type Az treatment leads to p53 stabilization, thereby raising its intracellular level (Yamada et al., 2002).

Not only the intracellular pathways underlying Az cytotoxicity are considered by the investigators, but also they tried to reveal the association between Az redox activity with its cytotoxic behavior. As it is discussed above Az has two redox centres: the Cu ion coordinating directly to amino acid residues and a disulphide bridge between Cys-26 and Cys-3 residues. The binding of Cu ions to ligand residues such as Cys-112 is essential for the redox activity of Az.
A group of investigators (Goto et al., 2003) inquired whether the oxidoreductase (redox) activity of Az of the involvement of copper is important for Az cytotoxicity in 2003. They isolated apo-Az lacking Cu and designed redox negative site-directed mutants of Az. The redox activity mutants was disturbed. In some of them a cysteine residue (Cys-112) was replaced so that the Cu coordination was altered. In some others, two methionine residues (Met 44 and Met 64) were replaced, resulting in inappropriate interaction of Az with cyt c-551. They demonstrated that, even though the wild-type and the Cys-112 Asp Az-mutant was capable of complex formation with the p53 and could generate high levels of reactive oxygen species (ROS), the redox-negative Met-44 Lys met-64Glu Az-mutant could not form complexes with p53, generated low levels of ROS and was defective in considerable cytotoxic action towards macrophages. Consequently, it was shown that complex formation with p53 and ROS generation, are important in the cytotoxicity of Az towards macrophages.

Another study (Yamada et al., 2002) showed that wild type Az exhibited significantly more cytotoxicity toward UISO-Mel-2 cells, comparing to the redox-negative M44K/M64E Az-mutant protein. This indicates the importance of electron transfer activity of Az in its cytotoxic behavior. Wild type Az had less cytotoxicity toward p53-null UISO-Mel-6 cells, suggesting that the presence of p53 may be important for Az-induced cytotoxicity. Moreover the M44K/M64E double Az-mutant protein failed to form a stable complex with p53 in UISO-Mel-2 cells. Considering its lack of cytotoxicity, it can be concluded that complex formation with p53 may be the primary cause for Az cytotoxic behavior.

A study in 2005 provided an evidence for p53 activation by Az which conquer p53 activation by Az which conquer p53 deactivation by oncogenes. It was found (Apiyo and Wittung-Stafshede 2005) that Az interacts with p53 in a four –to-one stoichiometry. So may protect p53 from degrading enzymes. This can explain the increased intracellular p53 from degrading enzymes. This can explain the increased intracellular p53 levels in the presence of Az. After Az binding, p53 tryptophan fluorescence is suppressed,
indicating that interactions occur in the N-terminal domain (NTD) of p53 (P53 has got 3 tryptophans in NTD that show weak emission). P53 NTD is also the binding site for many oncogenes. MDM2 binding to NTD results in p53 inactivation and subsequent degradation; it is overactive in many tumours. Given that the affinity of Az for p53 is higher than that of MDM2, and the binding sites overlap, it was proposed that by physical displacement of oncogenes binding to NTD, Az might be able to activate p53 in vivo. Computational methods are used lately to simulate P53-Az interaction (Taranta et al., 2009). A high degree of geometric fit was demonstrated between these two proteins which are connected by numerous hydrogen bonds and several hydrophobic interactions. The computational result confirmed that Az binds to p53 NT domain, in the region that MDM2 binds.

Azurin is a multi-targeted anticancer agent that acts through three pathways:

1) Inhibition of cell cycle progression through extracellular binding to Eph receptor tyrosine kinases,

2) Induction of apoptosis through intracellular stabilization of p53 protein and

3) Prevention of angiogenesis through inhibition of VEGFA.

These pathways are independent and together lead to the inhibition of cancer growth.

1. **Inhibition of Cell Cycle progression**: Ephrins are a family of receptor tyrosine kinases that bind to their ephrin ligands generating signals at sites of cell-cell contact controlling cell morphology, adhesion, migration and invasion. In many tumours, Eph and ephrins are over expressed and promote cancer growth and angiogenesis. Ephrins can be divided into two classes: A-type, that are cell membrane-linked and B-type, which possess highly conserved intracellular domains and transmembrane domain (Chaudhari et al., 2007, Pasquale, 2010).
Azurin has structural similarity with ephrinB2, EphB2 ligand, and binds strongly to EphB2, interfering with the ephrin/Eph signaling system. This interference contributes to the induction of cancer cell death and tumour regression (Chaudhari et al., 2007).

In 2007, Chaudhari et al showed that azurin and plastocyanin, other cupredoxin, have a remarkable structural similarity with the extracellular domains of some ephrins. Surface plasmon resonance (SPR) analysis showed that azurin has the highest affinity to EphB2, followed by EphA6, EphA4 and A7. The azurin derived peptide Azu 88-113 showed similar affinities and demonstrated to interfere in EphrinB2 signaling pathway by competing with ephrinB2 on binding to the tyrosine kinase.

2. Induction of Apoptosis: Yamada et al., demonstrated that azurin and more specifically p28, can enter cancer cells and induce p53 mediated apoptosis in murine J774, human breast cancer melanoma and osteosarcoma but not in normal liver cells or p53-negative osteosarcoma cells (Yamada et al., 2005; Yamada et al., 2009).

The azurin entry into cancer cells is mediated by its protein transduction domain (PTD), p18 through a caveolin-mediated pathway. Caveolins (caveolin-1, caveolin-2 and caveloin-3) are membrane proteins that act as regulators of signal transduction and are present in a subset of lipid raft invaginations called caveolae. The increased caveolin-1 expression in cancer cells over normal cells partly explains the preferential entry of azurin into cancer cells. Although, kinetic studies show that this is not the only entry pathway of azurin. (Taylor et al., 2009; Yamda et al., 2005).

After internalization, azurin interacts strongly with the p53 forming a stable complex of four azurins binding to one p53 monomer. This complex stabilizes and shields p53 from degrading enzymes like ubiquitin ligases, increasing its intracellular levels (Apiyo and Wittung-Stafshede, 2005; Yamada et al., 2009). Regulation of p53 expression is mainly done through ubiquitin proteosmal pathways. Because some ubiquitin ligases are overexpressed in some cancers, like breast cancers, p53 levels
are naturally low and its apoptic and cycle arrest action is reduced (Yamada et al., 2009).

In 2004, Punj et al., showed that azurin-induced apoptosis in MCF-7 cells occurs partly because of p53 stabilization and consequent down regulation of bcl2 and up regulation of bax genes. P53 is a transcriptional regulator of Bax and Bcl2, a proapoptotic and antiapoptotic proteins respectively. The down regulation of Bcl2 levels and up regulation of Bax levels triggers Bax translocation to mitochondria and release of cytochrome c that will activate a caspase cascade that induce apoptosis (Punj et al., 2004).

As referred before p28 is an important fragment of azurin that can, by itself, enter cancer cells and induce apoptosis and cell arrest through stabilization of p53 tumour suppressor, p28 is also able to inhibit angiogenesis (Mehta et al., 2011).

In 2009, Yamada et al., studied the mode of action of p28 in p53-positive breast cancer cells. Confocal microscopy showed that p28 preferentially enter tumourigenic cells (adenocarcinoma MCF-7, ductal T47D and carcinoma ZR75-1) comparing to non-tumourigenic cells (epithelia MCF-10A cells). The induction of MCF-7 and MCF-10A cells with caveolae disrupting agents (methyl-β-cyclodextrin and filipin) and microtubule disrupting agents (nocodazole) inhibited significantly p28 entry, confirming the involvement of a caveolae-mediated endocytosis in p28 penetration. Clathrin-mediated endocytosis inhibitors (chlorpromazine, amiloride and sodium azide) did not affect p28 entry. MCF-7 cells were more sensitive than MCF-10A cells to caveolae disrupting agents, revealing that MCF-7 cells may have more entry sites at cell surface. Incubation of MCF-7 and ZR-75-1 cells in medium supplemented with p28 showed that p28 has a time and dose dependent anti proliferative effect. 30 days treatment of MCF-7 tumour xenograft in athymic mice with intraperitoneal injection of p28 significantly inhibited tumour growth without reducing the body weight and changing mice behavior.
MCF-7 treatment with p28 elevated p53 intracellular levels. GST-pull down assays using p28, p18 (amino acids 50 to 67) p18b (amino acids 60 to 77) and p12 (amino acids 66 to 77) showed that p28, p18 and p18b pulled down p53, suggesting that the azurin region responsible for binding to p53 is located between azurin amino acids 60 and 67. MCF-7 cells treatment with p28 enhanced the DNA-binding activity of p53. Reverse transcription PCR and studies of p53 ubiquitination in presence of p28, MDM2 (main responsible for p53 ubiquitination) and MG132 (proteasome inhibitor) showed that p53 increased levels due to p28 presence result from post transcriptional stabilization of p53 by p28 and that, although p28 does not compete with MDM2, it inhibits other ubiquitination and proteasomal pathways. Therefore, p28 stabilizes p53 decreasing its ubiquitination and proteasomal degradation. Computational analysis and western blotting assays with specific antibodies for different regions of p53 suggest that p28 binds to p53 in its DNA binding domain (DBD), between amino acids 80 and 276.

To evaluate the extent of p28 treatment effect in the cellular cycle and apoptosis, levels of some proteins transcriptionally regulated by p53 (p21 and p27) were analyzed in treated MCF-7, and MDD2 cells. In the case of MCF-7 cells, p21 (inhibitor of CDK activity) levels were elevated, inhibiting cell cycle progression through G2-M phase. P21 binds to cyclin A, inhibiting CDK2 (Cell cycle progression at G1 regulator) and induces cdc2 (cell cycle progression at G2-M regulator) inactivation through phosphorylation. Therefore, the levels of the natural activator of cdc2, cyclin B1, are elevated in p28 treated cells and levels of CDK2 and cyclin A are reduced over time of p28 exposure. P27 levels were also elevated. In MDD2 cells p27 was not detectable and p21 levels were not altered, as well as the levels of the analyzed cyclins and CDKs. (Yamada et al., 2009).

CDG Therapeutics inc, an American biotechnology company, is developing a drug consisting in the synthetic p28 peptide for the treatment of p53 –positive cancer.
Several patents have been issued on this matter and at the date p28 ended Phase I clinical trials (CDG, 2011).

3. **Inhibition of Angiogenesis**: Mehta et al., very recently showed that p28 preferentially enter endothelial cells and inhibit angiogenesis and tumour growth (Mehta et al., 2011).

VEGFA is an angiogenic factor that activates ECM (Extra cellular matrix) proteins that induce quiescent endothelial cells to form a tube-like structure. The angiogenic VEGFA effect is essentially mediated by one of its receptors, VEGFR-2. This binding activates several proteins like FAK (focal adhesion kinase) that are involved in cell motility processes. P28 co-localizes with caveolin-1 and VEGFR-2 and inhibits VEGFR-2 activity. (Mehta et al., 2011).

P28 possesses anti angiogenic properties by interfering with different VEGFR-2 and VEGFA target proteins and pathways like F-actin, a stress fiber that is related to cell motility and migratory ability; FAK, a non-receptor protein tyrosine kinase associated with supra molecular focal adhesion complexes that is important in cell attachment and movement; and PECAM-1 (platelet/endothelial cell adhesion molecule-1), responsible for endothelial cell contact maintenance (Mehta et al., 2011).

Different observations suggest that p28 induces reduction of cell motility, through increasing of cell rigidity and ultimately impeding migration and capillary tube formation, therefore inhibiting angiogenesis (Mehta et al., 2011).

**Azurin as a scaffold protein:**

As mentioned previously, azurin acts as scaffold protein because it mediates different high-affinity interactions with several proteins. These high-affinity interactions are due to azurin’s structural similarity with those proteins, enabling the formation of complexes between azurin and the target proteins (Fialho and Chakrabarty 2010).
Different protein interaction analysis show that azurin is capable of forming stable complexes (KD in the nM range) with the different proteins referred before: p53, EphB2, ICAM-3, DC-SIGN, gp120, MSP-1 and SAG1 (Fialho and Chakrabarty, 2010). Azurin derived peptide of the 88-113 region, Azu 88-113 is involved in azurin’s interaction with EphB2 and with DC-SIGN. This peptide includes the F-G loop, which is the responsible region for the complex formation with EphB2 (Fialho and Chakrabarty, 2010; Fialho et al., 2008).

Azurin interaction region with p53 was identified as being its hydrophobic region, which includes the amino acids Met-44 and Met-64 (Yamada et al., 2002a). De Grandis et al., proposed that the hydrophobic region of azurin bind to the p53’s DNA binding domain (DBD) involving the L1 and s7-s8 loops (De Grandis et al., 2007).

The different regions involved in azurin binding to EphB2 and p53 strongly suggests that different regions of azurin are specialized in interacting with different proteins, i.e., particular domains of azurin bind to particular proteins (Fialho et al., 2008).

**Azurin derived peptides as therapeutic agents:**

As referred before, the synthetic p28 peptide ended Phase I clinical trials and acts as an anticancer agent against p53 positive cancer (Jia et al., 2010). Another study is being conducted using other azurin derived peptides as a targeted delivery system of radiosensitizers (Micewicz et al., 2011).

Based on azurin affinity with Ephs, Micewitz et al. studied the development of azurin derived peptides as targeted delivery systems of nicotinamide, a potent radiosensitizer currently used in cancer treatment (Micewicz et al., 2011). As previously said, azurin region responsible for binding with Ephs is the 96-113 region (Chaudhari et al., 2007). The developed peptides were based on the C-terminal region of azurin and were modified to increase their stability. These modifications include the addition of D- and other unusual aminoacids.
Nicotinamide was conjugated in the N-terminal of the most stable and binding effective peptide. The final product was a linear peptide named AzV36-NicL. Because some types of cancer (lung, breast and prostate cancers) overexpress Ephs at the cell surface, conjugating a radiosensitizer to a peptide with affinity to these receptors will target the radiosensitizer to cancer cells. Having a targeted way of delivering the radiosensitizer may increase the radiotherapy efficacy and reduce the required dose of radiation. (Micewicz et al., 2011).

In vitro and in vivo studies showed that using this peptide-based-radiosensitizer-targeting strategy conjugated with radiotherapy increases the efficacy of the treatment. This strategy increases radiotherapy efficacy about ~13-fold in a artificial metastasis mouse model and delays the tumour growth in about 2.5 days in solid tumour engraftment mouse model. Other invitro studies also showed that this peptide has low cytotoxic effect in the in vivo models and is very stable in human serum. (Micewicz et al., 2011).

Altogether, these observations show that this azurin derived peptide has promising therapeutic properties and that the study of other azurin derived peptides can open new doors in cancer treatment whether using azurin anticancer activity or azurin high affinity targeting.

Action of Azurin on Mammalian cells:

Bacterial pathogens such as Pseudomonas aeruginosa (Tang.H, 1999) and many others (Clark V.L et al., 1994; Salyers, A.A et al., 1994) produce a range of virulence factors that allow the bacteria to escape host defense and cause disease. Some of these virulence factors induce apoptosis in phagocytic cells such as macrophages to subvert the host defense (Monack, D. M et al., 1997; Zychlinsky, A., and P. Sansonetti. 1997.) Very little information is available, however, about the role of any purified bacterial virulence factor in triggering apoptosis in mammalian cells. An important inducer of mammalian cell apoptosis is the tumour suppressor protein p53 (Agarwal, M. L et al., 1998; Schuler, M., and D. R. Green. 2001; Vogelstein, B et al., 2000). A model for p53-induced apoptosis envisaged three critical steps: (i) the transcriptional induction of redoxrelated genes; (ii) the formation of reactive oxygen species (ROS); and (iii) the oxidative degradation of mitochondrial components, culminating in cell
death (Polyak, K et al., 1997). The release of cytochrome c from the mitochondria, resulting in complex formation with Apaf-1 and the activation of caspases such as caspase 9 (Adrain, C., and S. J. Martin. 2001), is a critical process in mammalian cell apoptosis, and p53-mediated apoptosis is known to involve caspase cascade activation (Schuler, M., et al., 2000; Schuler, M., and D. R. Green. 2001; Soengas, M. S, et al., 1999).

However, the level of p53 is quite low in normal mammalian cells due to its short half-life of a few minutes, primarily due to its degradation through the ubiquitin proteasome pathway (Maki, C. G., et al., 1996). Stabilization and consequent higher levels of intracellular p53 can be achieved either through DNA-damaging agents such as UV radiation, which inhibits p53 ubiquitination (Maki, C. G., and P. M. Howley. 1997.) or through complex formation with specific proteins such as simian virus 40 T antigen (Tiemann, F., J. Zerrahn, and W. Deppert. 1995) or with mammalian redox proteins such as NADH quinone oxidoreductase, oxidoreductase 1 (NQO1), which may act through accumulation of intracellular NAD - (Asher, G., et al., 2001) In addition to stabilization of p53 by NQO1, which inhibits its degradation, redox proteins may contribute to the activation of p53 by stimulating its DNA-binding activity as a transcriptional activator. For example, the oxidized form of p53, which is inactive for DNA binding, is greatly stimulated in its DNA-binding activity in the presence of a dual-function redox and repair protein, Ref-1, particularly in the presence of reducing agents (Jayaraman, L et al., 1997). Since p53 transcriptionally regulates the level of pro-apoptotic proteins such as Bax, NOXA, and P53AIP1 (Oda, E et al., 2000, Reed, J. C. 1999), mammalian redox proteins such as Ref-1 play an important role in p53-mediated induction of apoptosis by enhancing the transcription of pro-apoptotic genes in mammalian cells (Gaiddon, C. et al., 1999). Redox proteins such as azurin and cytochrome c551 are involved in electron transfer during denitrification in P. aeruginosa (van de Kamp, M., et al., 1990). A great deal is known about the structure of these proteins, and many site-directed mutants are available (Cutruzzola, F. et al., 2002; Farver, O. et al., 2000; van Pouderoyen, G. et al., 1997). Azurin is a type I blue copper protein with a molecular mass of 14 kDa, while cytochrome c551 (9 kDa) is a haem-containing cytochrome.
Azurin possesses a relatively large hydrophobic patch close to the active site, and two residues in this hydrophobic patch, Met-44 and Met-64, are believed to be involved in its interaction with the redox partners cytochrome c551 and nitrite reductase (van Pouderoyen, G. et al., 1997). Similarly, amino acid residues such as His-46 and His-117 are important for electron transfer, since the site-directed mutants His-46Gly (H46G) and His-117Gly have only 34 and 15% of the activity of the wild-type azurin (Farver, O. et al., 2000). The double mutant Met-44Lys/Met-64Glu (M44K/M64E) is deficient in binding to its redox partners and demonstrates 3.4 and 3.3% of the electron transfer activity of wild-type azurin towards cytochrome c551 and nitrite reductase, respectively (Farver, O. et al., 2000). Although a great deal is known about these two redox proteins with regard to their electron transfer activity, nothing is known about any cytotoxicity of these two proteins towards phagocytic or other mammalian cells. It is demonstrated that purified azurin and cytochrome c551 from \textit{P. aeruginosa} exhibit cytotoxicity towards macrophages.

Additionally azurin forms a complex with the tumour suppressor protein p53, generates reactive oxygen species (ROS), and induces apoptosis in macrophages. That the cytotoxicity is due to azurin and not due to contaminating cellular constituents such as cell wall lipopolysaccharides is clear from their absence in the purified wild-type or mutant azurin preparations and from the fact that several mutant azurins isolated by the same purification procedure as the wild type demonstrated very low cytotoxicity, even though they might have been contaminated by the same cellular constituents present in the wild type (T.Yamada et al., 2002).

**P53 Stabilization in Breast Cancer:**

Among cancer incidence breast cancer comprises 10.4% among women than men alarmingly high. With such intense spread, treatment for breast cancer is indispensable. Since azurin has been reported to be a potential anticancer protein against breast cancer cell lines (Goto M, Yamada T, Kimara K, Horner J, Newcomb M, et al., 2003), researchers are searching for novel methods to enhance its production of azurin. Synthesis of a pure microbial
metabolite like azurin from *P. aeruginosa* strain 2453 reduces toxic effects in regression of cancer treatment.

Azurin-induced apoptosis revealed some important evidence of its potent anticancer effect: (1) azurin inhibits proliferation of breast cancer cells and induces cell cycle arrest at sub-G1 phase, (2) Commotion of mitochondrial cytochrome c by triggering p53 and Bax as Punj et al. explained. (Punj V et al., 2004) ROS generation indicates activation of p53, (4) p53 induction modulates the level of expression of apoptosis-inducing proteins like Bcl-2 and Bax, and (5) caspase-3 also induces apoptosis in breast cancer cells treated with azurin. Several studies (Chaudhari et al., 2006; Chaudhari et al., 2007; Yamada et al., 2002) demonstrated that azurin has anticancer, antiparasitic and antiviral activity and that azurin behaves like a scaffold protein, establishing high affinity interactions between different molecules using different regions (Fialho et al., 2008). These characteristics make azurin a good source of therapeutic peptides for Oncology and microbial infections.

Sankar Ramachandran et al, 2011 findings indicate that azurin exerts a significant Anti-proliferative effect on breast cancer cells. DNA content of azurin-treated breast cancer cells was examined using flow-cytometry; the results revealed an elevated cell population accumulating in sub-G0-G1 phase, which denotes induction of apoptosis. ROS generation in cells plays a vital role in both cellular toxicants and signaling molecules (Gotto M. Yamada T. et al., 2004). ROS synthesis in azurin-treated breast cancer cells implies p53 induction for programmed cell death. Tumour suppressor protein p53 is a redox vibrant transcription factor, which eventually decides the cellular response to range of stressed that persuades genomic instability in the cancerous cells. Increased ROS and p53 levels leads to apoptosis in the azurin-treated cells were revealed in our study. Previous studies (Punj V et al., 2004) have indicated that p53 may facilitate apoptosis by triggering genes including BH3, a proapoptotic protein that regulates Bcl-2 proteins. Our study illustrated that azurin-induced apoptosis of breast cancer cells was accompanied by down regulation of Bcl-2 and up regulation of Bax, which are the downstream objectives of p53 (Raffo AJ et al., 2000).
Azurin-treated cells showed elevated caspases-3 levels with due time to evoke apoptosis for programmed cancerous cell death. (Masip S et al., 2003) Finally, it’s confirmed by S. Ramachandran et al. 2011, apoptosis using scanning electron and light microscopes of azurin-treated cells. Treated cells displayed characteristic features of apoptosis, like cell blebbing and membrane shrinkage in SEM. Azurin-induced apoptosis cells in a light microscope showed granule formation, which accumulates exclusively in the perinuclear zone. The DNA fragments and granules localized in the cytoplasm showed an increase in apoptotic induction. (Ramachandran S et al., 2011).

**Oral Cancer:** The use of bacteria in the treatment of cancer has a long and interesting history. The use of live bacteria in this way however has a number of potential problems including toxicity. Purified low molecular weight bacterial proteins have therefore been tested as anticancer agents to avoid such complications. Oral cancer is a widely occurring disease around the world and these lesions are typically very resistant to anticancer agents. In our present study we investigated the effects of purified recombinant azurin from *Pseudomonas aeruginosa* against YD-6 (P-53 positive) human oral squamous carcinoma cells. Azurin showed cytotoxic effects against these cells in a dose dependent manner. The cell death accompanied by this treatment was found to be characterized by chromatin condensation and apoptotic bodies. Azurin treatment was further found to increase the expression of p53. The stabilization of p53 and induction of apoptosis in YD-7 cells by azurin suggests that it has potentially very strong anticancer properties in oral squamous carcinoma. (Uk-Kyu Kim et al., 2010).

Since three decades many efforts have been made focused on understanding how cancer grows and responds to drugs. Based on the dominant drug-development paradigm “one drug, one target,” the two main targeted therapies developed to combat cancer include the use of tyrosine kinase inhibitors and monoclonal antibodies. The limitations are Development of drug resistance and side effects for their use in cancer treatment. Nowadays, a new paradigm for cancer drug discovery is emerging wherein multi-targeted approaches gain ground in cancer therapy. Therefore, it is clear that a new generation of drugs are very much needed to overcome resistance to therapy (Ananda M. Chakrabarty et al., 2014). Regarding
the concept of multi-targeted therapy, Ananda M. Chakrabarty et al., 2014 presented the challenges of using bacterial proteins and peptides as a new generation of effective anti-cancer drugs.

The primary treatment method for any type of cancer involves surgical resection of the tumor(s) followed by radiation and chemotherapy. In some cases where the cancer, particularly pancreatic cancer, has advanced to a stage that precludes surgery, chemotherapy remains the standard of care (Stathis A, Moore MJ. 2010). There are mainly two types of drugs that are used in chemotherapy, both types usually guided by rational or structure based drug design. The first type are the small molecule drugs that target a single or limited number of key steps in cancer progression pathways, thereby significantly slowing down their growth. An advantage of such drugs is that they can often be administered orally. A second group of drugs comprises human and/or mammalian (but humanized) proteins, often monoclonal antibodies (Fialho AM, Chakrabarty AM 2010). A typical example of a structure-guided small molecule drug that inhibits a tyrosine kinase BCR-ABL is imatinib (Gleevec, Novartis) that was approved by the US FDA for the treatment of chronic myelogenous leukemia (CML), a cancer of bone marrow white blood cells triggered by the loss of regulation of a proto-oncogene protein tyrosine kinase. As CML cells became increasingly resistant to imatinib, other inhibitors such as dasatinib, nilotinib or bosutinib were developed and were approved by the US FDA for CML therapy (Lambert GK, et al., 2013).

There are two problems associated with quick resistance development. They are significant toxicity and side effects, while encouraging new drug development to combat resistance. They have encouraged the development of medium to high molecular weight drugs, such as peptides and proteins, with larger number of targets and with a less stringent mode of action. The goal is not just to strongly inhibit a single type of pathway such as cell signaling mediated by the tyrosine kinases, but other pathways as well with less stringent mode of inhibition, so as not to elicit a quick and strong response from the cancer cells for resistance development. Modest inhibition of multiple pathways in the growth progression of cancer cells, while still contributing to significant growth inhibition, may elicit less toxicity-related problems,
thereby addressing the dual problem of drug resistance and toxicity (Avner BS, Fialho AM, Chakrabarty AM. 2012).

CDG Therapeutics has an exclusive licensing agreement for the development and commercialization of cupredoxin-derived peptides. T. Yamada is one of the inventors and potentially receive a share of the royalty received by University of Illinois at Chicago through the licensing of the technology. C.W. Beattie is Chief Scientific Officer of CDG Therapeutics. All terms of the sponsored research agreements are managed by University of Illinois at Chicago in accordance with its conflict of interest management policies. T.K. Das Gupta is a cofounder of CDG Therapeutics and shareholder in it.

A major problem with the current approved therapeutic peptides is that none of them target intracellular proteins, thus limiting their usefulness (Milletti F. 2012), particularly in cancer therapy. Even though extracellular domains of receptor tyrosine kinases or similar signaling molecules promote cancer growth, many of the key components such as tumor suppressors or signaling proteins involved in cancer growth regulation are intracellular.

So one key need for peptide therapeutic development for cancer therapy is the development of cell-penetrating peptides (CPP) that can cross the cellular membrane to modulate key intracellular proteins involved in cancer growth regulation. Well-known CP peptides have mostly been derived from heparin-, RNA- or DNA-binding proteins, antimicrobial or viral proteins as well as various natural proteins, a few of which are currently in clinical trial. Two such peptides which are azurin derivatives, a helical peptide with a stretch of hydrophobic amino acids, termed p18, and an extended form of p18 with 10 additional amino acids, termed p28, have the unique capacity to enter preferentially to cancer cells but not the corresponding normal cells (Taylor BN et al., 2009).

P28 is a part of the bacterial protein azurin which not only enters preferentially to cancer cells but demonstrates strong anticancer activity as well (Fig.7 Fialho AM, Chakrabarty AM 2010). Once internalized in cancer cells, p18 (azurin amino acids 50–67) has the protein transduction domain (PTD) but very little anticancer activity. Thus fluorescently-labeled p18 can...
be a good diagnostic marker to locate tumors inside the body where it accumulates because of selected entry (other than the kidney and/or liver). P28 (azurin 50–77), on the other hand, has not only preferential entry to the cancer cells but it forms a complex within the p53 DNA binding domain, inhibiting its ubiquitination and proteasomal degradation via an HDM-2 independent pathway (Bizzarri AR et al., 2011). Such inhibition of p53 degradation raises the intracellular levels of this tumor suppressor, inducing apoptosis and cell cycle arrest in breast and other cancer cells. (Yamada T, Mehta RR, et al., 2009, Fialho AM, Chakrabarty AM 2010).

Stabilization of p53 is not the only mode of action of the cell-penetrating peptide p28. In HUVEC cell model, p28 has been shown to inhibit angiogenesis in tumor cells by inhibiting phosphorylation of VEGFR-2, FAK, and AKT, and thus affecting tumor cell growth by inhibiting multiple independent pathways (Fig. 7).

Apart from inhibition of tumor cell growth and induction of apoptosis in tumor cells because of its preferential entry and binding with intracellular p53 and angiogenesis-inducing proteins, p28 is also involved in preventing cancer induction in normal mouse ductal or alveolar mammary cells, when such cells were exposed to a potent carcinogen 7,12-dimethyl-benzanthracene (DMBA). Treatment with DMBA allows induction of pre-cancerous lesions in the growing normal cells, and such lesion formation was shown to be inhibited by increasing concentrations of p28, as well as azurin, up to 70–75%, demonstrating both therapeutic and cancer preventive activity of p28 (Mehta RR et al 2010, Richards JM et al 2011, Fialho AM, Chakrabarty AM. 2012).

Does p28 work in cancer patients? This question was addressed in phase I clinical trials in Chicago with 15 stage IV cancer patients. When given as an intravenous bolus in 5 escalating doses in 15 stage IV cancer patients with refractory metastatic solid tumors (7 melanoma, 4 colon, 2 sarcoma, 1 pancreatic, and 1 prostate) 3 times per week over a 4 week period with a two week break before the next, very little immunogenicity or toxicity was observed. Of the 15 patients with an average life expectancy of less than 6 months, and who had solid tumors resistant to conventional drugs, 2 patients showed partial regression while 2 patients showed complete regression of their tumors at the end of the trial (Richards JM et al., 2011) ( Further
follow up for another year and half after the termination of the trial demonstrated 3 patients with a partial response and one patient with a complete response for 139 weeks were reported. Three patients were reported alive at 158, 140, and 110 weeks post therapy completion, demonstrating a clear anticancer activity without significant toxicity symptoms in stage IV cancer patients with drug-resistant tumors who had an average life expectancy of 26 weeks or less (Warso MA et al., 2013).

The above diagram depicts (Fig. 4) Bacterial proteins with anticancer properties. The anticancer activity of azurin, the primary focus of this article, resides, at least in part, within an extended 28-residues α helix termed p28. Full azurin as well as the p28 peptide induce apoptosis and impair angiogenesis through multiple mechanisms. The phase I clinical study indicates that p28 peptide is safe and should be further considered as a promising anticancer therapeutic peptide.
AZURIN AND NANOMEDICINE:

It goes without saying that advances in biological and engineering sciences have contributed greatly to the advances made in our quality of life and wellbeing. Molecular processes are the basis of modern biology and engineering. In the 20\textsuperscript{th} century, engineering approaches to medicine started its alteration from a merely rational science to a scientific technology with fully molecular basis. At first, antibiotics which meddled with pathogens at the molecular level were engineered and introduced. Then, the ongoing developments in genomics, proteomics, bioengineering, bioinformatics and now nanobiotechnology have been providing precise knowledge of the functions of the different organs and mechanisms underlying infections and diseases at the molecular level (Vaziri et al., 2006). In other words, understanding of life advanced from understanding the functions of organs and tissues to the functions of cells and finally molecules as well as nanoscale systems.

Nanotechnology developments were initiated during the last decade of the 20\textsuperscript{th} century when we had already achieved profound molecular awareness about the living systems in general and human body in particular. Generally, nanotechnology is the science of dealing with molecular scale systems and matters (Mansoori 2005). Such systems and matters being worked on through nanotechnology have the following three important features:

1. They have at least one of their three dimensions in between 1 to 200 nm
2. There are some techniques with control on the physical and chemical characteristics of structures in molecular scale.
3. They are able to be assembled together to generate larger structures (Ramezani and Mansoori 2007)

In addition, these features are applicable to all natural and microbiological systems including biosystems such as cells, bacteria, enzymes and viruses.

The simultaneity between the launch of nanotechnology development and our molecular awareness of living systems has resulted in introducing a molecular-based technological medicine in which the molecular basis of life is manipulated to construct specific desired results through nanotechnology methods and devices. In other words,
the knowledge of human molecular structure is used for missions of cellular inspection, repair and reconstruction. Therefore, the 21st century treatment through nano medicine is expected to entail the proliferation of efficacious therapeutic molecular tools to establish and maintain a continuous state-of-health for humans. Generally, the focus of the nanotechnology therapeutic approaches has been on early disease detection, drug discovery and monitoring, controlled release of therapeutic agents, and targeted drug delivery. Targeted drug delivery is being more researched and it is especially fundamental for reaching stronger therapeutic effects with lower side effects.

Azurin- A novel molecule for cancer treatment through nanotechnology:
Az has gained much attention due to its apoptosis induction activity with evidences supporting P53 involvement in the mechanism of its cytotoxic effect (Yamada et al; 2002). Investigations on Az properties and nanotechnology approaches indicate that we may use nanotechnology targeting and delivery systems to eradicate tumour cells using Az. Recently, several patents are released which take the advantage of Az in combination with other cytotoxic agents in cancer diagnosis and treatment (Das Gupta et al., 2008; Das Gupta and Chakrabarty 2008; Chakrabarty et al., 2008). In what follows we report investigations, which are in support of the use of Az in nanotechnology treatment of melanoma, breast cancer, bone cancer and brain tumour cells. In all such in vitro investigations it is shown that Az, when delivered to the cancerous cells, can kill cancer cells. To achieve the same results in vivo we may utilize nanotechnology approaches discussed below for Az targeting and delivery to cancerous cells. The success of in vivo cancer therapy by Az relies basically, however, on delivering Az to target organs.

Melanoma cell treatment:
It is shown by Yamada et al., 2002 that Az was shown to be internalized in UIISO-Mel-2 cells (like macrophages) and was localized mainly in the cytosol and in the nuclear fraction. Meanwhile, the level of Bax (B-cell leukemia/lymphoma associated X protein)
which promotes apoptosis was also increased in mitochondria, and led to significant release of mitochondrial cyt c into cytosol, consequently initiated the apoptosis.

To achieve this, UISO-Mel-2 cells were incubated with Az for twelve hours, and the amount of residual p53 was determined in the cell extracts for the next two hours. Very little p53 remained 2 hours after cycloheximide addition (Cycloheximide was added to the cell suspensions to stop protein synthesis) in the extracts of untreated control or Az-mutant –treated UISO-Mel-2 cells. In contrast, substantial p53 was still present in the extracts of wild-type Az-treated cells. Therefore, it can be concluded that wild type Az treatment leads to p53 stabilization, there by raising its intracellular level. The Az-mutant, which was deficient in complex forming with p53, was also deficient in stabilizing p53 in UISO-Mel-2 cells. Given that Az exerts cytotoxicity to human melanoma UISO-Mel-2 cells in vitro, experiments were continued with the study of the effect of flanks of nude (athymic) mice and when small tumours appeared, the animals were divided into Az-treated mice received 0.5 mg wild type Az (intraperitoneal) daily for 22 days. Az treated mice demonstrated tumour growth inhibition comparing to untreated control group. Finally, the mean tumour volume in Az-treated mice was 59% lower than that in Az-untreated mice.

It is known that metastatic melanoma can be one of the most difficult forms of cancer to treat. Considering the above mentioned findings and using appropriate nanotargeting and nanodelivery techniques we may be able use Az for nanotreatment of melanoma. However, a nanotechnology drug delivery method for melanoma treatment is already developed (Lesinski et al., 2005). Novel nanochannel delivery system was used to directly deliver IFN-α (an antitumour agent) to the tumour microenvironment. This nanochannel system eliminates the toxicity of systemic drug administration. By taking advantage of this method we may be able to deliver and target Az to melanoma cells.

**Breast Cancer cell treatment:**

The effect of Az on breast cancer is investigated and it is shown that Az is significantly cytotoxic to the MCF-7 cell line (Human breast adenocarcinoma cell line) and
interestingly less cytotoxic toward p53-negative breast cancer cell line (MDA-MB-157) or cell lines with non-functional p53 such as MDD2 and MDA-MB-231 (Punj et al 2004). Like in melanoma cells, Az enters into the cytosol of MCF-7 cells and moves to the nucleus, enhancing the intracellular levels of p53 and Bax, and triggering the release of mitochondrial cyt c into the cytosol. This process turned on the caspase pathway (including caspase-9 and caspase-7), so it initiated the apoptotic process. In order to examine the role of Az in vivo, a nude mouse model with xenotransplanted MCF-7 cells was utilized. Athymic mice were treated daily with 1 mg Az for 28 days, and then compared with control animals. The data indicated that there was significant difference in tumour growth rates between Az treated animals and control animals. The treated animals did not show weight loss or other commonly observed signs of toxicity during the 28 days of treatment. After necropsy, all viscera were histologically examined, and no detectable alterations were found when comparing the viscera of Az-treated animals to those of Az-untreated mice.

Bacterial DNA, particularly the unmethylated CpG dinucleotides, was previously shown to trigger activation of specific Toll-like receptors (TLRs) in immune cells, leading to various cytokine and chemokine production that caused cancer cell death and tumor regression. But, for the first time in 2007, it was reported that pseudomonas aeruginosa, senses the presence of cancer cells and releases a specific protein or extrachromosomal DNA, which inhibits cancer cell growth (Mahfouz et al 2007).

This property of P. aeruginosa was examined in the presence of MCF-7 breast cancer cell line. P. aeruginosa strain 8822 was observed to see if it releases genomic DNA in addition to Az in its growth medium. Very little Az was produced in the absence of exposure to MCF-7 cells. The DNA amount was elevated in the presence of the cancer cells; suggestive of enhanced release as is the case with Az. Also, for the first time the release of “CpG-rich DNA harboring the Az gene” from P. aeruginosa was described, that resembled “the Az gene from Neisseria”, demonstrating 95% nucleotide sequence
identity with it. Accordingly, not only this DNA fragment has antitumour activity and is able to activate TLR-9 promoted NF-kB, but also it harbors the Az gene from Neisseria (laz) exerting stronger cytotoxicity (Mahfouz et al., 2007; Gupta et al., 2008).

In order to apply the above mentioned findings for the effective treatment of human breast cancer using Az we can utilize nanotechnology methods of targeting and delivery. For example, the recent development in biodegradable self-assembled nanoparticles for the treatment of breast cancer has opened the doors for the nanoscale drug delivery devices. These nanodevices are able to carry large doses of therapeutic agents or genes near malignant cells and away from healthy tissues (Sinha et al., 2006).

**Bone cancer cell treatment:**

The effect of Az on human osteosarcoma (the most common type of malignant bone cancer) cell lines is already investigated (Yang et al., 2005). It is found that the growth of U2OS cells (Osteosarcoma cell line) is significantly inhibited by Az in a dose dependent manner. Furthermore, U2OS cells showed typical apoptotic morphological features after treatment with Az. This research has indicated that Az induced apoptosis is strongly associated with down-regulation of Bcl-2, up-regulation of Bax and activation of caspase-3.

Recently, nanotechnology came into play when functionalized biodegradable nanomaterials (less than 50nm in size) were formulated to specifically attach in vivo to bone cancer cells to form an implant used to treat bone cancer (Balasundaram and Webster 2006). After attachment, sustained release of the anti-cancer agent (like Az) could then occur at targeted sites. Specifically, inorganic biodegradable nanomaterials (including ceramics like hydroxyapatite or HA) can be functionalized with anti-cancer drugs (such as Az using covalent chemical attachment). The outer coating of the embedded nanoparticle systems will also be created to have different biodegradation rates for the controlled release of anticancer agents to the target site. In this study, the investigators provided the evidence of synthesizing highly degradable nanoamorphous...
calcium phosphate and slowly degradable nanocrystalline HA as drug delivery carriers to treat bone cancer.

**Prostate Cancer Cell treatment:**
Lately, a group of investigators (Chaudhari et al., 2007) demonstrated that Az has structural similarity to a ligand known as ephrinB2 and binds to its receptor EphB2 with high affinity. Signaling through ephrin B2 and EphB2 is known to be involved in cancer progression. They localized a C-terminal domain of Az (Azu 96-113) that shows structural similarity to ephrin B2 at the G-H loop region known to be involved in receptor binding. Then they designed and synthesized a peptide (Azu 96-1130 on the basis of the structural similarity alignment with the high affinity binding G-H loop domain of ephrinB2. Azu 96-113 as well as a GST fusion derivative GST-Azu 88-113 decreased the viability of various human cancer cells.

It is shown that Az and a C-terminal domain Azu 88-113 specifically bind to EphB2, which leads to interference in the autophosphorylation of the tyrosine residue in its kinase domain in the presence of ephrinB2. This process interferes in upstream cell signaling and contributes to cancer cell growth inhibition.

The ability of GST-Azu fusion peptides to interfere with cell growth was tested in breast cancer MCF-7 cells and DU145 prostate cancer cell line. GSTAzu 36-128 or GST-Azu 88-113, containing the Az region capable of interfering in ephrin B2/EphB2 binding, significantly inhibited MCF-7 cell growth in a dose dependent manner. Also DU145 cells were incubated with Az, GST, ephrin B2, and GST-Azu fusion proteins. In contrast to EphB2-negative DU145 cells, Az, GST-Azu 36-128, and GST-Azu 88-113 showed significant growth inhibition in EphB2-positive DU145 cells in a dose dependent manner (Chaudhari et al., 2007).

In application of nanotechnology methods for prostate cancer treatment using Az two approaches are worth mentioning. In one approach a group of investigators (Parti et al., 2004) have recently designed a dendrimer for targeted therapy of prostate cancer. They synthesized J591 anti-PSMA (Prostate Specific membrane antigen) antibody dendrimer conjugatges holding fluorophores on the dendrimer. They showed that in vitro, the
conjugates specifically bind to cells expressing PSMA and were internalized in such cells. Further studies utilizing bioconjugates like antibody-dendrimer-drug with Az as the cytotoxic drugs are suggested to be examined for prostate cancer treatment. Interactions of Az with dendrimers are discussed later in this report.

Simultaneously in another approach for nanotechnology-targeted therapy of prostate cancer another group of investigators designed a bioconjugate composed of controlled release polymer nanoparticles and aptamers (Nucleic acid ligands). They used RNA aptamers that bind to the prostate-specific membrane antigen (PSMA). They confirmed that these bioconjugates could efficiently target and be internalized in a prostate LNCaP epithelial cells, which express the PSMA protein (Farokhzad et al., 2004). Az encapsulated nanoparticles conjugated with aptamers would, likewise, make great therapeutic candidates for many types of cancers.

**Treatment of malignant brain tumours:**

Az had been the subject of some studies involving brain tumour cells and is examined in angiogenesis inhibition (Hong et al., 2006).

For example increasing concentrations of Azu 96-113 synthetic peptide led to the reduced –cell-viability in glioblastoma LN-229 cells (Chaudhari et al., 2007)

Another group of investigators have synthesized a nanodevice named PEBBLE (probes encapsulated by biologically localized embedding) to tackle brain tumours (Kopelman et al., 2005). They have designed nanoparticles with 20 to 200nm diameter that are able to carry a variety of agents on their surface, each with a unique function, One agent (a target molecule) immobilized on the surface is able to direct the PEBBLE to a tumour. Another agent helps to visualize the target using magnetic resonance imaging, while the third agent attached to the PEBBLE could deliver drugs or toxins efficiently nearby cancer cells. These functions can be combined in a single tiny polymer sphere to make a powerful destructive weapon against cancer. Interestingly, PEBBLE has a protective coating polyethylene glycol (PEG) that is able to help it cross the blood-brain barrier. PEBBLE bear photo catalysts which can be stimulated by light and convert “O2” into so-
called “O” singlet state. Which efficiently bleaches and destroys nearby cells. A light source is inserted into the skull through a micrometer-sized fiberoptic probe. Using this technology the PEBBLES are harmless unless the light is turned on. Az internalized in these multifunctional nanodevices would increase efficacy for the treatment of glioblastoma as PEBBLEs have the better chance of passing through the blood brain barrier.

In another, study (Hong et al., 2006) it is demonstrated that although Az is deficient in entering glioblastoma cells, exhibiting low cytotoxicity, an Az paralogue, laz (lipid modified Az) shows a higher level of cytotoxicity and is able to enter glioblastoma cells more efficiently. Neisserial Az , know as laz, has a 39 aminoacid epitope in its N-terminal region which is called H.8epitope. H.8-Paz (P. aeruginosa Az with H.8 epitope in its N-terminal) and Paz-H.8 (P. aeruginosa Az with H.8 in its C-terminal), both had high cytotoxicity for glioblastoma cells and a higher level of internalization. It is also shown that H.8 moiety plays a role in disrupting the entry barrier in brain tumour cells, so facilitating killing of such brain tumours as glioblastomas.

Considering the fact that Az and laz must be delivered to the brain for targeting and delivery to glioblastomas, nanotechnology approaches, which are being developed for drug delivery through blood brain barrier (BBB), need to be employed. These applications are quite remarkable and challenging in respect to diseases of the brain especially for malignant brain tumour and the Alzheimer’s disease (Nazem and Mansoori 2008). The subject of targeted drug delivery, for example, is appreciably complicated for brain due to the additional obstacle of the BBB against the entry of a variety of molecules into the brain tissues. With respect to drug discovery and monitoring, the histological complexity of the brain is a restricting factor. However, the potential capabilities of nanoparticles, and nanodevices, including their controllable size and suspendability (based on modifiability of the nanoparticles outer layer), multifunctionality and remote controlled functionality show promise in overcoming the BBB restrictions. Nevertheless, there are many challenges regarding the biocompatibility
of nanoparticles and nanodevices especially in a complex biological milieu like brain with a huge concentration of cells and intercellular communications (Nazem and Mansoori 2008)

**Leukemia treatment:**

Az and laz are recently examined for their cytotoxic effect on K562 which is a chronic myelogenous leukemia (CML) cell line and HL60, an acute myeloblastic leukemia (AML) cell line (Kwan et al 2009). Az (or laz) with the concentration of 10μM, reduced viability of the mentioned cells lines by more than 90%. It was also shown that these two proteins did not enter normal peripheral blood mononuclear cells (PBMCs), but significant entry of laz and Az in to both K562 and HL 60 cells was reported. Two laz like proteins were cloned with H.8epitope of N-terminal and C-terminal of Az, named H8-Az and Az-H8. These two proteins showed similar or rather higher cytotoxicity than Az, even at lower concentrations (1.0-2.5M) but their cytotoxicity was comparable to laz. H8-A and Az-H8 demonstrated higher level of entry than Az in K562 cells, however comparable to laz. This indicated a role for H.8 epitope in facilitating entry of Az or laz into leukemia cells. Considering the selective entry of Az and laz in malignant cells, these proteins are not known to cause any cytotoxic effect in normal cells.

Furthermore, Az had demonstrated significant cytotoxicity towards ovarian adenocarcinoma cell line SKOV3 while showing little cytotoxicity towards normal ovarian HOSE6-3 cells (Kundu et al., 2009).

Regarding the fact that melanoma, breast cancer, bone cancer, brain tumour leukemia and ovarian cancer cell ines are killed by Az and the existence of nanotechnology approaches for the in vivo drug delivery and targeting to those same cells as discussed above, it is now obvious that Az bears several unique characteristic that make it an interesting molecule in cancer treatment though nanotechnology.