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Prospects for Cancer Nanotechnology Treatment by Azurin

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ABSTRACT

Bacterial redox protein azurin (azurin for short, or Az) belongs to a class of copper-containing proteins known as cupredoxins. We report here our detailed studies and findings on prospects for application of Az for treatment of cancer through various approaches of nanotechnology. We first present the structure and characteristics of Az protein molecule, its electronic properties and its electron transfer capabilities. The details of how Az induces apoptosis including the mechanisms of its entry into mammalian cells and its cytotoxicity are also presented. Past studies on Az for controlling cancer cell treatment are enumerated. They include melanoma cell treatment; breast, bone and prostate cancer cells treatment; and malignant brain tumor and leukemia cells treatment. Then other applications of Az in nanomedicine and in molecular detection are presented. They include: i) the uses Az-specific antibody to detect *Pseudomonas* bacteria in a patient's blood; ii) application of anti-oxidative properties of laz (an Az paralogue) in different biomedical situations that involves oxidative stress; iii) nanoscale liposomal formulation of Az to target tumor-associated macrophages and immune system cells; iv-nano-technology approaches to use Az as inhibitor of parasitic growth. The prospects of facilitating anticancer function of Az in conjunction with various nanoentities including gold nanoparticles, magnetic nanoparticles, dendrimers, folic acid and carbon nanotube are presented in detail. We conclude there are a number of outstanding challenges to be met in using Az for nanotechnology treatment of cancer.

Keywords: apoptosis, Az, bacterial redux protein, blue-copper protein, cancer, chemotherapy, copper-containing protein, cupre-doxin, nanomedicine, nanobiotechnology, nanobioelectronics, *Pseudomonas*

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INTRODUCTION

The term 'cancer' describes a wide range of malignant tumors, which may affect almost every tissue and organ of the body (Mansoori *et al.* 2007). Cancer is a major cause of mortality and morbidity worldwide (Ovesná and Horvá-thová-Kozics 2005). Cancer incidence and mortality have risen greatly throughout the world since records began around the mid 20th century (McCarter *et al.* 2004). In 2003, cancer accounted for 7.1 million deaths annually worldwide with more than 70% of cancers occurring in ages over 65 years. The cancer prevalence keeps on increasing because of the increasing old population. By 2020, there could be 15 million new cases of cancer and 10 million deaths per year in the entire world (Parkin 2001).

Existing cancer treatment methods may be divided into four main categories: surgery, radiation therapy (including photodynamic therapy), chemotherapy (including hormonal therapy and molecularly targeted therapy), and biological therapy (including immunotherapy and gene therapy) (Sausville and Longo 2005). We may use these treatment methods singly or in combinations based on the assessment of the drug toxicity and antitumor efficacy (Humes 2001).

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Invited Review

Cancer and apoptosis

Accumulating evidence suggests that lack of balance between proliferation and apoptosis may cause cancer emergence (Kuttler *et al.* 2002). Suppression of apoptosis hinders many forms of cancer therapeutic strategies, including radiation therapy, cancer immunotherapy, and chemotherapy (Kanwar *et al.* 2001). Elimination of cancer cells by early apoptosis is preferred over other forms of cell growth inhibition. Apoptosis directly leads to tumor regression and reduces risks of selecting more aggressive and/or drug-resistant phenotypes (any observed quality of an organism) that are often responsible for tumor re-growth and treatment failure (Woynarowska and Woynarowski 2001). Apoptosis also plays a critical role in the cytotoxic activity of a wide range of anticancer agents, including chemotherapeutic agents, biological agents and hormones. Defects in apoptosis pathways are associated with drug resistance in many cancers (Kaufmann and Earnshaw 2000).

Mechanisms by which cells undergo programmed cell death through apoptosis are as follows:

1) Generation of signals within the cell which is also known as the intrinsic (mitochondrial, stress induced) pathway; these signals are induced and activated by DNA damage, UV radiation, activation of oncogenes and hypoxia.

2) Triggering death activators which bind to death receptors at the cell surface; this pathway is also called the extrinsic (death receptor) pathway. Death receptors include a group of transmembrane proteins that are represented by tumor necrosis factor receptors (TNF-R), and FAS (Johnstone *et al.* 2002).

P53 tumor-suppressor protein

The p53 (53-kDa protein) tumor-suppressor protein is a transcription factor that regulates the transcriptional rate of several genes known to play a critical role in signal transduction from damaged DNA. The p53 protein senses DNA damage and can halt progression of the cell cycle at the G1 checkpoint. As a transcriptional regulator, it regulates the expression of several genes including Bax (B-cell leukemia/lymphoma associated X protein which promotes apoptosis), the gene p21, and several other genes like peg3, Apaf-1, caspase-9, and PUMA (Vogelstein *et al.* 2000). Moreover, it can suppress several anti-apoptotic genes like Bcl-2. Bcl-2 overexpression activates endogenous antioxidants (e.g., glutathione or superoxide dismutase) and consequently inhibits ROS-mediated apoptosis (Hockenbery *et al.* 1993).

In mammalian cells during induction of apoptosis by DNA-damaging agents such as hypoxic stress, a fraction of P53 rapidly concentrates to the mitochondria of cells. According to a postulate (Schuler and Green 2001), p53 localization in mitochondria causes oxidative damage to start apoptosis. P53 localization in mitochondria occurs besides the nuclear transcriptional activation by P53.

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are superoxide anions, which are both ions and radicals. Actually, they can be very strong radicals, like the hydroxyl radicals, or molecules like hydrogen peroxide and hypochlorite molecules.

Variable effects of ROS on cell death depend on the level of ROS within the cell. High levels of ROS can lead to lipid peroxidation, damage to cellular membranes, inactivation of caspase enzymes, and finally necrotic cell death. On the other hand, low levels of ROS may induce activation of protein kinases and phosphatases, mobilize Ca^{2+} stores, activate or inactivate transcription factors, and lead to apoptotic cell death. Thus, high levels of ROS destroy cells by means of direct damage. Variable effects of ROS on cell death depend on the level of ROS within the cell. On one hand, high levels of ROS may cause cellular membrane damage via lipid peroxidation. It also causes caspase enzymes to be inactivated, and eventually, necrotic cell death occurs. On the other hand, low levels of ROS may induce activation of protein kinases and phosphatases, inactivates or activates transcription factors, mobilizes Ca^{2+} stores, and leads to apoptotic cell death. Thus, while high levels of ROS directly destroy cells, low levels of ROS affect gene expression and intracellular pathways leading to apoptosis (Kannan and Jain 2000).

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 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 defense 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 (Hoitink Carla and Canters 1992) and eradicates the host defense system by encouraging apoptosis in phagocyte 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 λ_{max}= *ca*. 600 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). Azs 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 enzyme (Ambler and Brown 1967). The family of blue copper-proteins 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 a combination of four or more copper ions per molecule, of which one or more are bound to the type-1 site. Their function is to shuttle electrons and to catalyze dioxygen reduction to water (Farver and Pecht 1991). In **Table 1** characteristics of eight well-known copper-containing proteins are presented.

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) as it is shown in **Fig. 1A**.

In 1988 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, His117 Nδ-atom and Cys112 Sγ-atom, and much weaker axial interactions with Met121 Sγ-atom and Gly45 C=O. The Sγ-atom of a methionine holds the axial position

Table 1 Characteristics of few copper containing proteins. For each protein, the type of Cu site, the redox potential and the weight is mentioned in addition to a brief description of its function. Data from Protein Knowledgebase (UniProtKB) website (http://www.uniprot.org/).

Copper metalloproteins	Mw [Da] No. of AAs	Copper site	Subunit	Redox potential	Subcellular location	Function
Azurin *Pseudomonas Aeroginosa*	16008 148	Type 1	Monomer	310 mV	Periplasm	Involves in electron transfer from cytochrome c-551 to cytochrome oxidase.
Plastocyanin *Anabaena variabilis*	11104 105	Type 1	Monomer	390 mV	Periplasm	Involves in electron transfer between P700 and the cytochrome b6-f complex in photosystem I.
Amicyanin *Bradyrhizobium* sp.	11240 104	Type 1	Monomer	220 mV	Periplasm	Electron acceptor from methylamine dehydrogenase. Passes those electrons on either a soluble cytochrome c or to pseudoazurin.
Pseudoazurin *Paracoccus denitrificans*	13337 123	Type 1	Monomer	280 mV	Periplasm	Required for the inactivation of copper-containing nitrite reductase in the presence of oxygen.
Rusticyanin *Thiobacillus ferrooxidans*	16446 155	Type 1	Monomer	680 mV	Peripalsm	Carries electron from cytochrome c552 to the A-type oxidase.
Stellacyanin *Cucumis sativus*	19313 182	Type 1	Monomer	184 mV	Plant cell wall	Exact function is not clearly known, it is suggested that it is involved in oxidative cross-linking reactions to build polymeric material making. up the plant cell wall.
Hemocyanin *Palinurus vulgaris*	75674 657	Type 3 (Dicopper protein)	At least four very similar subunits	120-190 mV	Secreted, extracellular space	Oxygen carriers occurring freely dissolved in the hemolymph of many mollusks and arthropods.
Ceruloplasmin *Homo sapiens*	122205 1065	Multi copper protein (three type 1, single type 2 and two type 3 copper site)	Monomer	The redox potential of the type 1 Cu sites are 490, 580 and ≈1000 mV	Secreted, extracellular space	Oxidizing iron (II) to iron (III) without releasing radical oxygen species (ferroxidase activity). Involved in iron transport across the cell membrane.

AA: amino acid; Da: Dalton

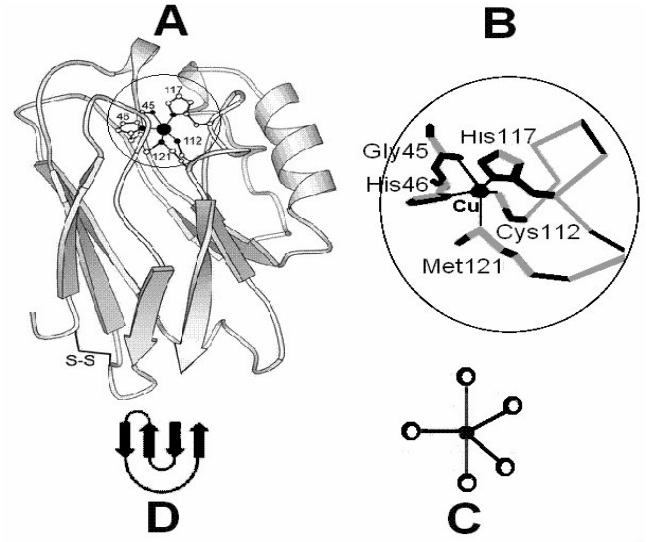


Fig. 1 (**A**) A schematic picture from Azurin molecule. It is composed of one α-helix and two β-sheets, which create a β-barrel motif. Cu ion is under the β-barrel motif. (**B**) The copper ion and the surrounding atoms. The copper ion is coordinated by a Sγ-atom of cysteine and Nδ-atoms of two histidines. The copper coordination is best described as distorted trigonal planar, with strong in-plane bonds to His46 Nδ-atom (nitrogen), His117 Nδ-atom and Cys112 Sγ-atom (sulfur), and much weaker axial interactions with Met121 Sδ-atom and Gly45 C=O. The Sδ-atom of methionine holds the axial position (fourth ligand). (**C**) A trigonal pyramidal geometry around the copper, (**D**) A Greek-key folding motif, is formed by eight β-strands, arranged in two β-sheets opposing each other in a β-sandwich.

(fourth ligand), resulting in a trigonal pyramidal geometry around the copper as it is shown in **Fig. 1B**.

Differently stated, there are three potential binding sites in Az (planar trigonal ligands): two imidazole nitrogen groups of histidines (His117, His46) and a thiolate group of cysteine (Cys112) (Messerschmidt. 1998), which are coordinated somewhat equatorially 2.0-0.22 nm from the copper.

Conformation of the copper ligands of Az is depicted in **Fig. 1B**. Alongside, there are two axial ligands, methionine 121 and glycine 45 occupying the more distant axial positions at 0.30-0.31 nm (Antholine *et al.* 1993), thus, giving the Cu site in Az a trigonal planar or trigonal bipyramidal (3+1+1) form as shown by **Fig. 1C**. 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 (**Fig. 1D**; referring to super-secondary structure of amino-acids sequence 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 backbone carbonyl oxygen (A-CO-B) of a glycine residue [H_2N-CH_2-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 a methionine [O-CO-CH(NH_3)-CH_2-CH_2-S-CH_3] or a glutamine [CO(NH_2)-CH_2-CH_2-CH(NH_2)-COOH]. However, in Az, opposite to the methionine ligand, there is a carbonyl oxygen (O=C) at a distance of ~ 0.21 nm from the copper ion. Additionally, the distance between the copper ion and the axial methionine ligand (~0.318 nm) is relatively longer comparing to other blue-copper proteins (0.28–0.29 nm) (Walter *et al.* 1996). A schematic picture of copper ligands of Az is illustrated in **Fig. 1B**.

Az is also unique in that it contain a disulfide bond [≡C-S-S-C≡] 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 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 amino acids (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).

Az contains a single tryptophan residue (see Glossary) 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 (see Glossary) known for a 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.* 1977).

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). Intra-molecular 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 strong charge-transfer absorption with the 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 (Fuentes *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 (see Glossary). 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°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 (see Glossary) 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 metalloproteins, are generally regarded as the physiological electron donors for *Pseudomonas* nitrite reductase (or *Pseudomonas* oxidase), which is known to react with both O_2 and NO_2- (Silvestrini *et al.* 1982).

Pseudomonas cyt oxidase is a bifunctional enzyme, consisting of two identical subunits each containing one heme c and one heme d_1 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 dioxygen (O_2) to water and the single-electron reduction of nitrite (NO_2-) 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 within the molecular complex of Az and *Pseudomonas* cyt oxidase became 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 in 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.* 2000). 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 (Malmström *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).

Electronic properties of azurin

Az is a protein with capability of electron transfer. The concept of a few atoms or molecules forming electronics circuits has always been very appealing to the semiconductor industry as it may improve the circuit speed and reduce the circuit heat significantly (Joachim *et al.* 2000; Rinaldi and Cingolani 2004). Recently, impressive progress has been made in realizing this concept by using macromolecules (Derycke *et al.* 2001; Postma *et al.* 2001). Also, metalloproteins are of great interest in the design of such circuits because of their special biophysical and biochemical properties. Particularly, Az, due to its electron transfer property, is shown to be applicable in the design of electronic nanodevices (Friis *et al.* 1999; Frascerra *et al.* 2005).

In 1998 high-resolution *in situ* scanning tunneling mic-

roscopy (STM) images of the *P. aeruginosa* Az adsorbed on gold (III) surface was produced (Friis *et al.* 1999). The images illustrated the unique well organized structures of molecular size which showed a brighter contrast in the sub-molecular level that signified a higher tunnel current inside the molecule where the Cu atom is placed. Based on these images, Az was suggested to be a proper candidate for two-dimensional protein adsorption as well as long-range electron transfer. This is in contrast with the crystallographic shape of Az which is smooth and ellipsoidal.

In 2000 Az was first anchored to the gold surface, and after examinations of the Az-Au (III) interface with STM images, it was discovered a closely packed protein monolayer was formed causing individual Az molecules to be resolved (Cavalleri *et al.* 2000). A distribution of bright spots, attributed to the tunneling enhancement around the Cu ion in Az, covers the surface. The gold surface was then coated with self-assembled monolayers of 11-mercaptoundecanoic acid, to facilitate detaching the protein layer from the metal. Atomic force microscopy (AFM), both in tapping mode and contact mode was used to investigate the changes in the sample morphology due to the protein adsorption. This showed a fairly uniform distribution of protein molecules over the surface. A protein diameter of about 20 nm, on average, was measured, due to the tip broadening effect.

In 2002, a biomolecular electron rectifier was implemented (Rinaldi *et al.* 2002) by interconnecting an Az monolayer immobilized on SiO_2 with two gold nanoelectrodes. This work highlights the importance of the orientation of the layers of Az that are deposited on gold substrates. The possibility of depositing oriented layers on gold substrates is made true since the surface disulfide bridge Cys3-Cys26 may be used to bind the protein to gold. Electric current-voltage measurements were performed on, and compared for, two different samples: a sample with randomly oriented Az layers, and another sample with oriented Az layers. It was shown that conductivity of the electron rectifier is significantly improved by orienting layers of Az. Apart from that, both setups of oriented and non-oriented Az exhibit the rectifying property: they allow no electric current for negative input voltages.

In 2004 the possibility of using Az as a "molecular wire" which promotes a strong coupling between the redox centers of two proteins or a protein and an electrode was explored (Biasco *et al.* 2004). By interconnecting an Az monolayer immobilized on SiO_2 with two gold nanoelectrodes, a biomolecular field effect transistor (FET) in the solid state was then constructed. The redox protein Az controlled the flow of electrons from the source to drain electrode by the voltage applied across the gate (silver back electrode) and source electrodes. In 2005 (Alessandrini *et al.* 2005) an electrochemically gated "single-molecule" transistor operating in an aqueous environment, using metalloprotein Az, hosted in a nanometer gap between two electrically biased gold electrodes was constructed.

The investigations presented above were only the fundamental studies in this field and later experiments have been involved in expanding such nanobioelectronical knowledge into technology. The newly emerging field of nanobiolectronics, and more specifically, biomolecular electronics is expected to largely evolve in the near future, due to all the promising experimental results that have been obtained thus far. In a recent advance, redox properties of Az and cytochrome c were utilized to develop a biomemory device capable of charge storage and memory function (Lee *et al*. 2010). Future studies are expected to focus on achieving more stable and efficient nanodevices. From the previously cited literature, one can deduce quite a few major barriers which still need to be further explored, and hopefully overcome soon. While these finding seem to have applications in electronics industry, they may have potential for application in nanobioelectronics approaches for cancer treatment.

How does azurin induce apoptosis?

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 tumor that arose in a female BALB/c mouse) and human cancer cells. While Az in Az-treated mouse, induced apoptosis *in vivo* in the tumor 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-tumorigenic epithelial cell line) was different from MCF-7 cells (Human breast adenocarcinoma 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 *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 (Yamada *et al.* 2002) that Az forms a complex with the tumor-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 signifi-

cantly increased when the macrophages were treated with the redox proteins for 12 hours. To see the subcellular localization of both Az and p53 after treatment of macrophages with Az-cyt c-551, the macrophage cell extract was fractionated to obtain cytosolic, mitochondrial, and nuclear fractions. The levels 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 the 1-hour 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 (PBS) (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, see Glossary) 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 BH_3 (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 positive melanoma cell line) with Az. In the UISO-Mel-6 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. Later the role of p53 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 centers: 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 or 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 in 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-44LysMet-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 significant 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 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 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 tumors. 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.

Intracellular processes involved in Az induced apoptosis are summarized in **Table 2**, based on the type of malignant cell line that was studied. Once we know the exact mechanisms, their application in drug design is feasible and we may predict probable causes of drug resistance or ineffectiveness.

AZURIN AND NANOMEDICINE

Nanomedicine is medicine in the 21st century

It goes without saying that advances in biological and engineering sciences have contributed greatly to the advances made in our quality of life and well being. Molecular processes are the basis of modern biology and engineering. In the 20th 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 molecu-

Table 2 Intracellular processes involved in azurin-induced apoptosis are summarized, based on the type of malignant cell line that was studied in.

Cancer type	Ref.	Cell line	Intracellular processes involved
Melanoma	(a)	UISO-Mel-2 cell line	1) P53 stabilization 2) Increased level of Bax in the mitochondria → release of mitochondrial cytochrome *c* into the cytosol → apoptosis
Breast cancer	(b)	MCF-7 cell line	1) P53 stabilization 2) Increased level of Bax, Bcl-2 expression level was decreased (increase in the ratio of Bax to Bcl2) → translocation of Bax from the cytosol to the mitochondria→ release of mitochondrial cytochrome *c* into the cytosol → activation of caspase-7 and caspase-9 → apoptosis
Bone cancer	(c)	U2OS cell line	1) Down-regulation of Bcl-2 2) Up-regulation of Bax 3) Activation of caspase-3
Prostate cancer	(d)	DU145	Azurin inhibited the ephrinB2-mediated autophosphorlyation of the EphB2 tyrosine residue → interfering in upstream cell signaling → cancer cell growth inhibition.
Brain tumor (glioblastoma)	(d)	LN-229	Azurin inhibited the ephrinB2-mediated autophosphorlyation of the EphB2 tyrosine residue → interfering in upstream cell signaling → cancer cell growth inhibition.
Leukemia	(e)	K562, HL60	Cell cycle arrest in G2/M phase in K562 cells
Ovarian cancer	(f)	SKOV3	Unknown

(a). Yamada *et al*. 2002; (b). Punj *et al.* 2004; (c). Yang *et al.* 2005; (d). Chaudhari *et al.* 2007; (e). Kwan *et al.* 2009; Kundu *et al.* 2009.

lar 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 20th 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 to design biomedically active microscopic devices in the 21st century. These devices will be used for missions of cellular inspection, repair, and reconstruction. Therefore, the 21st century treatment through nanomedicine 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 tumor 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 tumor 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: As was discussed above, Az, a redox protein produced by *P. aeruginosa*, has demonstrated significant cytotoxic activity towards certain cell lines. It is also shown that Az enters melanoma UISO-Mel-2 cells harboring a functional tumor-suppressor protein p53 and induces apoptosis (Yamada *et al.* 2002). Az was shown to be internalized in UISO-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 the 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, thereby 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 Az *in vivo*. UISO-Mel-2 cells were injected into the right flanks of nude (athymic) mice and when small tumors appeared, the animals were divided into Az-treated and Az-untreated groups. Az-treated mice received 0.5 mg wild-type Az (intraperitoneal) daily for 22 days. Az treated mice demonstrated tumor growth inhibition comparing to untreated control group. Finally, the mean tumor 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 to use Az for nanotreatment of melanoma. However, a nanotechnology drug delivery method for melanoma treatment is already developed (Lesinski *et al.* 2005). Novel nanochan-

nel delivery system was used to directly deliver IFN-α (an antitumor agent) to the tumor 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 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 tumor 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 twenty-eight 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 causes 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 "CpGrich 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 antitumor activity and is able to activate TLR9-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 nonodevices 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 (human 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 bioresorbable nanomaterials (less than 50 nm 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 (see Glossary), and binds to its receptor EphB2 with high affinity. Signaling through ephrinB2 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 ephrinB2 at the G-H loop region known to be involved in receptor binding. Then they designed and synthesized a peptide (Azu 96-113) 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 (see Glossary) 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 ephrinB2/EphB2 binding, significantly inhibited MCF-7 cell growth in a dose dependent manner. Also DU145 cells were incubated with Az, GST, ephrinB2, 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 (Patri *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 conjugates 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 drug 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, see Glossary). 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 the 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 tumors: Az has been the subject of some studies involving brain tumor cells and is also examined in angiogenesis inhibition (Hong *et al.* 2006). For example, increasing concentrations of Azu 96-113 synthetic peptide (as presented above) led to 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 tumors (Kopelman *et al.* 2005). They have designed nanoparticles with 20 to 200 nm 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 tumor. 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 all 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 photocatalysts which can be stimulated by light and convert "O_2" oxygen into a 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, known as laz, has a 39 amino acid epitope in its N-terminal region which is called H.8 epitope. 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 tumor cells, so facilitating killing of such brain tumors as glioblastomas.

Considering the fact that Az or 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 tumor 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), multi-functionality 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 cell viability of the mentioned cell 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 into both K562 and HL60 cells was reported. Two laz-like proteins were cloned with H.8 epitope of N- and C-termainal 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-Az 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 has 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 that melanoma, breast cancer, bone cancer, brain tumor, leukemia and ovarian cancer cell lines 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 characteristics that make it an interesting molecule in cancer treatment through nanotechnology.

Other applications of Azurin in nanomedicine and in molecular detection

Here we present a brief description of some other applications of Az nanomolecule in medicine:

- The U.S. National Aeronautics and Space Administration uses Az-specific antibody to detect *Pseudomonas* bacteria in a patient's blood (U. S. Patent No. 5,210, 019). This can be used to rapidly detect early and evolving sepsis (Margalit and Marino 1993). This is in the category of nanoscale lab-on-a-chip. Biological tests measuring the presence or activity of selected substances or microorganisms become quicker, more sensitive and more flexible when certain nanoscale particles (antibodies in this case) are put to work as tags or labels.
- Laz, an Az paralogue, has been recognized in both *Neisseria meningitidis* and *Neisseria gonorrhoeae*. As it was discussed before, laz proteins contain an N-terminal domain of 39 amino acids, encoding the H.8 epitope, which distinguishes them from other Azs. Also they are modified with lipid (Woods *et al.* 1989). Recent studies reported that the neisserial laz mutants are more sensitive to detect H_2O_2 molecule than their parent wild-type strains, suggesting that laz might be important in H_2O_2 stress responses in both *N. meningitidis* and *N. gonorrhoeae*. *N. gonorrhoeae* can survive and replicate inside epithelial cells while infecting the genitourinary tract. The wild-type *N. gonorrhoeae* and laz mutant strains were examined if they they are able to attack and maintain within primary human ectocervical epithelial cells (pex cells). During the assays, the laz mutant strain could not survive inside pex cells as much as the wild-type strain. Thus, the role of laz in the survival of *N. gonorrhoeae* inside pex cells is possibly due to its role in protection against H_2O_2 stress and/or copper storage (Wu *et al.* 2005). To apply anti-oxidative properties of laz in different biomedical situations that involve oxidative stress (infections, autoimmune diseases, malignancies), nanotechnology approaches need to be employed.
- Lately, cupredoxins are being used to inhibit angiogenesis in mammalian cells, tissues, animals, and particularly the angiogenesis that relates to tumor development in humans. The compositions comprising the cupredoxin(s), and/or peptides that are derivatives, variants or structural equivalents of cupredoxins can potentially be used to treat any pathological condition that inappropriate angiogenesis involves in it as a cause, specially inappropriate angiogenesis related to tumor development (Mehta *et al.* 2007).
- By nanoencapsulation of Az and other cupredoxins we can develop a nanoscale liposomal formulation of

the drug to target tumor-associated macrophages and immune system cells that actually promote tumor growth and angiogenesis. For example, a drug now is used for a similar application using this nanotechnology approach (Zeisberger *et al.* 2006). Clodronate encapsulated in liposomes was able to effectively inhibit tumor growth and its blood vessel density by targeting phagocytic cells in the murine F9 teratocarcinoma and human A673 rhabdomyosarcoma mouse tumour models.

o In a study in 2006 it was reported (Chaudhari *et al.* 2006) that Az binds to several envelope- or surface-proteins of parasites and viruses such as *P. falciparum* or HIV-1. Specific interactions of the *Plasmodium falciparum* merozoite surface proteins (PfMSP1-19 and PfMSP1-42 proteins) with Az, H.8-Az (Az with the H.8 epitope in the N-terminal) and laz were discovered. All the latter three proteins showed significant inhibition of parasitemia at relatively high concentrations (about 50 μM) in a dose-dependent manner. While these studies were performed successfully, we may utilize nanotechnology approaches to use Az and other cupredoxins as inhibitors of parasitemia and other forms of parasitic growth.

It must be mentioned that other nanotechnology approaches have been used for control and inhibition of parasitemia which can be applied with Az: i) Lipid nanoemulsion has been used to control parasitemia. Ii) Primaquine (Singh and Vingkar 2008) and Chloroquine (Owais *et al.* 1995) (effective drugs against *Plasmodium bergheii*) were incorporated in lipid nanoemulsion having particle size in the range of 10–200 nm, decreasing significantly parasitemia for *P. bergheii*; iii) PEG-coated liposomes and other nanocapsules for prolonged circulation of medicine are excellent tools to inhibit and diminish parasitemia of malaria (Bakker-Woudenberg 2002; Mosqueira *et al.* 2004). So, PEG-coated liposomes and nanocapsules loaded with Az and even full lipid nanoemulsion by Az would be the possible new candidates to halt or slow down parasitemia.

Az is structurally similar to fab fragment of the monoclonal antibody that makes complex with PfMSP1-19 (one of the two above-mentioned *Plasmodium falciparum* merozoite surface proteins); moreover it has demonstrated similarity to the same molecules involved in HIV entry and viral growth. These molecules include surface glycoprotein CD4 and the extracellular domain of the intercellular adhesion molecule ICAM-3. Az readily bound to HIV-1 gp120, and to the dendritic cell-specific adhesion receptor DC-SIGN. In these cases Az mimics the function of the intercellular adhesion molecule ICAM-3 which it also binds readily with Az (Chaudhari *et al.* 2006).

Other nanotechnology approaches are found helpful in preventing HIV-1 proliferation in the body. For example, pH-sensitive liposomes loaded with antisense oligodeoxynucleotides have been applied against HIV-1 (Ropert *et al.* 1992; Düzgünes *et al.* 2001). Antisense oligonucleotides are synthesized DNA/RNA oligonucleotides which could bind to a messenger RNA of an infectious agent and inactivate it efficiently (see Glossary). In comparison with free drugs, these loaded liposomes not only protect oligonucleotides against degradation by nucleases, but also help increase their retention time in the blood circulation, inhibiting significantly HIV-1 activity (Bochot *et al.* 2000). Also, utilizing nanoparticles loaded with antisense oligonucleotides against HIV-1 led to strong inhibition of the viral production both *in vitro* (Berton *et al.* 2001) and *in vivo* models (De Jaeghere *et al.* 2000). These achievements may also be attained using liposomes and other nanoparticles loaded with Az(s), and their trial is expected to maximize the effect of anti-HIV drugs.

Az, H.8-Az and the Neisserial protein laz, showed high inhibitory activity against the growth of HIV-1 Bal, the most predominant HIV-1 subtype B (clade B) circulating in the US and Western Europe. They are also effective against a clade B African isolate RW/92/008/RE1 and a clade C Indian isolate IN/2167 D15 (Chaudhari *et al.* 2006).

Az and laz from gonococci/meningococci are recently demonstrated to have activity against toxoplasma (*Toxoplasma gondii*), a parasite that causes opportunistic infection like toxoplasmosis in immunocompromised individuals (Naguleswaran *et al.* 2008). Computer structural analysis showed that Az has common structural features with the predominant surface antigen SAG1, which plays an important role in parasite attachment. SAG1 interacts with laz strongly and to a lesser extent with Az. Thus it is revealed that the mechanism of action for laz is to interfere with the ability of toxoplasma to adhere to host cells, leading to toxoplasma growth inhibition.

Facilitating anticancer function of Az in conjunction with nanoentities

There exist a number of nanoentities (molecules, nanoparticles and nanotechnology molecular building blocks), which have found applications in cancer treatment through nanotechnology. In this section we report past attempts and future prospects of combining these various nanoentities with Az in order to facilitate the anticancer function of Az. When these nanotechnology platforms are joined with Az they may help in Az delivery, Az targeting and triggering the body's immune system by coating cancer cells with a high affinity antigen, and additional cancer-cell destruction capability like thermal ablation (or hyperthermia).

Az with gold nanoparticles (AuNP): Gold nanoparticles (AuNPs) are synthesized by reducing an Au salt in an aqueous solution. Although chemical methods of AuNP synthesis have been known for a long time, accurate control of nanoparticle size, monodispersivity and shape has become possible only in recent years (Turkevich 1985). One of the interesting properties of AuNP is its optical property (Anjali 2004; Armendariz *et al.* 2004). AuNPs are used in diagnostic medicine in several studies, for example as optical imaging contrast agents (Chen *et al.* 2005), for vital reflectance imaging (Sokolov *et al.* 2003), plasmon resonance scattering imaging or surface plasmon resonance (SPR) absorption spectroscopy (El-Sayed *et al.* 2005) and in immunetargeted imaging (Loo *et al.* 2005). Also, AuNPs have been the subject of several studies for therapeutic purposes like enhancing the effect of radiotherapy (Hainfeld *et al.* 2004), for effective drug delivery (Paciotti *et al.* 2004) and conjugated to antibodies targeting tumors (Patri *et al.* 2004; Chen *et al.* 2005; Loo *et al.* 2005).

A hybrid system obtained by conjugating Az with a 20-nm sized AuNPs was investigated (Delfino and Cannistraro 2009). Binding of Az molecule to AuNP surface results in the red shift of AuNP resonance plasmon band and in the quenching of the Az single tryptophan fluorescence signal. These findings together with the estimate of the hydrodynamic radius of the nanoconjugate are consistent with the formation of a monolayer of Az molecules, with preserved natural folding, on AuNP surface. It is expected that nanocancer therapy using AuNPs in association with Az may facilitate Az function. Not only they may reduce the amount of Az needed, the localized drug delivery would be enhanced, reducing probable side effects.

By conjugating or binding the gold nanoparticles to an antibody for EGFR (epidermal growth factor receptor), suitably named anti-EGFR, a group of researchers (El-Sayed *et al.* 2006) were able to get AuNPs to specifically attach themselves to the cancer cells; so that the malignant cells required less than half the laser energy to be killed than the benign cells. In addition, no photothermal destruction of any type of cell in the absence of AuNPs at these low laser powers was observed.

Az with magnetic nanoparticles (MNP): Magnetic nanoparticles (MNPs) are biodegradable particles, which are powerful and versatile diagnostic tools in biology and in medicine (Pankhurst *et al.* 2003). Nowadays, MNPs are applied to label specific molecules, structures, or microorga-

nisms by binding to them through a suitable antibody (Molday and Mackenzie 1982). Particular techniques have been developed like magnetic cell separation which makes use of magnetic field gradients to control and isolate magnetically labeled cells (Högemann *et al.* 2000), or magnetic immunoassay techniques where the magnetic field generated by the magnetically labeled targets is sensed directly with a sensitive magnetometer (Lübbe *et al.* 1996).

MNPs are gaining a great deal of attention in drug delivery. In this regard, a cytotoxic drug is attached to biodegradable MNP-carriers and then these conjugates (drug-carrier) are injected into the body. The particles are circulating through the bloodstream, but high-gradient magnetic fields are applied externally, in order to focus the complex at a definite target place into the body. When concentrated at the specific site, the drug may be released either via modification in physiological conditions like pH and temperature, or by enzymatic activity, and finally be taken up by the tumor cells (Alexiou *et al.* 2000; Pankhurst *et al.* 2003).

The magnetic component of the MNP is usually coated by a biocompatible polymer such as polyvinyl acetate (PVA) or dextran. The coating acts to protect the MNP from the surrounding environment and may also be functionalized by attaching to it carboxyl groups, biotin, avidin, carbodi-imide, etc. (Kolczak *et al.* 1999; Pankhurst *et al.* 2003). These molecules then act as attachment points for the coupling of cytotoxic drugs to the carrier complex. The carriers usually have one of the following two structural patterns: 1) a MNP core (usually magnetite (Fe_3O_4) or maghemite (Fe_2O_3)) covered with a biocompatible polymer, and 2) a spongy biocompatible-polymer where MNPs are deposited inside the pores (Pankhurst *et al.* 2003).

Iron-oxide MNPs have also been used to nanotarget drugs for nanodelivery to selective sites, rather successfully. The first clinical trial using iron-oxide MNPs of 100 nm was conducted to transfer epirubicin (an anthracycline drug used for chemotherapy). It consisted of the intravenous infusion of the MNP-bound drug, and a course of chemotherapy. A magnetic field was set up as close to the pretreated tumor as possible, and the ferrofluid was shown to be directed to the tumor inside the body of patients (Lübbe *et al.* 2001). Other useful applications of MNPs are bonding them with other metallic nanoparticles (Caruntu *et al.* 2005) and as "bioprobes" (DeNardo *et al.* 2005). Similar procedures using Az instead of epirubicin as the cytotoxic drug bound to MNPs may be suggested, to enhance Az cytotoxicity with minimal side effects on normal tissues.

It is noteworthy that in 2004 a patent was issued for certain magnetically modified electrodes which possess, both, electrically conducting and catalytic capabilities. The electrode is claimed to be consisted of three main parts: at least one catalyst component mediating a subatomic particle transfer process, several magnetic and/or magnetizable particles, and at least one ion conducting material. The electrode is also claimed to be comprised of a metalloprotein like Az. The oxidation/reduction reactions with this invented electrode may have potential applications in medicine and pharmaceutics (Leddy *et al.* 2004).

Blue ferrocenium Az (FcAz) was introduced in 2005 as an artificial organometalloprotein (Hwang *et al.* 2005). It was designed by the attachment of ferrocenium species to the active site of copper-depleted Az. Ferrocenium is the oxidative form of ferrocene $Fe(C_5H_5)_2$, an organometallic compound. This metalloenzyme demonstrated increased water solubility, more stability and tunable redox activity. Hence, it is claimed to have potential applications in design of biosensors and other biological electron transfer processes (Hwang *et al.* 2005). Combined employment of a FcAz with electromagnetic field (which shows antineoplastic therapeautic effect) is an exceptional tool to be used against cancers which are aimed to be targeted selectively with electromagnetic field (Badawi and Hafiz 2005).

Az with dendrimers: Dendrimers are synthetic macromolecules, which consist of branched repeated units in layers originating radically from a core (see Glossary). The word dendrimer comes from a Greek word "dendron" meaning tree because of its similarity to branches of the trees (Hecht *et al.* 2001). The properties of a dendrimer are defined by the functional groups on its surface. Dendrimers can have a hydrophilic external surface while have a hydrophobic internal core, so they would allow carrying hydrophobic drugs in their interiors (Fischer and Vögtle 1999).

In recent years, assortments of studies are performed on DNA-dendrimer nanoclusters for gene and drug delivery with such applications as for cancer therapy. Dendronized polymers may be used as targeted vectors for DNA and drug delivery purposes. The effect of environmental parameters on dendronized polymers is also studied (Nikakhtar *et al.* 2005, 2006). Such studies may assist the understanding of how they can be used for gene therapy (Nikakhtar *et al.* 2007). Dendrimers' unique structure allows us to generate "the nanotechnology equivalent of a Trojan horse" to deliver anti-cancer drugs directly into tumor cells (Kukowska-Latallo *et al.* 2005). Since dendrimers are typically less than 5 nm in diameter, they are small enough to pass through small pores in cell membranes, allowing easy passage into these cells.

Lately, in an experiment trying to shed light on the potential interaction of dendrimers with biological components, apoAz (copper-depleted Az) interaction with 5th generation PAMAM dendrimers was examined (Gabellieri *et al.* 2006). In this study three types of PAMAM macromolecules (G4.5, G5–OH and G5), containing carboxyl, hydroxyl and amine, respectively, as the end groups were used to inspect the effect of the chemical properties of the dendrimer surface on the protein–dendrimer interaction. It was shown that dendrimers interact with studied proteins in solutions making stable complexes. In some proteins the structure was significantly altered, especially in superficial, flexible regions of the polypeptide. In the case of apoAz the globular fold of the protein remains intact. Any changes in protein flexibility in the outer layer caused by stable or transient interaction between polymer and protein do not affect the interior. Further investigations are needed to verify if the interaction of dendrimer and Az could assist the anticancer function of Az.

Az with folic acid: Folic acid, or folate (salt of folic acid), is an important vitamin required for healthy functioning of all cells. Folate is essential for DNA synthesis and cell division (see the chemical formula of folic acid in the Glossary). Thus, cancer cells which divide more rapidly appear to need much more than average amounts of folate. Folate receptors on a cell surface transport folate into the cytosol of the cell for the synthesis of thymine by dihydrofolate reductase (Hashemian *et al.* 2009; Shakeri-Zadeh *et al.* 2009, 2010a, 2010b).

Folate has been conjugated with gold nanoparticles to deliver them into cancer cells (Hashemian *et al.* 2009; Shakeri-Zadeh *et al.* 2009, 2010a, 2010b). Similar conjugation of folate with Az may be achieved for its delivery into cancer cells. We propose a possible folic acid conjugation with Az by using a thiol molecule with the chemical formula (SH_2-R-NH_2) as the linker as shown in the Glossary.

As it was mentioned above, Az by itself enters cancer cells preferentially. Accordingly, if it is conjugated to folate, it may be more specifically localize at the tumor site and exert its apoptosis-inducing function, harming much less normal cells.

Az with carbon nanotube (CNT): The CNT structure is like a cylindrical roll-up of one or more graphene sheets in which carbon atoms are arranged in honeycombs fashion (Kim *et al.* 2007). The tubes may be closed at both ends with caps containing pentagonal carbon rings (Harris 2005). There are two categories of CNTs, single-walled and multi-walled nanotubes (Dai 2002). CNTs are being increasingly

utilized in nanomedicine. Single-walled carbon nanotube (SWNT) immunosensors were fabricated for clinical screening of prostate cancer and demonstrated highly sensitive and selective electrochemical detection of a protein cancer biomarker (PSA, see glossary) (Yu *et al.* 2006). In 2008, it was reported that using monoclonal antibodies absorbed on the SWNT field effect transistors, the detection of live breast cancer cells was possible (Teker *et al.* 2008). Moreover, different methods of cancer cell destruction are examined using CNTs. *In vitro* and *in vivo* exposure to radiofrequency results in significant heat release by single-walled CNTs that actually exhibited cytotoxicity towards several human cancer cell lines and hepatic VX2 tumors in rabbits (Srinivasan 2008, Gannon 2007).

SWNTs could be loaded with doxorubicin (a cancer chemotherapy drug, see glossary). It was shown that doxorubicin-loaded SWNTs induced significant cell death and apoptosis in U87 cell line (Liu *et al.* 2007; Srinivasan 2008). Later, it was demonstrated that doxorubicin- multi-walled CNT complex exerts enhanced cytotoxicity compared to doxorubicin alone (Ali-Boucetta *et al.* 2008). Similarly SWNTs may be also loaded with Az to enhance Az cytotoxicity.

In 1997, in the first report of nanotube electrodes for bioelectrochemistry, Az and cyt c were reported to be adsorbed onto nanotubes. It was demonstrated that these redox proteins on and within the nanotubes provided reproducible voltammetric response (Davis *et al.* 1997). Later in 2006 Az was anchored onto ion-irradiated CNT segments. It was proved that although anchoring onto CNT alters Az structure and polarization, its redox activity would be retained (Raghuveer *et al.* 2006).

All together, through CNTs anchored to Az and joined with cancer-specific antibodies, we may take better advantage of the Az anti-tumor activity, making its action more selective, more efficient and less toxic to other tissues.

DISCUSSION AND CONCLUSIONS

One of the ongoing and important objectives of biomedical sciences is to find an effective strategy for cancer treatment. For this to become a reality, laboratories worldwide are performing research to find effective methods to eradicate tumor cells. These activities have resulted in a number of drugs, some of which are effective if properly targeted. We are now faced with the severe challenges of drug delivery and targeting. Nanotechnology can greatly help us to meet these challenges.

Az protein molecule is a member of the blue-copper proteins family found in several bacterial species. It stabilizes the tumor-suppressor p53 protein and induces apoptosis in cancer cells. This may very well be because of electron transferring characteristic of Az (Sánchez-Pulido *et al.* 2004; Paraskevopoulos *et al.* 2006), as recently has been utilized in nanoelectronics. Az is able to enter malignant cells more readily than healthy cells and ultimately destroys the cell. Az may be considered as an effective anti-cancer agent when targeted appropriately.

Az as an anticancer candidate with the special features presented in this report, and with the assistance of nanotechnology methods potentially may help us to unravel some problems of cancer treatment and may overcome some of the therapeutic challenges. Some limitations of the peptides instability *in vivo* have been recently addressed as the researchers reported the synthesis of "thioether-bridged Az peptide fragment" which is aimed to resist the proteolytic degradation (Kuipers *et al.* 2009).

By using appropriate nanoparticles for carrying Az as an anti-cancer agent, not only the tumor cells drug resistance may be defeated, but also other dilemmas like drug toxicity and problems in drug targeting, drug release and drug dosage adjustment could be eliminated. Therefore, by applying nanotechnology for the delivery of Az joined with other nanoparticles, more effective elimination of cancer cells may be achieved.

There are a number of outstanding challenges in using nanotechnology for cancer treatment by Az. There exist several nanoengineering and biophysicochemical problems with materials at such a small scale to be solved. Materials could have different and sometimes peculiar behavior and interactions with Az in nanoscale. Also, because of large nanomaterials surface relative to their volume, surface effects are much more serious in nanosystems than they are in large systems. Therefore further studies seem essential to determine the interaction between various nanotechnology platforms and Az and their combined effect on healthy cells and tissues before their use. Nevertheless, the fact that we are potentially able to overcome chemotherapeutic limitations of Az through nanotechnology is undeniable.

GLOSSARY

Adenocarcinoma: A cancer that originates in the cells of glandular tissues.

Alternating magnetic field (AMF): The area in which the magnetic force is exerted to move electric charges and magnetic dipoles. Alternating indicates that these fields are not static but alternate or change their polarity or direction regularly.

Angiogenesis: A physiological process involving the genesis of new blood vessels from older vessels.

Antisense oligonucleotides (ODNs): Antiviral compounds that have shown potential therapeutic application against HIV-1.

Apaf-1 (apoptotic protease activating factor 1): A cytosolic protein involved in apoptosis. After cytochrome c release from the mitochondria, it interacts with Apaf-1 and dATP to form the apoptosome, which can activate caspase 9.

Apoptosis: A normal cellular process which involves a programmed series of intracellular events leading to the cell death. A distinctive morphological change will happen during this form of cell death.

Aptamer: Peptide or oligonucleic acid molecules that bind a specific target molecule. They can be used for basic research and also clinical purposes as macromolecular drugs.

Atomic force microscope (AFM): A type of scanning probe microscope to image atoms and molecules, with fractions of an Angstrom resolution and working based on interatomic forces.

Bacteria: Unicellular microorganisms. Their length is a few micrometers and has several shapes like spheres, rods, and spirals.

Bax: A Bcl-2-homologous protein. It promotes apoptosis.

Bcl-2: The prototype for a family of mammalian genes that govern mitochondrial outer membrane permeabilisation (MOMP). They can be pro-apoptotic (Bax, Bak, Bok, etc.) or anti-apoptotic (like Bcl-xL, Bcl-w, etc).

Beta sandwich (barrel): A large β-sheet that coils and twists to form a closed structure in which the first strand is bonded to the last. Beta-strands in beta-barrels are aligned in an antiparallel fashion.

Beta sheets: The β sheet (also named as β-pleated sheet) is a form of regular secondary structure in proteins in which beta strands are linked by at least three hydrogen bonds to make a twisted sheet.

BH3-domain (Bcl-2 homology-domain 3): Bcl-2 family share one or more of four characteristic domains of homo-

logy entitled the Bcl-2 homology (BH) domains (named BH1, BH2, BH3 and BH4). They can form hetero-, or homo-dimers. Bcl-2 family acts as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities.

Bid protein: A pro-apoptotic member of Bcl-2 family. See Bcl-2.

Binding site: A region on a molecule, to which specific other molecules and ions, could specifically form a chemical bond.

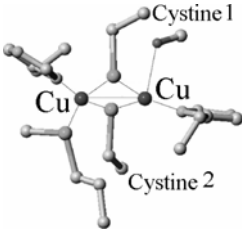
Caspases: Protein-cutting enzymes which are the executioners of apoptosis.

Cell nucleus: A spherical structure in which the cell's DNA is located and is the control center for all cell functions.

Chemotherapy: A disease treatment method using chemical substances. It mainly indicates the use of cytotoxic drugs to treat cancer.

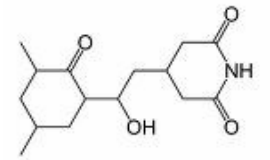
Clade: Combined biological species (or organisms) that have a common ancestor and all the later generation.

CuA center: A dinuclear $Cu_2(Cystine)_2$ electron transfer center that can be found in nitrous oxide reductase and cytochrome c oxidase.

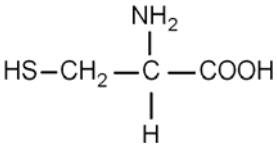


Cupredoxin: A copper protein (like Azurin) that contain one or more copper ions as prosthetic groups.

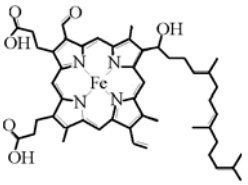
Cycloheximide: $C_{15}H_{23}NO_4$



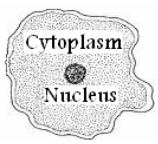
Cysteine: An amino acid, containing a thiol (SH) group.



Cytochrome c (cyt c): A small protein, associated with the inner membrane of the mitochondrion. It is a fundamental component of the electron transfer chain.

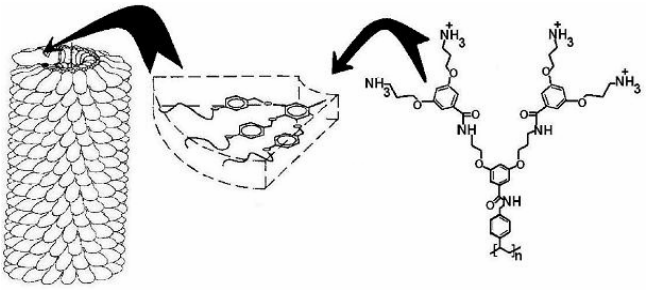


Cytoplasm: The fluid that fills the cells.



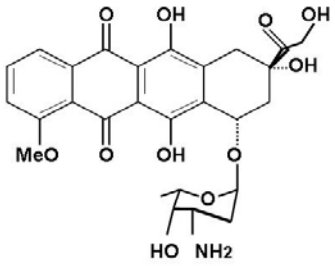
Cytotoxicity: The level of being toxic to cells.

Dendrimer: Synthetic macromolecules, which consist of branched repeated units in layers originating radically from a core. Here we show (Nikakhtar *et al.* 2005) the chemical structure of the second generation of poly(amido amine) (PAMAM) dendrimers and formation of dendronized polymer nano-cylinder. From Nikakhtar *et al*. (2005).



DNA (deoxyribonucleic acid): The carrier of genetic codes. Nearly all cells in the body contain DNA.

Doxorubicin (hydroxyldaunorubicin): A drug that is extensively used in chemotherapy.



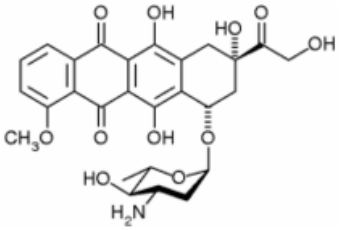
DU145 cell line: Derived from brain metastasis.

Endogenous substrate: Produced naturally in the body.

Endothelium: The monolayer of cells composing the interior blood vessels surface.

Ephrin: Ephrins are eukaryotic proteins divided into two classes (A and B) on the basis of their sequence homology.

Epirubicin: An anthracycline drug used for chemotherapy.



Epitope: The part of a molecule that is recognized by the immune system agents (antibodies, B cells, or T cells).

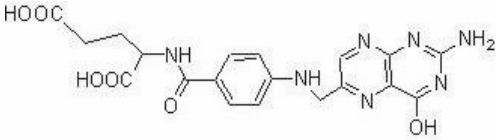
E. coli: *Escherichia coli*, the bacteria which lives in the lower intestines of mammals.

Fas ligand: A type II transmembrane protein - a member of the tumor necrosis factor (TNF) family.

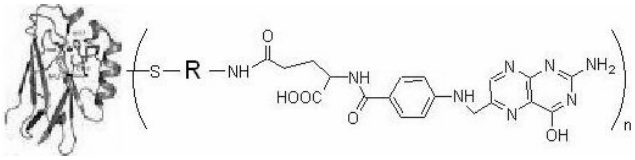
Fibroblast: A connective tissue cell found in every part of the body.

Fluorescence: A luminescence, mostly found as an optical phenomenon in cold bodies, in which the molecular absorption of a photon induces the emission of another photon with a longer wavelength.

Folic acid: An important vitamin required for healthy functioning of all cells.



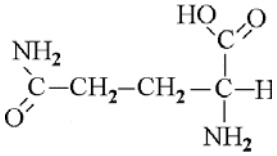
Folic acid conjugation with azurin: Folic acid may be conjugated with azurin by using a thiol molecule with the chemical formula SH_2-R-NH_2 as the linker.



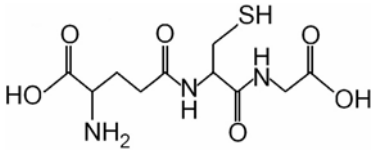
G1 checkpoint: Specific points in eukaryotic cells cycle.

Glioblastoma: The more common and aggressive type of primary brain tumor, accounting for 52% of all primary brain tumor cases and 20% of all intracranial tumors.

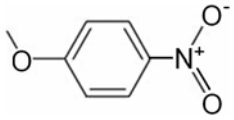
Glutamine (Gln, Q): An amino acid containing an amide side chain.



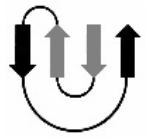
Glutathione (GSH): A tripeptide. Protector of cells from toxins like free radicals.



Glycin: is derived from the amino acid glycine.

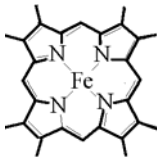


Greek-key folding motif: This structure forms easily during the protein folding process. It is composed of four adjacent antiparallel strands which three of them are linked by hairpins. It was named after a pattern common to Greek ornamental artwork.

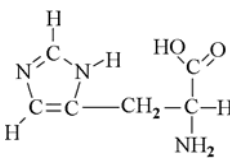


GST (glutathione *S*-transferase): A family of enzymes capable of reactions with a large numebr of endogenous and xenobiotic substrates.

Haem (or heme): A prosthetic group that is comprised of an iron atom enclosed in porphyrin ring which is a heterocyclic organic ring.



Histidine (His): An amino acid present in proteins.



Hormone: A chemical compound which serves as a signal from one cell (or group of cells) to another. Its action is determined by the secretion pattern and the signal transduction of the receiving tissue.

Hypoxia: A reduction of oxygen in the body.

Hydrogen peroxide: H_2O_2.

Hydroxyl radicals: HO– or –OH.

In situ: Phenomenon exactly in place where it takes place.

In vitro: Phenomenon studied in lab environment outside a living organism.

In vivo: Phenomenon studied inside an organism.

J774 cell line: A murine macrophages cell line derived from a tumor of a female BALB/c mouse.

Kinase: A group of enzymes that serve as the catalizers for the phosphate groups transfer from high-energy donor molecules, such as ATP, to specific receiving molecules.

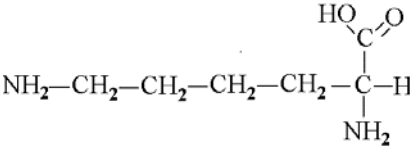
Kinetics: Study of reaction in progress.

L02 cell line: A cell line derived from normal liver cells.

LNCaP cell line: Androgen-sensitive human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis. These cells hold Prostate Specifeic Membrane Antigen (PSMA).

Lymphocytes: An important group of white blood cells in the immune system. There are two main categories of lymphocytes: the large granular lymphocytes (or natural killer cells) and the small lymphocytes. The small lymphocytes are divided into the T cells and B cells.

Lysine: An essential amino acid. It contains a 4-aminobutyl (primary amine) side chain.



Macrophage cell: An important type of white blood cell that plays a key role in immune system by surrounding and killing microorganisms, removal of dead cells and inducing the action of other parts of immune system.

MCF-10F cell line: A non-tumorigenic epithelial cell line. The line was produced by long term culture in serum free medium with low Ca^{++}.

MCF-7 cell line: Human breast adenocarcinoma cell line.

MDA-MB-157: Human Negroid breast medulla carcinoma cell line.

MDA-MB-231: Human Caucasian breast adenocarcinoma cell line.

MDD2 cell line: A variant derived from MCF-7 cell line by transfection.

MDM2: An important negative regulator of the p53 tumor suppressor. It is the name of a gene as well as the protein encoded by that gene.

Mel-2 cell line: Female human embryonic stem cell line.

Melanoma: A malignant tumor of melanocytes which are

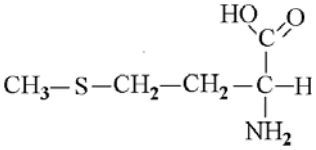
found predominantly in skin but also in the bowel and the eye.

Metalloprotein: A group of proteins that contain a metal cofactor. The metal is either an isolated ion or is coordinated with a nonprotein organic compound, like the porphyrin found in hemoproteins.

Metal–oxide–semiconductor (MOS): A widely used type of field effect transistors (FETs).

Metastasis: Spread of cancer cells from the original site to other body organs.

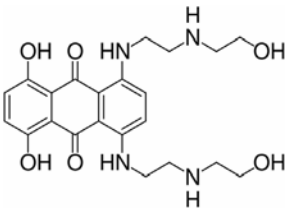
Methionine: An essential amino acid that like cysteine is sulfur-containing.



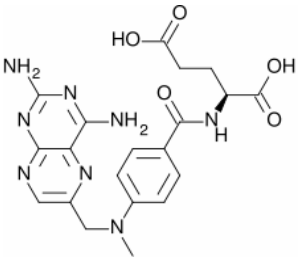
MG63 cell line: Human osteosarcoma cell line.

Mitochondria: Rod-shaped membrane-enclosed organelle considered the cell's power generator by converting oxygen and nutrients into ATP.

Mitoxantrone: A chemotherapy agent used in treatment of several types of cancerincluding metastatic breast cancer, non-Hodgkin's lymphoma, and acute myeloid leukemia.



Methotrexate: An antimetabolite and antifolate drug used in treatment of cancer and autoimmune diseases. It acts by inhibiting the metabolism of folic acid.



Mutation: Changes to the nucleotide sequence of the genetic material of an organism.

Necropsy: A post-mortem examination performed on an animal or inanimate object.

Necrosis: Unprogrammed cell death (versus apoptosis that is programmed cell death). Cell death due to acute cell injury (swelling, breaking open, releasing cell contents).

Neisseria: Parasitic bacteria growing in pairs and tetrads in animal body or serum media.

N-MOS FET and P-MOS FET: Negative- and positive-metal–oxide–semiconductor field-effect transistor. A device to amplify or switch electronic signals.

Nude mouse: A generic mutant that has a deteriorated or removed thymus gland.

Oncogenes: Genetic materials that carry the ability to induce cancer.

P53 (or TP53): Protein 53 (pr tumor Protein 53) is a 53-kD tumor suppressor protein.

PACA nanospheres: Polyalkylcyanoacrylate nanospheres of around 150 nm which can be generated by an emulsion polymerization process, which emulsifies droplets of water-insoluble monomers in an aqueous phase.

Pathogen (or infectious agent): A term often used for agents that disrupt the normal physiology of an animal or plant.

Parasitemia: The quantitative content of parasites in the blood.

PBS: Abbreviation for phosphate buffered saline.

Peptide: A short compound formed by two or more amino acids. Proteins are made of peptides.

Peritoneal: Related to peritoneum which is a sheet of body tissue that lines the inside of abdomen.

Phagocyte: A cell (like macrophage, monocytes, and neutrophils) that engulfs and digests debris and invading microorganisms. Phagocytosis is the action of a phagocyte cell.

Pharmacodynamics: The study of biochemical and physiological effects of drugs on the body or on microorganisms or parasites within or on the body and the mechanisms of drug action and the relationship between drug concentration and effect

Pharmacokinetics: (in Greek: "pharmacon" meaning drug and "kinetikos" meaning putting in motion, the study of time dependency) is a branch of pharmacology dedicated to the determination of the fate of substances administered externally to a living organism

Phosphatase: A group of enzymes that transfer a phosphate ion from its substrate like phosphoric acid monoesters into a molecule with a free hydroxyl group, the opposite action of phosphorylases and kinases, which attach phosphate groups to their substrates.

Photodynamic therapy (PDT): A kind of cancer treatment which involves three key components: a photosensitizer, light, and tissue oxygen. It is also being studied as a treatment for psoriasis and acne, and is approved for treatment of wet macular degeneration.

Plasmodium falciparum: a protozoan parasite, one of the species of *Plasmodium* that cause malaria in humans.

Prostate-Specific Membrane antigen (PSMA): A well-known prostate cancer tumor marker which is overexpressed on prostate acinar epithelial cells.

PUMA (p53 upregulated modulator of apoptosis): A pro-apoptotic member of the Bcl-2 protein family. Its expression is regulated by the tumor suppressor p53, and PUMA has been demonstrated to be involved in p53-mediated apoptosis.

Radiation therapy (radiotherapy): A kind of treatment using high energy radiation to shrink tumors and remove

cancer cells in the area. The radiation source may be from a machine placed near the patient, called external beam radiation therapy, or from a source of radiation inside the body, so called internal radiation therapy.

Redox: shorthand of oxidation/reduction reaction, describes all chemical reactions in which the oxidation number (oxidation state) of atoms are changed.

Reduction: The process of lowering the positive valence condition of an element (e.g., reducing a salt to metal).

RNA (ribonucleic acid): A molecule similar to DNA which is located both in the nucleus and cytoplasm of cells. Its primary function is protein synthesis within a cell.

Scanning tunneling microscope (STM): A non-optical microscope that scans and gives images of the electrically conducting surfaces at micro and nanoscale levels.

Secondary structure: The general three-dimensional form of local segments of biopolymers such as proteins and nucleic acids.

Self-assembly: A process of self-organization of one or more components in a way that the total energy of the system is lowered to let to a more stable state.

Serum: It is the same with blood plasma, with clotting factors removed.

Signal transduction: Any kind of converting signals or stimuli within the cell, which most often involves ordered sequences of biochemical reactions inside the cell that are carried out by enzymes.

Superoxide dismutase (SOD): An enzyme which catalyzes the dismutation (simultaneous oxidation and reduction) of superoxide (a reactive anion and free radical) into oxygen and hydrogen peroxide. An important antioxidant in nearly all cells exposed to oxygen.

Super-secondary: is formed when nearby secondary structure elements are combined in specific arrangements called motifs.

Surface plasmon resonance (SPR): The excitation of surface plasmons by light is denoted as a surface plasmon resonance (SPR) for planar surfaces/localized surface plasmon resonance (LSPR) for nanometer-sized metallic structures.

Therapeautic index (or therapeutic ratio): The ratio of the amount of a therapeutic agent that brings about the therapeutic effect to the amount that is toxic to the organism.

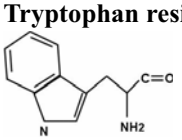
Thiol: An organic compound that contains the functional group composed of a sulfur atom and a hydrogen atom (-SH).

TNF: Tumour Necrosis Factor, TNF-R: The TNF receptor.

Transcription (in genetics): The process in which messenger RNA is synthesized from a DNA template resulting in transfer of genetic information from DNA to RNA.

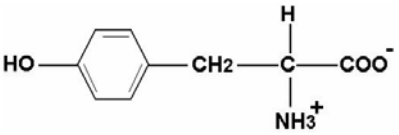
Transistor: A semiconductor device used in amplifiers, control circuits, and oscillators. In this device, current flow is modulated by voltage or current applied to electrodes. Most transistors take the advantage of silicon.

Tryptophan residue: An essential amino acid.



Tumor: (or tumour) is primarily used to refer to abnormal tissue growth. This growth may be malignant or benign. It has a similar meaning to "neoplasm".

Tyrosine: is one of the 20 amino acids, used in protein synthesis. It contains a phenol side chain with a hydroxyl group.



U2OS cell line: U2OS is a cell line derived from the bone tissue.

UISO-Mel-2: A cell line derived from metastatic melanoma from human pleural fluid.

Vital reflectance imaging: A photographic process that captures views of a surface under varying lighting conditions.

Virus: A microscopic particle that can infect the cells and replicate itself in that host cell. Their size is ranging from 20-300 nm.

Xenobiotic substrate: A substance foreign to the body.

Xenograft: A surgical graft of tissue from one species to an unlike species (or genus or family).

Xenotransplantation: The transplantation of living cells, tissues or organs from one species to another such as from pigs to humans.

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