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Review

Exploring the anticancer potential of the bacterial protein azurin

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Abstract: Bacterial proteins and their derivative peptides have emerged as promising anticancer agents. Nowadays they represent a valuable set of candidate drugs with different origins and modes of action. Among these, monomeric cupredoxins, which are metalloproteins involved in the electron transport chain of prokaryotes, have been shown to possess potent anticancer activities. In particular, much attention has been focused on azurin produced by the pathogenic bacterium *Pseudomonas aeruginosa*. More recently, several *in vitro* and *in vivo* studies have reported the multi-targeting anticancer properties of azurin. Moreover, p28, a peptide derived from azurin, has completed two phase I clinical trials in cancer patients with promising results. In this updated review, we examine the current knowledge regarding azurin's modes of action as an anticancer therapeutic protein. We also review the clinical trial results of p28 emphasizing findings that make it suited (alone or in combination) as a therapeutic agent for cancer treatment. Finally we discuss and address the challenges of using the human microbiome to discover novel and unique therapeutic cupredoxin-like proteins.

Keywords: anticancer bacterial proteins; cupredoxins; azurin; p28 peptide; clinical trials; human microbiome

1. Cupredoxins: A Family of Copper Proteins with Anticancer, Antiviral and Antiparasitic Activities

The cupredoxin fold has a widespread occurrence in nature including bacteria, plants, and mammals. The known members of this family are azurins, pseudoazurins, rusticyanins, auracyanins, amicyanins and halocyanins [1,2]. With an ancient origin, it groups copper-binding proteins that have many cellular functions associated with their ability to shuttle electrons between proteins [1,2]. Overall, the prototype structure defining the cupredoxins is composed of two main stable β-sheets made up of 7 or more parallel and antiparallel strands (Greek key β-barrel structure), a feature interpreted previously as being an immunoglobulin-like fold [3] (Figure 1). In fact and despite their diverse origin and function, several studies indicated that cupredoxins and immunoglobulins have an evolutionary link and might have evolved from a common ancestor [3,4]. One argument used to support this theory is based on the fact that as observed in the immunoglobulin variable domains, cupredoxins have a tyrosine corner-like motif, a unique signature of the immunoglobulin Greek key structure [3,4]. Moreover, for both classes of proteins, the interaction surfaces include common features such as the existence of hydrophobic clusters surrounded by polar and charged residues. Altogether, this correlation is consistent with the ability of cupredoxin members to mediate specific high-affinity interactions (at sub-nanomolar range) with various unrelated proteins, conferring on it the property of a natural scaffold protein and enabling its use for therapeutic purposes.

Azurin, produced by *Pseudomonas aeruginosa*, is a representative member of the cupredoxin family. It is a periplasmic copper-containing protein composed of 128 amino acids (14 kDa). Its structure comprises eight antiparallel-strands connected by four loops linked by a disulfide bridge (Figure 2). Azurin can bind to multiple sites on a ligand, specifically through the existence on its surface of three distinct binding regions. It contains on one face two charged clusters (one large negative nearby to one small positive). Moreover it presents, in another region, a prominent neutral aromatic-rich hydrophobic patch (Figure 2). This arrangement, centered on Phe114, occupies a region around the copper center [5,6]. Based on these observations, we propose that azurin has scaffold properties driven by electrostatic and hydrophobic interactions.

Several studies have shown that bacterial cupredoxins, particularly azurin, can serve as a source for the development of emerging therapeutic drugs to treat cancer as well as against various infectious agents, such as viruses and parasites. While most of these studies were focused on the anticancer effects, the antiviral (HIV) effect has been proven for the bacterial protein azurin produced by *Pseudomonas aeruginosa* or a modified lipid azurin termed Laz produced exclusively by *meningococci/gonococci* [7]. Laz is very similar to *P. aeruginosa* azurin and has a lipidated epitope (H.8; 39 amino acids) at its N terminal that is responsible for its attachment to the outer membrane [7,8]. Moreover, azurin and rusticyanin (produced by *Acidithiobacillus ferrooxidans*) have been found to display activity against the malarial parasite *Plasmodium falciparum* [9,10]. Azurin and Laz have also been implicated in inhibiting the growth of the toxoplasmosis-causing parasite *Toxoplasma gondii* [11]. Overall, these findings reveal that azurin and the other cupredoxins studied serve as scaffold therapeutic tools having the ability to promote functional inhibition of molecular/cellular events relevant for cancer progression and virus/parasite infection.

Numerous applications in oncology and diagnostics have been reported for non-Ig scaffolds [12]. Azurin, like the others non-Ig, is small in size, remarkably stable and water-soluble. Moreover it can be easily produced/purified in a bacterial host. Ultimately, azurin appears to have

selective capacities to be associated (i.e. internalized) with cancer cells. This selective internalization seems to be dependent on the cholesterol-enriched microdomains, termed lipid rafts and commonly over-represented in cancer cells. The advantages of such cell entry specificity open new possibilities for azurin use, i. e. the exploitation of combined targeted therapies as well as the design of nanoparticles decorated on the surface with azurin and encapsulated with a known effective anticancer drug.

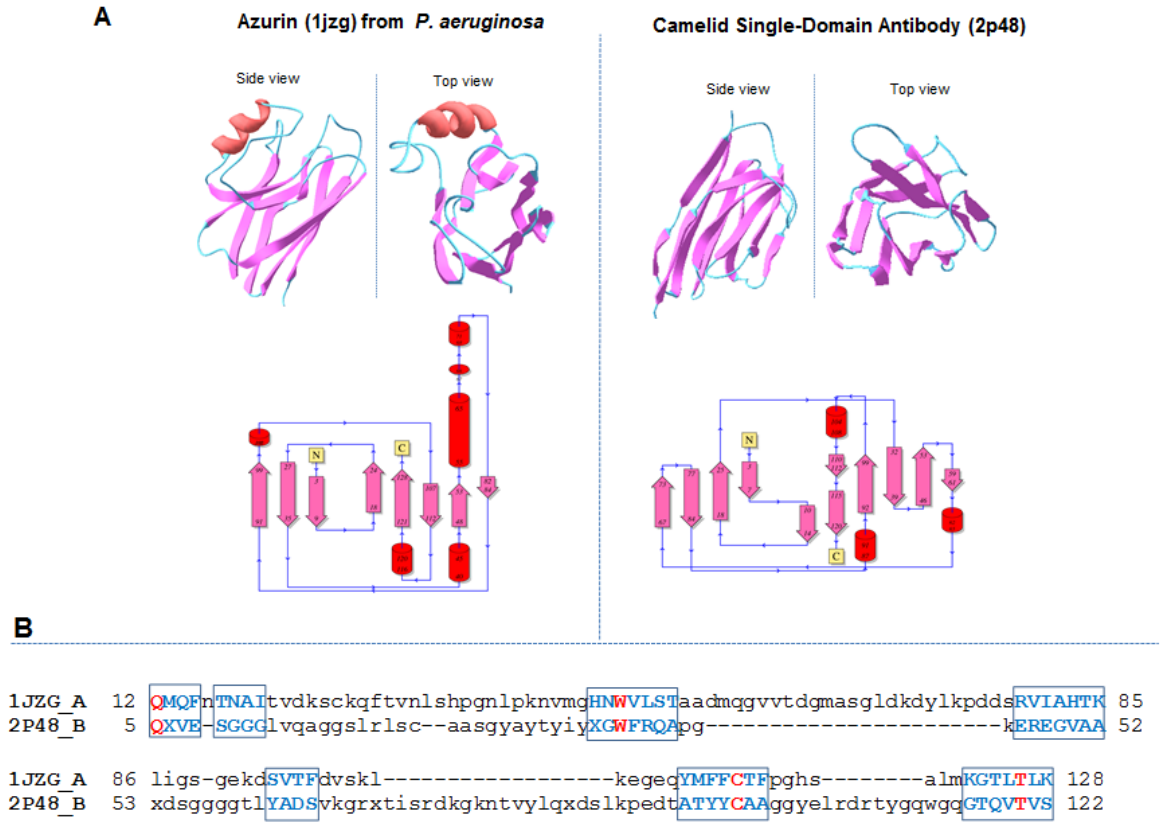

Figure 1. (A)—(Top) Comparison among the structures of azurin from *Pseudomonas aeruginosa* (1jzg_A) and a single domain antibody from *Camelus dromedaries* (2p48_B). These figures were produced using Swiss Pdb viewer [40]. (Bottom) The topology diagram of each protein generated by PDBSum (www.ebi.ac.uk/pdbsum/) is also shown. Cartoons represent the structure as a sequence of secondary structure elements (SSEs): β-strands (depicted as arrows) and helices (depicted as cylinders), their connection in a sequence from amino to carboxyl terminus, and their relative spatial positions and orientations. The direction of the elements can be deduced from the connecting lines. (B)—Structural alignment of azurin (1jzg_A) from *P. aeruginosa* with camelid single-domain antibody (2p48_B), as computed by the VAST algorithm [41]. Superimposed secondary structure elements are denoted by capital red and blue letters. Dashes and lowercase lettering indicate where there are no alignments.

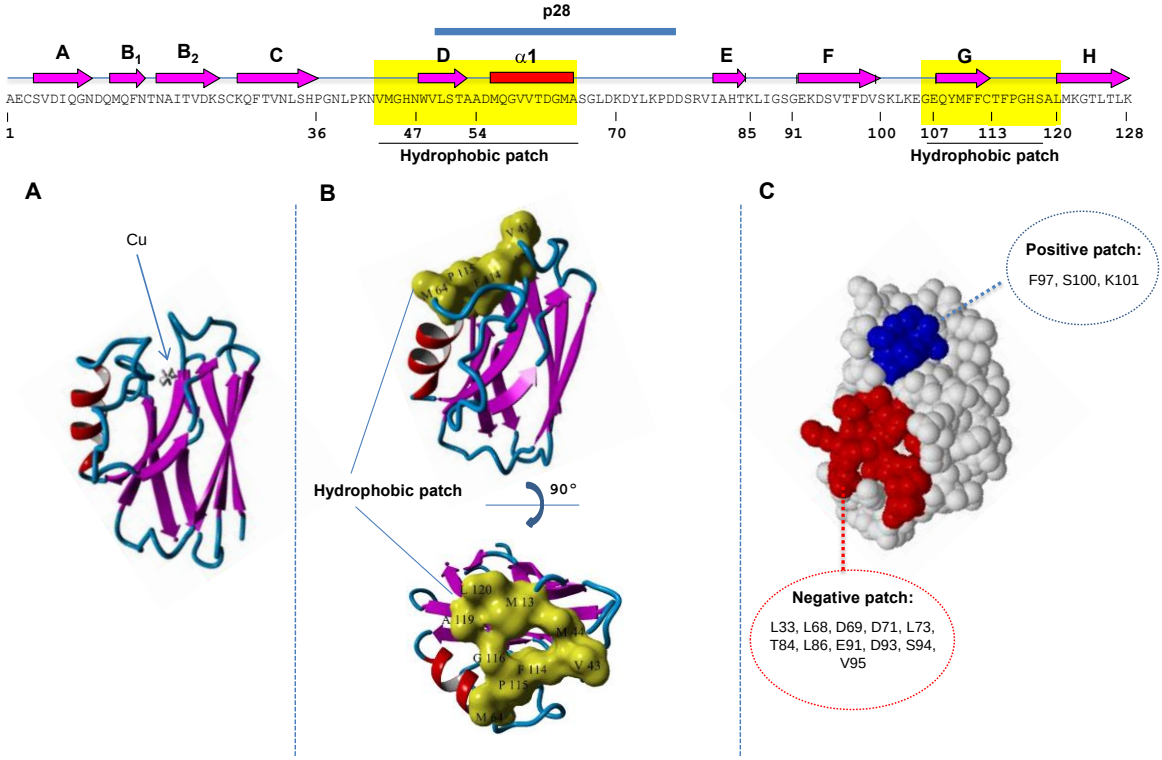

Figure 2. [Top]—Primary structure (128 aa) of azurin (holo form) from *Pseudomonas aeruginosa*. Secondary structure elements are illustrated as pink arrows for β-sheets and red rectangles for helices. The yellow areas denote the existence of hydrophobic regions. (A)—Ribbon drawing of azurin (1jzg_A) from *P. aeruginosa*. (B)—Ribbon drawings of azurin (1jzg_A) from *P. aeruginosa* denoting the existence of a surface hydrophobic patch (yellow). These figures were produced using Yasara (http://www.yasara.org/products.htm#view). (C)—Illustration of the largest positive (blue) and negative (red) patches calculated on chain A of 1jzg (computed by the BindUP program) [42]. Azurin is represented with atoms as spheres and their relative orientation is the same as that of panel A.

To further explore the use of azurin as a scaffold for cancer therapy purposes, a phage display technology can be applied. This approach can be attained by randomized substitution of selected residues within the pre-existing binding sites of azurin aiming to generate azurin variants displaying multiple picomolar affinities to pre-defined targets relevant in cancers. The novel properties of the azurin engineered variants will be supported by an overall structural integrity of the β-sandwich scaffold that will remain conserved. Successful completion of these studies would lead to directed studies of azurin-engineered variants in cancer therapy, either alone or in conjunction with chemotherapeutics.

2. Azurin: An Old Cupredoxin with New Functions

Besides its defined function as a redox partner in electron transfer reactions, azurin has been actively explored as a new anticancer agent [13,14,15]. Upon preferential entry in various cancer

cells, azurin interferes in cell growth by multiple mechanisms including complex formation with the DNA-binding domain (DBD) of tumor suppressor protein p53 [13,16], stabilizing it and enhancing its intracellular level (both nuclear and cytoplasmatic fractions), which then allows induction of apoptosis [13]. Using isothermal calorimetry assays it has demonstrated that azurin exibits subnanomolar affinity for the p53-DBD with the four to one stoichiometry [16]. Moreover, site directed mutagenesis indicates that the hydrophobic patch of azurin is determinant for this interaction [17]. Intravenous injections of azurin in xeno-transplanted mice harboring melanoma and breast tumors led to significant tumor regression, without adverse side effects [14]. The presence of azurin is likely to increase the mRNA levels of pro-apoptotic molecules via p53, such as the levels of BAX, thereby leading to an imbalance of BCL2-BAX levels and enhanced cell death or growth arrest [18]. It is also worth noting that Laz from *Neisseria* species, unlike all other known azurins, can disrupt the blood-brain barrier and kill brain tumors [19].

Besides its interaction with p53, azurin also targets a cell proliferation pathway mediated by the EphB2 tyrosine kinase, a family of extracellular receptor proteins known to be upregulated in many tumors. Azurin exhibits competitive binding towards this receptor and can prevent the tumor progression caused by the binding of the natural ligand ephrinB2 [20]. In addition, azurin decreases the phosphorylation levels of VEGFR-2, FAK and Akt allowing inhibition of angiogenesis in cancer cells, thus inhibiting cancer cell growth [21]. Azurin can also interfere in oncogenic transformation to prevent precancerous lesion formation in mouse alveolar and ductal mammary glands exposed to a carcinogen DMBA [22]. While azurin itself has not been tested in clinical trials for its cancer regressing activity, p28, a peptide derived from azurin (a unique 28 amino acid extended α-helix in its middle part) (Figure 2), has recently completed two phase I clinical trials in cancer patients with promising results [section 4; 23,24].

Azurin and its peptide p28 can penetrate cancer cells faster than when compared to normal cells. This process was identified as being dependent on cholesterol, since its depletion significantly reduced its cellular penetration [25]. Preclinical evaluation of pharmacokinetics, metabolism and toxicity of azurin-p28 established it as non-immunogenic and non-toxic in mice and non-human primates [26]. Several international patents have been issued to cover the use of azurin-p28 in cancer therapies [27], and azurin has shown significant activity, as well as enhancement of the activity of other drugs [28,29]. Recent studies have shown that p28 in combination with lower concentrations of DNA-damaging drugs like doxorubicin, dacarbazine, temozolamide, and antimitotic agents such as paclitaxel and docetaxel, increased their cytotoxicity by stabilizing the tumor-suppressor protein p53. Taken together, these results highlight a new approach to maximize the efficacy of chemotherapeutic agents while reducing dose-related toxicity [29].

The mechanism mediating cell entry of azurin and its derived peptide has been studied. It is known that such entry is not dependent on membrane bound glycosaminoglycans nor on clathrins. However, it is possible that N-glycosylated proteins may have a role at least in the initial steps of recognition and the depletion of cholesterol from the membrane significantly inhibited the penetration of p28 (~60%), suggesting involvement of the caveolae-mediated endocytic route [25]. Once inside the cancer cells the apo and holo forms of azurin are similar in their effects, supporting a copper-independent mechanism of action [17].

3. Gaining Insights into the Azurin Anticancer Mode of Action

At the time of this writing, a PubMed search for "azurin + cancer" returns nearly 60 results. Since the discovery of unusual anticancer properties of azurin [30], an increasing amount of knowledge has been gained regarding its potential therapeutic use and mode of action (Figure 3). Here we intend to extend and update the review undertaken by Bernardes et al. in 2013 [15] regarding the use of azurin and its derived peptide p28 as novel and promising anticancer agents from bacterial origin. In our laboratory, we have reported that azurin targets P-cadherin overexpression in a subset of breast cancers, antagonizing its pro-invasive effects [31]. P-cadherin overexpression occurs in about 30% of all breast carcinomas representing one of the most aggressive sub-type of breast cancer, being associated with poor patient prognosis [32]. We determined that azurin treatment reduces the levels of P-cadherin at the cell membrane, whereas E-cadherin remains unaltered with high expression levels and with normal membrane localization. Thus, reduction in the level of aberrant P-cadherin concomitantly impairs the levels of the hyperphosphorylation forms of FAK and Src non-receptor tyrosine kinases [31]. These proteins regulate a wide number of signaling pathways involved in adhesion, migration, invasion, survival, and angiogenesis linking cellular responses to environmental stimuli (Figure 3). Altogether, these results show that azurin exhibits a specific preference for P-cadherin, abrogating its invasive effects and, therefore, may have a potential role in the treatment of breast carcinomas overexpressing this protein [32].

In another study, we employed a microarray-based approach to analyze the genetic pathways modulated by azurin that are important contributors to cell proliferation in breast cancer, in particular pathways that regulate cell-cell adhesion and cell-matrix interactions [33]. Our genetic analysis was used to infer about the signaling pathways that are altered in a p53 wild-type and P-cadherin overexpressing breast cancer cell model upon azurin treatment. The results strengthen the hypothesis that azurin has a multivalent action towards cancer cells, promoting the endocytosis of cell surface receptors and the interruption of signaling pathways hyper activated in cancer cells (Figure 3) [33]. The blockage of these signaling pathways in P-cadherin overexpressing breast cancer cell models leads to the abrogation of tumor cell invasion, providing a possible new therapeutic approach to this specific type of breast cancer. For some of these receptors it has been reported that their localization within lipid rafts is essential for their aberrant constitutive signaling, even in the absence of their ligand external stimuli [34]. Azurin up-regulated endocytosis and the lysosomal pathway in treated cells, suggesting that the mechanism by which it acts may involve up-regulating the degradation of membrane proteins required for signaling in cancer progression which localize to lipid rafts.

4. Clinical Trials of Azurin-Derived Peptide p28 in Cancer Patients

While as mentioned above, azurin has been shown to have significant cancer regressing effect through interference at multiple steps, it has the disadvantage of being a protein and not a small molecule. For clinical trials to determine the efficacy of a drug, proteins go through more stringent regulatory processes since the technology for chemical synthesis of a protein, as opposed to small molecules or even peptides, is not optimum and therefore the purity and cost of synthesis are often prohibitory for small start-up companies. Peptides, on the other hand, are more amenable to chemical synthesis to a high degree of purity, allowing for their easier regulatory approval for clinical trials. Thus a start-up company CDG Therapeutics Inc. (www.cdgti.com) approached the USFDA for

regulatory approval of conducting clinical trials in cancer patients not with azurin but with the 28 amino acid peptide p28 (azurin 50–77). P28, similar to azurin, has entry specificity in cancer cells and anticancer activity.

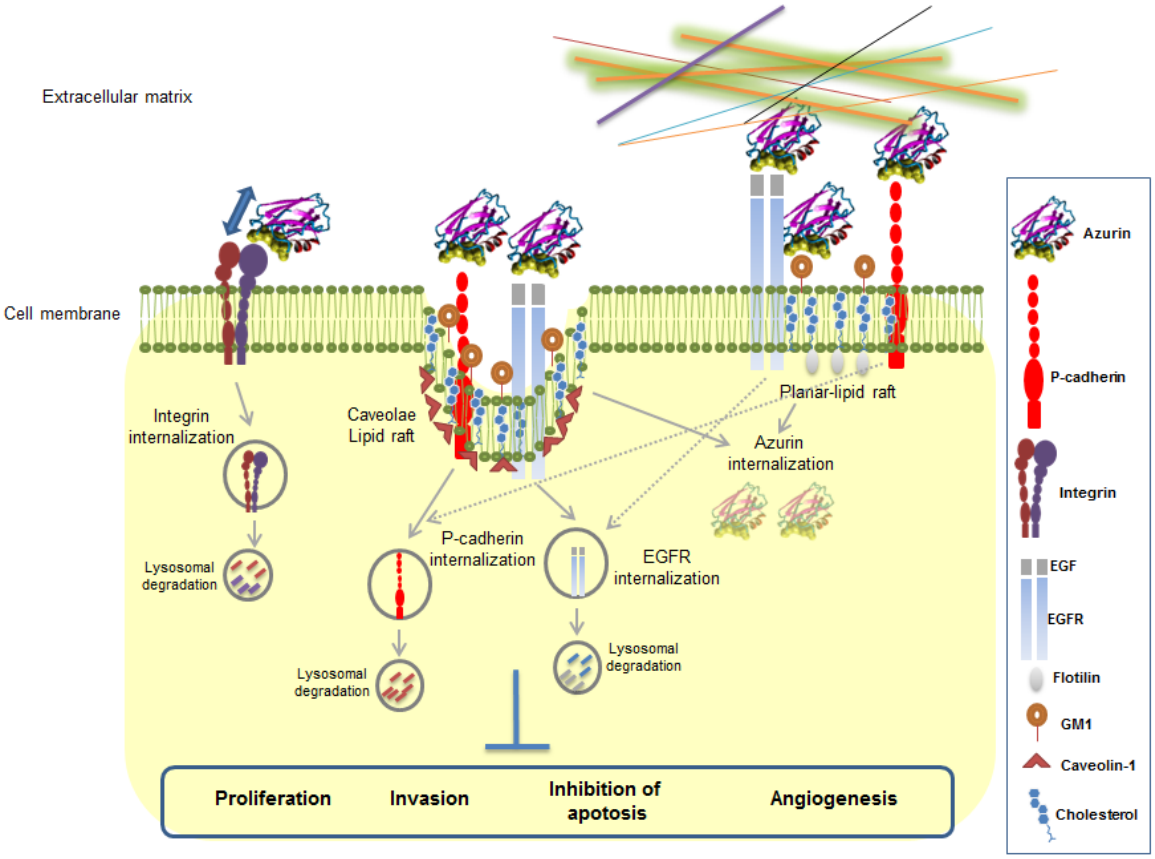

Figure 3. Proposed mechanisms of action of azurin against cancer cells. Azurin binds to cancer cells through binding to lipid micro domain (raft) components and cell-surface receptors. Upon entry, azurin interferes in cancer cell growth by multiple mechanisms.

Upon entry in the cancer cells, p28 binds within the DNA binding domain of p53 (both wt and mut) where it blocks the binding of the E3 ligase Cop1, inducing a major increase in the level and activity of the wild type and mutant p53, thereby upregulating p21 and p27 [35,36] and allowing apoptotic cell death in the cancer cells and growth inhibition (Figure 4).

In preclinical trials with animals, p28 showed no toxicity, thus gaining approval from the USFDA for a phase I trial in 15 stage IV cancer patients with multiple solid tumors such as melanoma, colon, sarcoma, prostate and pancreas. These patients had tumors that were resistant to all conventional drugs and the patients were terminally ill with a life expectancy of about 6 months. P28 was given in 5 escalating doses starting at 0.83 mg p28 per kg body weight of the patient, and subsequent doses of 1.66, 2.5, 3.33 and 4.16 mg per kg body weight of the patients. P28 was administered through intravenous bolus to the first 3 patients at the lowest concentration, and if no side effects were detected, 3 more patients were recruited and all the 6 patients received the next higher dose. If still no side effects were observed, 3 more patients would be recruited with the next higher dose and the process would continue until all the surviving patients received the highest dose.

Both the side effects and any beneficial effects were noted. No significant side effect was noted even at the highest concentration of p28. When given at low to medium concentrations, the tumor growth was arrested in 7 patients. At higher concentration, while no significant side effects were observed, 3 patients showed partial regression of their tumors while 1 patient had complete regression of the drug-resistant tumors. Three of these patients were still alive, one disease free, 2 to 3 years after the termination of the trial [23].

Encouraged by such results, the National Cancer Institute (NCI) and the Pediatric Brain Tumor Consortium sponsored a second phase I trial (http://clinicaltrials.gov/ct2/show/NCT01975116) in 11 Children's Hospitals in the US in pediatric brain tumor patients starting in October, 2013. Brain tumors are often highly invasive and difficult to treat because very few drugs can cross the blood-brain barrier to reach the brain tumors. P28 was given intravenously to such patients, age 3 to 21. Since the p28 was given at the adult dose as was administered during the first phase I trial, the sponsors stipulated that if p28 was found to be toxic to the pediatric brain tumor patients at this dose, or if p28 had no efficacy in reducing the growth of the tumors, they would stop the trial. As reflected in the recently released publication [24], the results of this second phase I trial suggest that p28 has shown acceptable toxicity and perhaps some tumor regressing effect in some of these pediatric brain tumor patients. Indeed, it is important to note that the USFDA has approved on December 02, 2015 the designation of azurin-p28 as an orphan drug for the treatment of brain tumor glioma.

5. Human Microbiomes: Exploring the Existence of Uncharacterized Cupredoxin-Like Proteins

Various tissues and organs from human origin are full of microbes. Overall they represent the human microbiome and include trillions of both culturable and nonculturable communities of eukaryotes, archaea, bacteria and viruses. Nowadays, the challenge of linking microbiome to human health and disease is undergoing a renaissance [37]. In this context, the Human Microbiome Project (HMP) (http://hmpdacc.org/) funded by NIH plans to make accessible, from various organs of the human body, the sequences of reference genomes of these organisms as well as metagenomics sequence data. To-date, there are available over 3000 genomes and 1265 metagenomic project data.

An exciting opportunity now exists to screen human microbiota reference genomes/metagenomes aiming to discover novel and unique cupredoxin-like protein encoding genes. To address this challenge, integrative bioinformatic approaches adjusted to either annotated genomes or metagenomic data need to be used. The final goal is to identify homologous cupredoxin associated genes that can be amplified from the reference DNAs or even be chemically synthesized. Then, after the expression and purification of the novel proteins of interest, studies on their putative anticancer activity can be carried out. A few studies have been conducted so far regarding the exploitation of the human microbiota as a source to identify novel genes/enzymes with biotechnological potential. Saikh et al. analyzed the genome of *Lactobacillus salavarius*, a commensal bacterium inhabiting the human intestine, with the aim of finding bacteriocin candidate genes [38]. More recently, Nguyen C and Nguyen VD [39] used a computational approach to identify azurin-like gene products across genomes of 66 dominant bacterial species present in the human gut microbiome. Extensive analyses of various protein parameters provided the authors with a list of 8 bacteriocins that demonstrated properties similar to azurin, providing an example of the usefulness of genomic scale screening of bacteriocins with potential anticancer activity from the human gut microbiome.

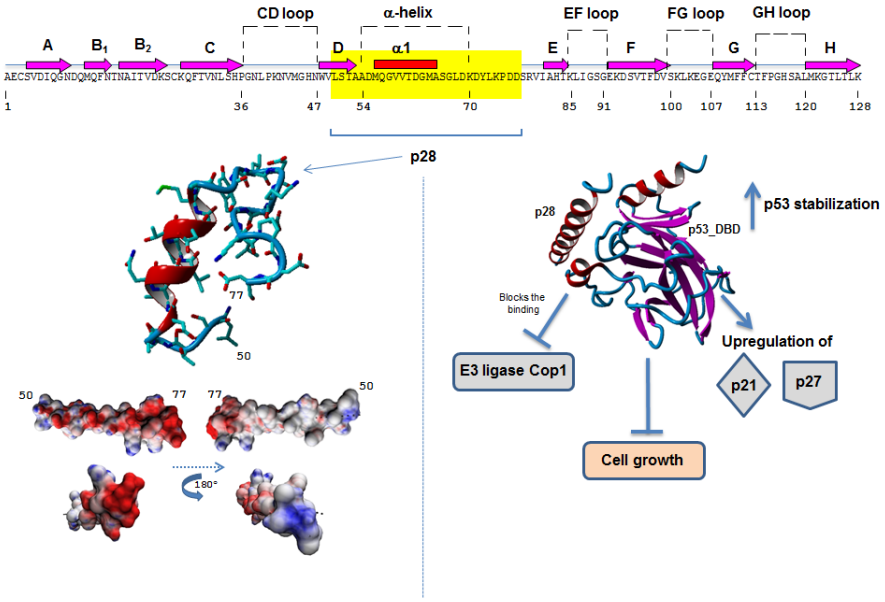

Figure 4. [Top]—Primary structure (128 aa) of azurin from *Pseudomonas aeruginosa*. The p28 azurin derived peptide (50–77) is highlighted in yellow. [Left]—Ribbon drawing of p28, an amphipathic peptide. P28 has a right-handed helical structure. p28 surface electrostatic potential as computed by the CHARMM program (+2 kcal/(mol e) in blue to−2 kcal/(mol e) in red) [43]. These figures were produced using Yasara. [Right]—p28 mode of action. p28 binds within the DNA binding domain of p53, where it blocks the binding of the E3 ligase Cop1, inducing a major increase in the level and activity of the wild type and mutant p53, thereby upregulating p21 and p27 [35,36] and allowing growth inhibition and apoptotic cell death in the cancer cells.

6. Final Remarks and Future Perspectives

Over the years, many different cytotoxic drugs have been developed to treat cancer. Most of them are based on the development of targeted therapies implying the use of synthetic small molecules or monoclonal antibodies. Initially they are efficient in killing cancer cells but then they become much less effective due to the acquisition of resistance, as reflected in stage IV cancer patients. In search of new concepts and paradigms to guide cancer treatment, it is interesting to note the return to the exploitation of cancer-fighting compounds from nature. For instance, the microbial world represents an extraordinary reservoir of novel molecules and may hold the key for finding new lead compounds for cancer treatment. Based on these considerations, in this paper we present an overview focusing on the anticancer properties of the bacterial protein azurin and its derived peptide p28.

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Conflict of Interest

The authors declare no conflict of interest.

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