REVIEW

Bacterial cupredoxin azurin hijacks cellular signaling networks: Protein–protein interactions and cancer therapy

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Abstract: Azurin secreted by *Pseudomonas aeruginosa* is an anticancer bacteriocin, which preferentially enters human cancer cells and induces apoptosis or growth inhibition. It turns out that azurin is a multi-target anticancer agent interfering in the p53 signaling pathway and the non-receptor tyrosine kinases signaling pathway. This suggests that azurin exerts its anticancer activity by interacting with multiple targets and interfering in multiple steps in disease progression. Therefore, azurin could overcome resistance to therapy. Besides azurin, putative bacteriocins that possess functional properties similar to those of azurin have been identified in more bacteria species. A systematic investigation on the anticancer mechanisms of azurin and the azurin-like bacteriocins will provide more and better options in cancer therapy. In this review, we summarize how azurin and the derived peptides hijack key cellular regulators or cell surface receptors to remodel the cellular signaling networks. In particular, we highlight the necessity of determining the structure of azurin/p53 complex and investigating the influence of post-translational modifications on interactions between azurin and p53. Therapeutic applications of azurin and derived peptides are also discussed.

Keywords: Anticancer drugs; bacterial proteins; non-receptor tyrosine kinases; p53; tumor suppression

Introduction

Azurin is a copper-containing redox protein secreted by *Pseudomonas aeruginosa*. It is involved in electron transfer during denitrification in *P. aeruginosa* by interacting with cytochrome c_{551} and nitrite reductase.[1] Azurin has attracted great attention in the past decade for its preferential entry into and cytotoxicity towards a variety of cancer cells.[2–6] Early evidences have shown that azurin is secreted into the growth medium of *P. aeruginosa* and leads to apoptotic death of peritoneal macrophages or mast cells.[7] Later, azurin demonstrates significant cytotoxicity towards human melanoma cells and human breast cancer

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cells.[8–10] In addition, injection of azurin into nude mice xenografted with human cancer cells leads to significant regression of the tumor and increase of survival rate.[8,9,11] Though azurin is deficient in entering glioblastoma cells, when the N-terminal H.8 moiety of the azurin-like protein from *Neisseria meningitidis* is fused with azurin, the fusion proteins have high cytotoxicity for glioblastoma cells.[12] Besides the full-length protein, peptides derived from azurin also retain the anticancer activity. A C-terminal segment of azurin (88–113) has been shown to induce growth inhibition of breast cancer MCF-7 cells and prostate cancer DU145 cells.[13] Another peptide, p28 (amino acids 50–77 of azurin), exhibits proapoptotic activity against a number of solid tumor cell lines.[14]

It is now emerging that azurin induces apoptosis or growth inhibition by interfering in the p53 signaling network and the non-receptor tyrosine kinases (NRTKs) signaling network. This suggests that azurin exerts its anticancer activity by interacting with multiple targets and interfering in multiple steps in disease progression. Although the p53-mediated pathway plays a central role in azurin's anticancer activity, the interactions between azurin and p53 are still poorly understood. In this review, we summarize how azurin and the derived peptides hijack key cellular regulators or cell surface receptors to remodel the cellular signaling networks. In particular, we highlight the necessity of determining the structure of azurin/p53 complex and investigating the influence of post-translational modifications on interactions between azurin and p53. Therapeutic applications of azurin and derived peptides are also discussed.

Selective Entry into Human Cancer Cells

Azurin preferentially enters human cancer cells and exerts cytostatic and cytotoxic effects. Truncation experiments identified a 28-amino-acid fragment (amino acids 50–77, p28) that is responsible for the entry of azurin into human cancer cells.[15] When fused to p28, cargo proteins such as GST-green fluorescent protein fusion protein were internalized in J774 cells and UISO-Mel-2 cells at 37°C but not at 4°C, suggesting that p28 mediated internalization of cargo proteins is an energy-dependent process.[15] Competition experiments as well as studies with inhibitors suggested that azurin may enter cancer cells via a receptor-mediated endocytic process.[15] Since azurin and p28 exhibit preferential internalization in cancer cells than the corresponding normal cells,[14,15] it is likely that the levels of such receptors are higher on the surface of cancer cells than on the surface of normal cells. Recently, Yamada et al. found that p28 preferentially entered human breast cancer cell lines mainly through a caveolin-mediated pathway as agents disrupting caveosome formation and caveolae-mediated endocytosis significantly inhibited the penetration of p28 into MCF-7 cells.[16] Understanding the cancer cell specificity of p28 will be beneficial for further development of targeted drugs.

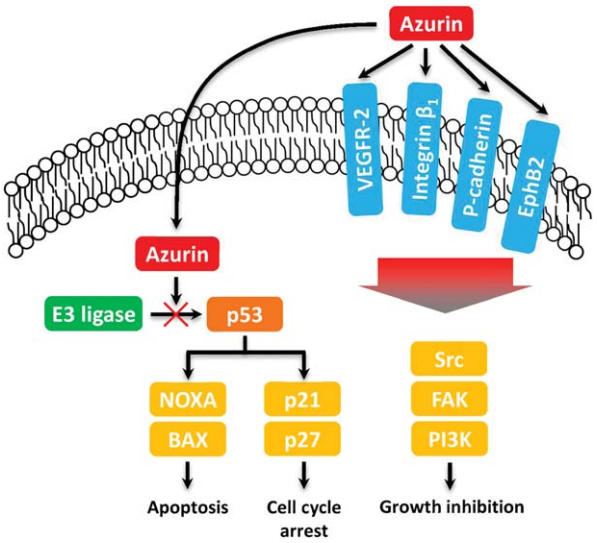

Figure 1. Mechanisms of azurin to induce apoptosis and growth inhibition of human cancer cells. Azurin enters cancer cells and forms complexes with p53, inhibiting ubiquitin-mediated degradation of p53 and increasing its level. The stabilized p53 travels back into the nucleus and transcriptionally induces proapoptotic genes such as *Bax* and *Noxa* or cell cycle inhibitors such as *p21* and *p27*. Azurin also binds to the cell surface receptors, including VEGFR-2, integrin β_1, P-cadherin, and EphB2, interfering in their signal transduction pathways that converge to NRTKs.

Mechanisms of Anticancer Activity

Azurin is a versatile protein and interferes in several independent signaling pathways associated with cancer progression. So far, several different mechanisms have been proposed to account for azurin's anticancer activity (Fig. 1): (i) azurin induces cancer cell apoptosis or growth inhibition by forming complexes with the tumor suppressor protein p53; (ii) azurin also inhibits cancer cell growth by interfering in the receptor tyrosine kinase EphB2-mediated signaling process; (iii) furthermore, azurin inhibits tumor growth by preventing angiogenesis through reducing VEGFR-2 tyrosine kinase activity; (vi) besides, azurin interferes with P-cadherin protein expression and inhibits the growth of breast cancer cells.

The tumor suppressor protein p53 mediated apoptosis or growth inhibition pathway is the most extensively studied mechanism accounting for the anticancer activity of azurin or p28. The p53 protein plays a central role in cancer suppression. It exerts its tumor suppressing activity through transcriptional regulation of downstream target genes. Under normal circumstance, the p53 protein has a very short half-life and the basal concentration is regulated via

ubiquitin-mediated pathways. Azurin has been shown to form complexes with p53 and inhibit the binding of E3 ubiquitin ligases to p53, thus increasing p53 levels through decreased ubiquitination and proteasomal degradation.[16] Mdm2 is a key E3 ligase negatively regulating p53. Although azurin has been shown to interact with the N-terminal domain (NTD) of p53 which also interacts with Mdm2, binding of azurin to p53 did not block binding of Mdm2 to p53[17] because the binding affinity of azurin with p53 transactivation domain (TAD) ($K_d \sim 5$ μM)[18] is about 25 folds weaker than that of Mdm2 with p53 TAD ($K_d \sim 0.2$ μM).[19] Recently, Yamada et al. identified potential binding sites of p28 on the p53 DNA-binding domain (DBD).[20] Interestingly, these potential binding sites for p28 overlap with the binding sites for E3 ligase COP1 (constitutively photomorphogenic 1). In addition to Mdm2, COP1 also negatively regulates p53 and COP1 is overexpressed in breast cancer, ovarian cancer, hepatocellular cancer, and gastric cancer. Yamada et al. found that p28 inhibited binding of COP1 to p53, leading to an increase in the p53 level.[20,21] Azurin may interact with p53 DBD in a similar way and stabilize p53 by interfering in the COP1/p53 interaction.

The p53/azurin complexes then travel back to the nucleus where p53 transcriptionally induces proapoptotic genes such as *Bax* and *Noxa* which interacts with mitochondria, triggering the release of mitochondrial cytochrome c into the cytosol.[8,9,22] This process activates the caspase cascade(s) and initiates the apoptotic process. In addition, p53 will also activate the expression of cell cycle inhibitors such as p21 and p27 which inhibit the activity of cyclin-dependent kinases, causing cell-cycle inhibition at the G_1 to S phase or G_2 to M phase.[16,23]

Besides via the p53 mediated mechanisms, recent studies revealed that azurin also inhibits cancer cell growth through interactions with various transmembrane receptors whose signal transduction pathways converge to NRTKs, such as Src, FAK, and PI3K. NRTKs play important roles in a number of fundamental biological processes and the activity of NRTKs is often up-regulated in cancer cells.[24,25] Therefore, azurin may interfere in the signaling pathways involving NRTKs and prevent the process of tumorigenesis.

Ephrins and their receptors, the Eph receptor tyrosine kinases, have been shown to be up-regulated in many tumors.[26–28] Upon binding, the Eph/ephrin molecules form a heterotetramers, activating the tyrosine kinase domain. Azurin exhibits remarkable topological similarity to ephrins and binds to EphB2 receptor tyrosine kinases with high affinity.[13] A C-terminal segment (amino acids 88–113) of azurin is found responsible for this tight binding. The azurin/EphB2 interaction interferes in EphB2 phosphorylation, resulting in cancer growth inhibition.[13] In addition, using human umbilical vein endothelial cells as a cancer model, p28 has been shown to inhibit angiogenesis and tumor growth by reducing VEGFR-2 tyrosine kinase activity.[29] Inhibition of VEGFR-2 kinase activity reduces the phosphorylation of the VEGFR-2 downstream targets FAK and Akt. As a result, the distribution of cell-motility and migration associated proteins are altered.[29] Furthermore, azurin was shown to reduce P-cadherin protein expression and inhibit the growth of highly invasive P-cadherin overexpressing breast cancer cells by abrogating P-cadherin mediated signaling processes.[10,30] Most recently, azurin was demonstrated to control the levels of integrin β_1 and its membrane localization in non-small cell lung cancer A549 cells.[31,32] Treatment with azurin decreases integrin β_1 protein expression and attenuates the phosphorylation levels of Src, Akt, and PI3K. Atomic force microscopy characterization indicated that membrane properties of A549 lung cancer cells were altered upon azurin treatment, enhancing the sensitivity of lung cancer cells to inhibitors specifically targeting the epidermal growth factor receptor.[32]

Azurin is a promiscuous protein that is able to interact with several unrelated targets. It is not surprising that azurin utilizes different segments to interfere in different cellular signaling pathways. So far, two segments have been identified responsible for interactions between azurin and the signaling targets. The central segment of azurin, p28, is involved in p53 binding.[33] The p28 segment has also been shown co-localizing with caveolin-1 and VEGFR-2;[29] however, direct physical interactions between p28 and caveolin-1 or VEGFR-2 have not been confirmed. Besides the p28 segment, a C-terminal segment of azurin is responsible for the tight binding between azurin and EphB2.[13] The same segment has also been found to bind to dendritic cells specific intercellular adhesion molecule 3-grabbing nonintegrin with high affinity, enabling azurin to interfere in the binding of HIV-1 with dendritic cells.[34]

Azurin/p53 Interaction: A Key to Understand Azurin's Anticancer Activity

Among the above discussed mechanisms, the p53 dependent mechanism is the most important and the most extensively studied one. However, our understanding on the molecular details of these interactions is far from complete (Fig. 2). Glycerol gradient centrifugation has shown that azurin forms complexes with full-length p53 (p53 FL), where p53 is a tetramer.[22,23] Therefore, one p53 tetramer is able to bind four azurin molecules. This stoichiometry has been confirmed by isothermal calorimetry.[35] There are evidences showing that azurin is able to interact with NTD, DBD, and the C-terminal domain (CTD) of p53.[18,20,36,37] Therefore, it is possible that azurin interacts with multiple p53 monomers simultaneously. Atomic force

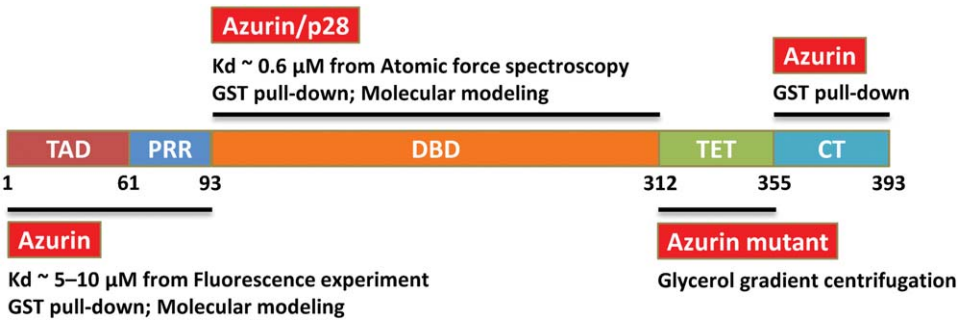

Figure 2. Interactions between azurin and p53. p53 FL consists of a TAD, a proline-rich region, a DBD, a TET domain, and an extreme C terminus. Interactions between specific p53 domain and azurin are shown by the black lines, where the binding affinities obtained and the experimental techniques used are indicated.

microscope and surface plasmon resonance were applied to determine the interactions between azurin and p53 FL monomer. The estimated dissociation constant (K_d) is about 1–6 μM,[17,38] consistent with the isothermal calorimetry determined K_d values for p53 tetramer and azurin,[35] suggesting that one azurin molecule is mainly interact with one monomer within the p53 tetramer.

The DBD domain of p53 is the main site for azurin or p28 to interact with p53 as the dissociation constant for interactions between p28 and p53 DBD is rather similar to that for interactions between p28 and p53 FL.[33] Computer simulations suggested that azurin and p28 bound to the same region of p53 DBD, locating around the L1 loop and the S7–S8 loop.[20,36] This potential binding site has been validated experimentally and T140, P142, Q144, W146, R282, and L289 of the p53 DBD are key residues for p28 binding.[20] Interactions between azurin and p53 are mainly hydrophobic.[39] Thus, mutating hydrophobic residues in the hydrophobic patches of azurin or p53 DBD abolishes interactions between azurin and p53 DBD.[21,40,41]

The p53 NTD is of particular interest as this region interacts with Mdm2, a key negative regulator of p53. Tryptophan fluorescence quenching experiment suggested that there were interactions between azurin and p53 NTD, which induced ~10% increase in p53's secondary structure content.[35] The dissociation constant for azurin/p53 NTD interactions is estimated in the range of 5–10 μM.[18] Docking and molecular dynamics simulations suggested that azurin bound to the two helical regions of p53 NTD, which are also binding regions for Mdm2.[42] Nevertheless, GST pull-down experiments showed that interactions between azurin and p53 NTD were very weak[37] and azurin was not able to block binding of Mdm2 to p53.[17] Therefore, the interactions between azurin and p53 NTD and their roles in reactivating p53 are still not determined.

Interactions between p53 CTD and azurin are the least studied and remain controversial. GST pull-down experiments have shown that interactions between azurin and p53 CTD are very weak.[43] On the contrary, nucleic acids mediated strong interactions between azurin and p53 CTD were observed in a recent study.[37] Glycerol gradient centrifugation has suggested that WT azurin forms complexes with p53 FL tetramer, whereas M44K/M64E azurin mutant interacts with p53 FL and inhibits p53 oligomer formation.[23] Therefore, it is possible that M44K/M64E azurin mutant interacts with the tetramerization (TET) region of p53 within the CTD.

The p53 protein is a scaffold molecule and exerts its function through intricate interactions with numerous targets.[44,45] More than 1000 binary interactions involving more than 400 proteins are found in the IntAct molecular interaction database for p53.[46] Many of these interactions are fine-tuned by post-translational modifications, for example, phosphorylation of Thr18 in p53 TAD reduces the affinity of Mdm2 with p53 TAD, while phosphorylation of Ser15, Ser20, Ser33, Ser37, Ser46, Thr18 or Thr55 increases the binding affinity of p53 TAD with various transcription factors. Therefore, it will be important to investigate the influence of post-translational modifications on interactions between azurin and p53 in future studies. Although interactions between azurin (p28) and p53 have been extensively characterized, information on the complex structures is still very limited and no structure of azurin/p53 or p28/p53 complex has been determined so far. We expect that the binding interface of azurin/p53 or p28/p53 complex will overlap with known p53 interactions (Fig. 3). Determination of structures for azurin/p53 and p28/p53 complexes will be valuable for further understanding the function of azurin and p28.

Azurin Derived Cancer Therapy

Live attenuated bacteria as vectors to deliver azurin

Azurin has been widely tested as a potential anticancer agent by specifically inducing azurin expression within the tumor regions to increase its toxicity. Live bacteria as potential cancer treatments and the

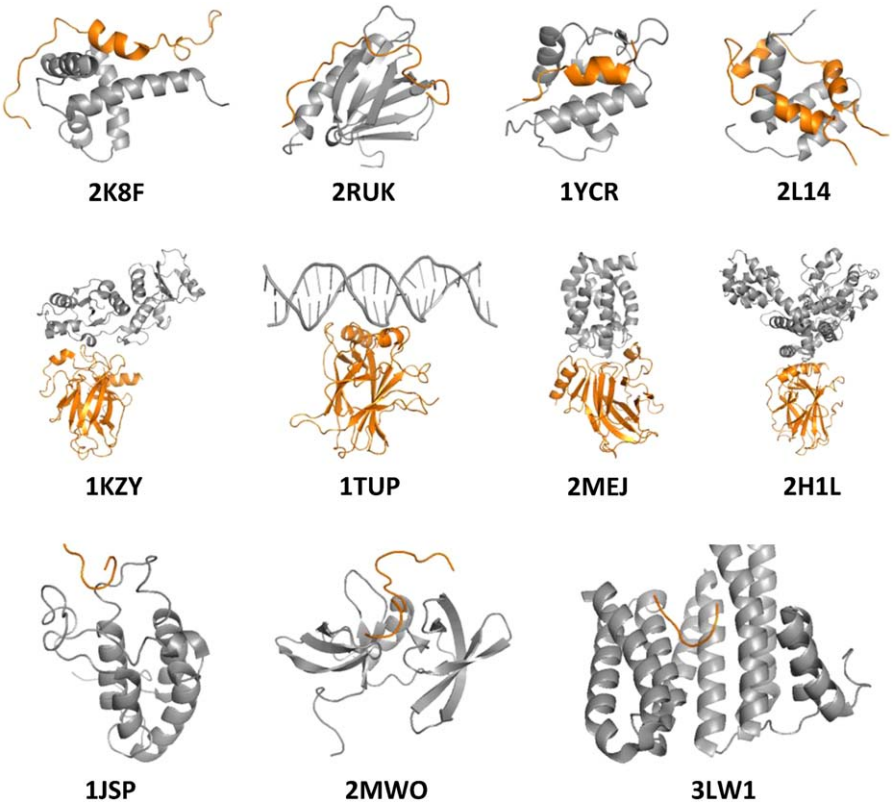

Figure 3. Illustrative structures of p53 bound to different partners. The first row: the p53 TAD binds to the Taz2 domain of p300 (2K8F), the PH domain of TFIIH subunit p62 (2RUK), MDM2 (1YCR), and the nuclear coactivator-binding domain of CBP (2L14). The second row: the p53 DBD binds to the brca1 CTD of 53BP1 (1KZY), DNA (1TUP), Bcl-xL (2MEJ), and oncoprotein SV40 large T-antigen (2H1L). The third row: the p53 CT binds to the bromodomain of CBP (1JSP), the tandem Tudor domain of 53BP1 (2MWO), and 14-3-3σ (3LW1). The p53 fragments are shown in orange and the interacting partners are shown in gray.

use of attenuated bacteria as vectors to deliver cytotoxic genes are promising. Previous studies have shown that some bacteria specifically and preferentially target solid tumors and can proliferate inside cancer cells.[47] Recently, combination of the azurin anticancer activity and bacterial vector was demonstrated in two studies. Zhang et al. employed the *Escherichia coli* Nissle 1917 to specifically target tumors and inhibit mouse B16 melanoma and 4T1 breast tumors through continuous expression of azurin.[48] They found that the growth of B16 melanoma and orthotopic 4T1 breast tumor was remarkably restrained and pulmonary metastasis was prevented in immunocompetent mice.[48] In the other study, Mehta et al. designed an avirulent strain of *Salmonella typhimurium* that induced apoptosis via simultaneous expression of p53 and azurin.[49] Expression of these two proteins was constrained within the tumor region by a hypoxic promoter *pflE*. In an xenograft model of human glioblastoma in rats, they found that bacterial carrier therapy significantly increased the survival rate.[49]

p28 as an anticancer agent

The antitumor efficacy of p28 has been assessed on human breast cancer, prostate cancer, and melanoma cells.[14,16,21,50] Recently, two phase I trials, one in adults and the other one in children have been carried out to investigate the safety, tolerability, pharmacokinetics, and activity of p28 in patients with solid tumors. In the first phase I trial, 15 adult patients with metastatic solid tumors enrolled. Dose-limiting toxicities, significant adverse events or immune responses to the peptide were not observed. Stable disease, partial response, and complete response were achieved after therapy.[51] In the other phase I trial for pediatric patients, 18 patients aged 3–21 years with recurrent or progressive central nervous system tumors enrolled. This phase I study demonstrated that although p28 may be not effective against pediatric central nevous system tumors as a single cytostatic agent, it is safe and well-tolerated in children.[52]

p28 as cancer-targeted vector for drug delivery

The p28 segment is responsible for the preferential entry of azurin into cancer cells and promote uptake of heterologous proteins.[15] Based on this, it is possible to design more complicated p28-based targeted drug delivery systems. p28 can be conjugated to a cargo (e.g., anticancer drug) by a linker which can be cleaved through enzymatic hydrolysis to release p28 and the cargo. p28 can also form noncovalent

complexes with the cargos via interactions with liposomes or nanoparticles. These p28-based targeted drug delivery systems will retain p28's anticancer activity and endow the cargo cancer-targeted specificity.

Azurin as anticancer agent enhancer

Azurin not only induces cancer cell death or growth inhibition by itself but also enhances the sensitivity of cancer cells to other anticancer drugs. Cho et al.[53] found that treatment of oral squamous carcinoma cell line YD-9 cells and human osteosarcoma cell line MG-63 cells with 5-fluorouracil (5-FU) alone only resulted in approximately 30% growth inhibition at 1 mM. In contrast, when 5-FU was used in combination with azurin, a much lower concentration of 5-FU (i.e., 10 μM) was able to induce 80–90% of cells growth inhibition.[53] Recently, azurin was found to enhance the sensitivity of lung cancer cells to gefitinib or erlotinib by reducing integrin β_1 levels and its membrane localization.[32] In a recent study, Yamada et al. found that p28 enhanced the cytotoxic activity of DNA-damaging drugs or antimitotic drugs in a variety of cancer cells through the p53/p21/Cdk2 pathway.[54] The ability of azurin to bind to ephrin receptors has been utilized to design a compound by conjugating the azurin derived peptide with nicotinic acid and the resulting compound showed a ~13-fold increase in the efficacy of radiotherapy.[55]

Conclusion and Future Perspectives

Investigations in the past 15 years have greatly improved our understanding on azurin's anticancer mechanisms. It turns out that azurin is a multitarget anticancer agent interfering in the p53 signaling pathways and the NRTKs signaling pathways. New functions of azurin such as interactions with p63 and p73 may be discovered in future studies.[56] Therefore, azurin could overcome resistance to therapy. In addition, putative bacteriocins that possessed functional properties similar to those of azurin have been identified in more bacteria species.[57] A systematic investigation on the anticancer mechanisms of azurin and the azurin-like bacteriocins will undoubtedly provide more and better options in future cancer therapy.

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