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Anticancer Actions of Azurin and Its Derived Peptide p28

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Abstract
Cancers are a great threat to humans. In cancer therapy, surgical removal of the tumor combined with radiotherapy and chemotherapy is the most routine treatment procedure and usually the most effective. However, radiotherapy and chemotherapy drugs that kill cancer cells efficiently also kill normal cells, thus exhibiting large side effects. Cancer-targeted drugs, which aim to specifically recognize proteins or signaling pathways associated with tumor proliferation and migration, have achieved marked progress in recent years. Azurin is a copper-containing redox protein secreted by *Pseudomonas aeruginosa*. Azurin and its derived peptide p28 preferentially enter a variety of cancer cells and induce apoptosis or cell cycle arrest. Mechanistic studies revealed that azurin and p28 target the p53 and receptor tyrosine kinase signaling pathways as well as other pathways. Two phase I trials of p28 have been carried out, with findings that p28 is safe and exhibits anticancer activity in both adult and pediatric patients. In this review paper, we provide an up-to-date summary of progress on the anticancer mechanisms and therapeutic strategies for azurin and p28.

Keywords Anticancer drug · Tumor suppression · Bacterial protein · Azurin · p28

1 Introduction

Malignant tumors (cancers) are a great threat to humans, and comprise the second leading cause of death after cardiovascular disease. Cancers and their complications greatly reduce the quality of life of patients and their families and increase medical and healthcare spending. Therefore, it is of much value to explore efficient anticancer drugs. For cancer treatment, surgical resection of the tumor combined with radiotherapy and chemotherapy is currently the most common treatment strategy, and often the most effective. However, traditional radiotherapy and chemotherapy drugs kill both cancer cells and normal cells simultaneously, and often show strong toxicity and side effects. With the aim of specifically recognizing proteins or signal transduction pathways related to tumor proliferation and migration, cancer-targeted drugs, which are expected to achieve the dual goals of tumor treatment and reduced side effects, have undergone rapid development in recent years [1–6].

Azurin is a copper-containing redox protein secreted by *Pseudomonas aeruginosa* [7]. Based on their ability for selective entry into and induction of apoptosis or cell cycle arrest in many cancer cells, azurin and its derived peptide p28 have attracted much attention in the last two decades [8–16]. At present, two phase I clinical trials of p28 have been completed, with findings that p28 is safe and exhibits anticancer activity in both adult and pediatric patients [17, 18]. Because azurin and p28 target many different signaling pathways [19], such as the p53 and receptor tyrosine kinase pathways, azurin and p28 may not easily induce resistance and have the potential to become new anticancer drugs. In this review paper, we summarize recent progress on the anticancer mechanisms and therapeutic strategies for azurin and p28.

2 Properties of Azurin and p28

2.1 Amino Acid Sequences and Structures

Azurin (UniProt ID P00282) is a copper-containing redox protein secreted by *P. aeruginosa* [7] that contains 128

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amino acids and a copper ion (Fig. 1). According to the SCOP classification, the three-dimensional structure of azurin belongs to the all-β folding class, which mainly comprises two groups of β-strands (4 strands per group) arranged in a sandwich structure (Fig. 1b) [20]. p28 is a fragment (Leu50-Asp77) of the azurin protein (Fig. 1a), encompassing a β-strand, an α-helix, a turn, and an irregular structure (Fig. 1b). It is worth noting that the structure of the p28 segment is separated from the sandwich structure of azurin. Although p28 is folded into a stable three-dimensional structure within azurin, it does not mean that the p28 fragment forms the same structure as an isolated peptide. In fact, molecular dynamics simulations showed that the α-helix of p28 was unstable after isolation [21, 22].

2.2 Preferential Entry into Cancer Cells

Azurin preferentially enters a variety of human cancer cells [23]. Truncation experiments revealed that the region of azurin mediating its penetration through the cell membrane is mainly composed of amino acids 50–77 (termed p28). Furthermore, fusion of p28 to cargo proteins, such as glutathione *S*-transferase and green fluorescent protein, enabled internalization of cargo proteins into macrophages, melanoma cells, or breast cancer cells [23]. Competition experiments and studies with inhibitors suggested that azurin may enter cells by a receptor-mediated endocytic process involving caveolin-1, the Golgi complex, and ganglioside GM-1 [23–25]. The membrane receptors that mediate azurin or p28 internalization often show higher expression levels in cancer cells compared with normal cells. Therefore, relative to normal cells, the contents of p28 in tumor cells are about 3–6-fold higher [24]. Although p28 is the main segment that mediates azurin cell penetration, hydrophobic residues adjacent to the p28 segment in space may also be involved. For example, alanine mutation of Phe114 significantly reduced the cell-penetrating activity of azurin [25].

2.3 Inhibition of Cancer Cell Proliferation and Tumor Growth

Besides cell membrane penetration, azurin and p28 can inhibit proliferation or induce apoptosis in various cancer cell lines (Table 1). For example, only 29% of MCF-7 breast cancer cells survived after treatment with 53 μM azurin for 72 h [26] and the cell number of ZR-75-1 breast cancer cells was reduced by 44% after treatment with 100 μM p28 for 72 h [27]. Many azurin/p28-sensitive cancer cells express p53 protein and the levels of p53 protein are elevated after treatment with azurin or p28. On the contrary, azurin or p28 do not effectively induce apoptosis or cell cycle arrest in control p53-null cells [22, 26–32]. Therefore, azurin and p28 mainly inhibit cell proliferation or induce apoptosis through the p53 signaling pathway. The p53 protein is a transcription factor that plays key roles in mediation of DNA damage repair, apoptosis, and cell cycle progression through transcriptional regulation of downstream gene expression [33, 34]. It was demonstrated that azurin/p28-stabilized p53 enters the nucleus and induces expression of proapoptotic genes like Bax and Bcl-2 [26, 32] and cell cycle inhibitors like p21 and p27 [27]. Meanwhile, studies on mouse models, including MCF-7 breast tumor mice, 4T1 breast tumor mice, and Dalton's lymphoma mice, showed that azurin and p28 can efficiently inhibit tumor growth [27, 35, 36].

3 Mechanism of the Anticancer Action of Azurin

3.1 Regulation of Redox Homeostasis

Reactive oxygen species (ROS), such as superoxide (O_2^-), hydroxyl radical ($HO^{\cdot}$), and hydrogen peroxide (H_2O_2), mediate redox signaling for numerous cellular functions [37]. High levels of ROS in cells usually cause cell death [38, 39]. Therefore, modulation of ROS levels may provide

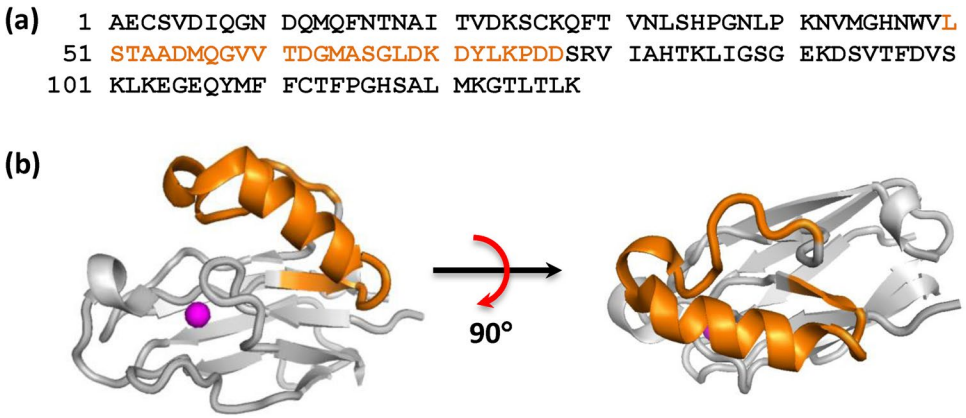

Fig. 1 Amino acid sequences and three-dimensional structures of azurin and p28. **a** Amino acid sequence of azurin. The region corresponding to p28 is shown in orange. **b** Three-dimensional structure of azurin (PDB ID 2xv2). The region corresponding to p28 is shown in orange. The copper ion is shown as a purple sphere (Color figure online)

Table 1 Responses of cancer cell lines to treatment with azurin or p28

Cancer	Cell line	p53 status	Response[a]	References
Breast cancer	MCF-7	WT	Azurin (+), p28 (+)	[22, 25–27, 30, 75, 76]
	MDA-MB-231	R280K	Azurin (+), p28 (+)	[22, 26, 29, 30, 75]
	MDA-MB-157	Null	Azurin (+)	[26]
	MDD2	Dominant-negative	Azurin (+), p28 (–)	[22, 26, 30]
	SK-BR-3	R175H	Azurin (+)	[75]
	SUM-149	M237I	Azurin (+)	[53, 54]
	T-47-D	L194F	p28 (+)	[30]
	ZR-75	WT	p28 (+)	[27, 29]
Cervical cancer	HeLa	WT	Azurin (+)	[25]
Colon cancer	HCT-116	WT	p28 (+)	[30]
	HT-29	R273H	Azurin (+), p28 (–)	[25, 30]
Dalton's lymphoma	DL	WT	Azurin (+)	[35]
Fibrosarcoma	HT-1080	WT	p28 (+)	[30]
Glioblastoma	LN229	K164E	p28 (+)	[29, 30]
	U87	WT	p28 (+)	[29, 30]
Leiomyosarcoma	HTB-88	G245S	p28 (+)	[30]
Lung cancer	A549	WT	Azurin (+)	[51]
Melanoma	UISO-Mel-2	WT	Azurin (+), p28 (+)	[24, 32]
	UISO-Mel-6	Null	Azurin (–), p28 (–)	[22, 30, 32]
	Mel-23	Δ178–183	Azurin (+), p28 (+)	[22, 24, 29, 30]
	Mel-29	WT	Azurin (+), p28 (+)	[22, 24, 29, 30]
Neuroblastoma	IMR-32	WT	p28 (+)	[29]
	SK-N-BE2	C135F	p28 (+)	[29, 30]
Oral squamous carcinoma	YD-9	WT	Azurin (+)	[31]
Osteosarcoma	MG-63	Null	Azurin (–)	[31]
	TE-85	R156P	p28 (–)	[30]
Ovarian cancer	ES-2	S241F	p28 (–)	[30]
Pancreatic cancer	MIA-Paca2	R248W	p28 (–)	[30]
Prostate cancer	LNCaP	WT	p28 (+)	[29]
	DU-145	P223L, V274F	Azurin (+), p28 (+)	[29, 30, 60]
Rhabdomyosarcoma	RD	R248W	p28 (–)	[30]

(+) indicates a positive response and (–) indicates no response

[a]The responses are inferred from the results described in the cited references

strategies for cancer treatment. Because azurin is a redox protein, treatment of macrophages with azurin generates a higher level of ROS. However, the cytotoxicity of azurin is not related to its redox activity because several redox-negative azurin mutants also generated ROS and induced macrophage apoptosis [40, 41]. Mechanistic analyses revealed that azurin and its mutants form complexes with p53 and increase its protein level, suggesting that azurin regulates redox homeostasis by a p53-mediated mechanism [40–42].

3.2 Stabilization of p53 Protein

Much evidence has indicated that the anticancer activity of azurin depends on the presence of p53 protein. For example, azurin readily induces apoptosis in cancer cells expressing functional p53, but to a much lesser extent in p53-null cells [26, 32]. The p53 protein levels in cancer cells are elevated, suggesting that azurin functions through the p53 pathway. MDM2 is a major E3 ubiquitin ligase that regulates p53 degradation by binding to its N-terminal transactivation domain (p53-TAD) [43]. Although azurin interacts with p53-TAD, the estimated dissociation constant of azurin/p53-TAD complex (K_d ~ 7 μM) is much larger than that of MDM2/p53-TAD complex (K_d ~ 34 nM) [44, 45]. Furthermore, azurin, MDM2, and p53 can form a ternary complex [46]. Thus, azurin is unable to inhibit MDM2 binding to p53-TAD. The interactions between azurin and the p53 DNA-binding domain (p53-DBD) were also characterized by molecular docking and Raman spectroscopy, revealing that azurin can bind to the flexible L1 and s7–s8 loops of p53-DBD and

increase its structural stability [47–49]. The structural stability of p53-DBD may be related to the protein level and anticancer function of p53.

3.3 Modulation of Cell Membrane Properties

Cell-surface receptors regulate the structural properties of the cell membrane by modulating cell attachment to the surrounding extracellular matrix, cell shape, cell migration, and membrane stiffness [50]. Therefore, the activity of anticancer drugs is related to the expression level of these cell-surface receptors. Bernardes et al. [51] found that azurin reduces the expression level of integrin β1 and disturbs its distribution on the cell membrane of A549 lung cancer cells. Recently, azurin was found to decrease the level of caveolin-1 and the order of the cell membrane in MCF-7 breast cancer cells and HeLa cervical cancer cells [25]. Direct physical interactions between caveolin-1 and azurin were confirmed by immunoprecipitation [25]. Changes to the surface structure of cancer cells by azurin treatment render the cells more vulnerable to anticancer drugs, such as epidermal growth factor receptor-specific inhibitors (e.g., gefitinib, erlotinib) and chemotherapeutic drugs (e.g., paclitaxel, doxorubicin) [25, 51]. Cadherins are crucial molecules that regulate cell–cell adhesion, and overexpression of P-cadherin is associated with increased cell invasion in many breast cancer cells [52]. Bernardes and colleagues showed that azurin decreases P-cadherin expression and inhibits P-cadherin-induced cell invasion [53, 54]. The modulation of cell membrane properties by azurin may also be associated with the intracellular signaling responses of non-receptor tyrosine kinases, because the phosphorylation levels of FAK, Src, Akt, and PI3K are usually attenuated [51, 53].

3.4 Interference with the Eph-Ephrin Pathway

Eph receptors comprise the largest family of receptor tyrosine kinases. The signaling pathways between Eph receptors and their ephrin ligands are known to be involved in cancer progression [55]. Therefore, Eph receptors are potential targets for cancer therapy [56–59]. Azurin shows remarkable structure similarity to ephrins and binds to EphB2 with an affinity ($K_d = 6$ nM) that is 5-fold higher than the affinity of ephrinB2 for EphB2 ($K_d = 30$ nM) [60]. Consequently, azurin efficiently competes with ephrinB2 for binding to EphB2 and interferes with tyrosine phosphorylation of EphB2. Truncation experiments identified a C-terminal segment (amino acids 88–113) of azurin that mediates the interactions between azurin and EphB2. Further experiments showed that azurin peptide (88–113) treatment leads to reduced cell viability in LN-229 glioblastoma cells and growth inhibition in MCF-7 breast cancer cells [60].

4 Mechanism of the Anticancer Action of p28

4.1 Stabilization of p53 Protein

The anticancer activity of p28 is also dependent on the p53 status in cancer cells [27, 28]. Similar to azurin, p28 does not compete with MDM2 for binding to p53 [27]. However, Yamada et al. [22] found that p28 interacts with p53-DBD. Utilizing a variety of biophysical characterization methods, the dissociation constant for p28/p53-DBD complex was reported to range from 7 μM to 0.7 nM [61–63]. p53-DBD is a hub domain that interacts with COP1, an E3 ubiquitin ligase that negatively regulates p53 and is overexpressed in many cancers [64–67]. GST pull-down experiments showed that p28 reduces COP1 binding to p53-DBD in a concentration-dependent manner, suggesting that p28 competes with COP1 for binding to p53-DBD [22]. Fluorescence resonance energy transfer-derived distance information was used to guide molecular docking and molecular dynamics simulations. In the simulated models, p28 binds to a pocket adjacent to Trp146 on p53-DBD [61], confirming that p28 competes with COP1 for binding to p53-DBD [22]. Recently, the dissociation constant for p53/COP1 complex measured by atomic force microscopy and surface plasmon resonance was determined as approximately 10 nM [68], and is thus in a comparable range to the K_d value of p28/p53-DBD complex. Therefore, one possible mechanism for the stabilization of p53 by p28 is through the COP1-mediated ubiquitination pathway, wherein p28 inhibits COP1 binding to p53-DBD. Besides wild-type p53, p28 also binds to p53 mutants and activates p53 mutants in a series of cancer cell lines [30, 63]. The affinity of p28 for p53 mutants was found to be positively correlated with the β-sheet content and negatively correlated with the random coil content of p53 [63]. Rational designs of p28 can be performed to achieve high affinity for various p53 mutants.

4.2 Inhibition of Angiogenesis

Vascular endothelial growth factor (VEGF) is a type of cytokine that promotes angiogenesis, and is often overexpressed in solid tumors or blood cancers [69]. The interaction between VEGFA and its receptor VEGFR-2 is a key regulator of angiogenesis in tumors [70]. Mehta et al. [71] found that p28 inhibits VEGF-induced migration, capillary tube formation, and neoangiogenesis in multiple xenograft models. Although p28 penetrates human umbilical vein endothelial cells and co-localizes with VEGFR-2, unlike other antiangiogenic agents that inhibit VEGFR-2 kinase

activity, p28 decreases downstream phosphorylation of FAK and Akt, resulting in abnormal distribution of cell migration-related proteins, such as F-actin, paxillin, and PECAM-1 [71]. Therefore, p28 may inhibit angiogenesis in a different manner from other antiangiogenic agents. Further studies on the interactions between p28 and VEGFR-2 will be valuable to clarify the antiangiogenic effect of p28 on endothelial cells. Inhibition of angiogenesis suppresses tumor growth in Mel-6 (p53 null) melanoma cell xenografts in athymic mice [71], while p28 has little effect on proliferation of these melanoma cells [30].

5 Therapeutic Strategies Based on Azurin and p28

Azurin and p28 preferentially enter cancer cells, and subsequently induce apoptosis or cell cycle arrest, or inhibit angiogenesis in tumors. Based on these anticancer activities, several therapeutic strategies have been designed.

5.1 Therapeutic Drugs

Azurin and p28 exhibit anticancer activity that has been verified in various cancer cells and mouse-based tumor models. Thus, azurin and p28 can be directly applied as anticancer drugs. *Escherichia coli* Nissle 1917 has been extensively used to treat acute diarrhea and possesses tumor-targeting activity [72]. Continuous expression of azurin in Nissle 1917 was shown to enable efficient inhibition of B16 melanoma and 4T1 breast tumor growth in mouse models [36]. Simultaneous expression of azurin with other anticancer agents has also been examined. For example, Ghasemi-Dehkordi et al. [73] designed a vector to express azurin and Mammaglobin-A and induce immune responses against breast cancer tumors, while Mehta et al. [74] designed a bacterial carrier that simultaneously expresses azurin and p53 under the control of a hypoxic promoter. Recently, two phase I clinical trials of p28 have been completed, with findings that confirm the anticancer activity and safety of p28 in human cancer patients. One clinical trial was carried out in 15 adult patients with metastatic solid tumors [17]. After p28 treatment, seven patients demonstrated stable disease for 7–61 weeks, three showed partial response, and one showed complete response. The other clinical trial was performed in children with brain tumors [18]. The results showed that p28 is safe and well tolerated, although its activity is not very high for central nervous system malignancies.

5.2 Cancer-Targeted Drug Carriers

The preferential entry of azurin and p28 into cancer cells enables them to function as cancer-targeted drug carriers. Azurin and p28 have been fused with several anticancer proteins/peptides to increase their activity. Granzyme B is released by the immune system and activates pro-apoptotic pathways. Paydarnia et al. [75] designed a granzyme B-azurin fusion protein and showed that the resulting protein induces significant apoptosis in several breast cancer cell lines. Upon fusion with p28, the NRC peptide and apoptin show higher anticancer activities toward breast cancer cells [76, 77]. Shahbazi et al. [78] fused HPV16 E7 protein with p28, and demonstrated that the resulting fusion protein efficiently targets cervical cancer cells and exerts immune activity. Furthermore, conjugation of the C-terminus of azurin to radiotherapy drugs enables ephrin receptor-targeted delivery, thereby improving the efficacy of radiation treatment for cancers overexpressing ephrin receptors [79]. p28 can also be conjugated to other cargos, such as liposomes and nanoparticles, enabling cancer-targeted drug delivery and release [5, 14].

5.3 Anticancer Drug Sensitizers

Besides directly inducing apoptosis and growth inhibition of cancer cells, azurin and p28 also disturb the membrane structure and inhibit cell migration, thus enhancing the sensitivity of cancer cells to anticancer drugs. For example, Bernardes and colleagues found that combined application with azurin enhances the sensitivity of A549 lung cancer cells to gefitinib and erlotinib [51], as well as the sensitivity of MCF-7 breast cancer cells, HeLa cervical cancer cells, and HT-29 colon cancer cells to paclitaxel and doxorubicin [25]. Yamada et al. [29] found that combined application with p28 improves the activity of DNA damage drugs and antimitotic drugs in a variety of cancer cells. Oral squamous carcinoma cells are resistant to many anticancer drugs. Choi et al. [31] showed that azurin treatment provides a way to enhance sensitivity to anticancer drugs, because the activities of 5-fluorouracil and etoposide in YD-9 oral squamous carcinoma cells are significantly increased after azurin treatment.

6 Conclusions and Future Perspectives

Cancer development is a complex process that involves many different factors. Consequently, it is difficult to achieve an ideal therapeutic effect using a single anticancer drug. Investigations on azurin and p28 during the last two decades have demonstrated that azurin and p28 are multi-target anticancer agents that can interfere with several different signaling pathways. Thus, azurin and p28 can induce apoptosis and cell cycle arrest by stabilizing p53 protein, inhibit downstream phosphorylation signaling pathways by binding with various receptor tyrosine kinases, and modulate the

cell surface structure by interacting with lipid raft components. Therefore, azurin and p28 can be applied as anticancer agents alone or synergistically with other anticancer drugs. Furthermore, after conjugation to liposomes or nanoparticles, p28 has the potential to achieve cancer-targeted drug delivery. Although binding affinities have been measured for azurin/p28 and several of their binding partners, structure information on azurin/p28 in complex with these binding partners is extremely limited. It is urgently required to obtain the structures of p28/p53-DBD complex and azurin/p53-DBD complex to further understand the functions of azurin and p28 as well as perform rational designs for p28 to improve its activity. In addition, the discovery of azurin-like anticancer proteins will provide valuable information toward understanding the activities of azurin and p28 and clues toward improving these activities [80, 81].

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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