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# High-throughput molecular profiling of a P-cadherin overexpressing breast cancer model reveals new targets for the anti-cancer bacterial protein azurin

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ABSTRACT

Azurin is a bacterial protein from *Pseudomonas aeruginosa* which exerts an inhibitory activity in cancer cells. In P-cadherin-overexpressing models, a bad prognosis marker in breast cancer increasing invasion and other malignant features, azurin decreases the invasion of cancer cells.

We performed a microarray analysis to compare the expression profile of azurin treated cells with different P-cadherin expression levels. Azurin up-regulated apoptosis mediated by p53 protein, endocytosis and vesicle-mediated transport. In the contrary, in invasive MCF-7/AZ.Pcad cells, azurin decreased the expression of genes associated with cell surface receptors and signal transduction, as well as biological adhesion. Further, azurin decreased adhesion of cells to proteins from the extracellular matrix (ECM) and altered protein expression of integrins α6, β4 and β1 and interfered with the ability of these cells to form mammospheres. Altogether, our results further enlighten the anti-cancer effects mediated by azurin in P-cadherin overexpression breast cancer models.



## 1. Introduction

Azurin is a protein secreted by *Pseudomonas aeruginosa*, which has already been demonstrated that harbors *in vitro* and *in vivo* anti-tumor progression properties (Yamada et al., 2002, 2004, 2009; Punj et al., 2003). This protein demonstrates the ability to penetrate preferentially in cancer cells compared to normal cells and induces a cytotoxic response that mostly relies in a p53-dependent apoptosis-induction. Recently, it has been demonstrated that some chemical compounds, commonly used to inhibit the caveosome or late endosome formation, or that remove cholesterol from cell membranes, were significantly effective inhibiting the azurin penetration in cancer cells (Yamada et al., 2009; Taylor et al., 2009).

P-cadherin is a cell–cell adhesion molecule that has been shown to promote aggressiveness in epithelial breast cancers and is recognized as marker for poor prognosis and worse patient survival (Paredes et al., 2005, 2007). In a context of wild-type E-cadherin, P-cadherin expression induces invasion and promotes the expression and activity of matrix metalloproteinases (MMPs) 1 and 2 (Ribeiro et al., 2010). In fact, P-cadherin exerts an inhibitory role for normal E-cadherin functional properties, which prevent tumor invasion. Recently, in mouse models of highly invasive breast cancers that co-express both cadherins, knocking down one of the cadherins rendered the cells to a less tumorigenic behavior when compared to their simultaneous presence (Ribeiro et al., 2012). Moreover, analyzing a series of tumor samples, the co-expression of both cadherins was associated with a poor clinical outcome of the patients (Ribeiro et al., 2012). In the subgroup of basal-like breast cancers, where P-cadherin expression is of particular importance, this protein is also associated with the phenotype of cancer stem cells, being co-expressed with CD44 and CD49f (α6 integrin). Cell populations enriched for P-cadherin demonstrated a higher ability to grow in anchorage-independent conditions, as mammospheres, than those with a lower P-cadherin expression; its presence still conferred resistance to X-ray-induced cell death (Vieira et al., 2012).

We and others have shown different levels of action for azurin or its derived peptide in different models. In a previous study, we addressed the possible effects in an invasive model of breast

*Abbreviations:* E-cad, E-cadherin (or epithelial cadherin); ECM, extracellular matrix; EGFR, epidermal growth factor receptor; CXCR1, chemokine (C-X-C) receptor 1; FAK, focal adhesion kinase; CCR4, chemokine (C-C motif) receptor 4; CXCR4, chemokine (C-X-C) receptor 4; MMPs, metalloproteases; P-cad, P-cadherin (or placental cadherin); VEGFR-2, vascular endothelial growth factor receptor 2.

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cancer that depends on P-cadherin. We demonstrated that azurin decreases cell invasion through Matrigel™ in P-cadherin over-expressing breast cancer cells, an effect that is associated with a specific decrease in P-cadherin protein levels and membrane localization in both p53 wild type and mutant cell lines, without affecting E-cadherin expression. Additionally, azurin decreased the activity of the extracellular protease MMP2, and the activity of Src and FAK non-receptor tyrosine kinases in the invasive cell models analyzed (Bernardes et al., 2013).

In this work, we have performed a microarray analysis 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, comparing with the mock condition. According to previous results, we showed that the entry of azurin in cancer cells most surely involves endocytic pathways that are dependent on vesicle trafficking and membrane organization, since genes associated with these cellular processes were found to be significantly up-regulated in azurin treated conditions. Also an enrichment in apoptosis-related signaling pathways was a common consequence, probably as a result of the p53 wild type status of the cell model used. Finally, we also showed that the addition of azurin to breast cancer cells, particularly to the transformed cell line that overexpresses P-cadherin, causes a down-regulation of genes coding for cell surface receptors, linked to a down-regulation of the their intracellular signaling cascades. The results suggested by the microarray analysis have been validated by some functional assays in both cell lines tested, as well as in another P-cadherin overexpressing breast cancer cell line – the triple negative basal-like SUM 149 PT, where we have also previous demonstrated that azurin reduces P-cadherin and invasion through Matrigel™ (Bernardes et al., 2013).

## 2. Methods

### 2.1. Antibodies

The following antibodies were used [integrin subunits α6 (1:200, sc-13543), β4 (1:200, sc-6629) and β1 (1:200, sc-18887) (Santa Cruz Biotechnologies)]. β-actin (1:1000, sc-1616) was used as a loading control. In order to evaluate azurin expression, an anti-azurin antibody was produced through immunization of one goat with purified azurin, obtained as described above (1:1000 dilution). The resulting immunized serum was then purified by protein A affinity chromatography (SicGen, Portugal) and purity was checked by SDS–PAGE.

### 2.2. Cell culture and growth conditions

The following human breast cancer cell models have been used in this study: MCF7/AZ [kindly provided by Prof. Marc Mareel (Ghent University, Belgium)] (Bracke et al., 1991) and SUM 149 [kindly provided by Prof. Stephen Ethier (University of Michigan, MI, USA)] (Willmarth and Ethier, 2006). Both cell lines were routinely maintained at 37 °C, 5% $CO_2$, in the following media (Invitrogen Ltd, Paisley, UK): 50% DMEM and 50% HamF12, supplemented with 10% heat-inactivated fetal bovine serum (Lonza, Basel, Switzerland), 100 IU/ml penicillin and 100 mg/ml streptomycin (Invitrogen). SUM149 medium was additionally supplemented with 5 μg/ml of insulin and 1 μg/ml of hydrocortisone (Sigma–Aldrich, St. Louis, MO, USA).

MCF-7/AZ cell line was retrovirally stable transduced to encode human P-cadherin cDNA together with EGFP (MCF-7/AZ.Pcad cell line), as previously described (Paredes et al., 2004). MCF-7/AZ.Mock cell line, encoding only EGFP, was used as a control. SUM149 cell line constitutively expresses high levels of P-cadherin. All the cell lines used express normal levels of E-cadherin.

### 2.3. Bacteria growth media and protein purification

Bacteria growth and protein expression and purification were performed as previously described in Bernardes et al. (2013).

### 2.4. cDNA microarray analysis

Total RNA from MCF-7/AZ cell lines (MCF-7/AZ.mock and MCF-7/AZ.P-cad), from treated with 100 μM of azurin or untreated conditions, was extracted with the RNeasy Extraction kit (Qiagen) (three independent samples per condition) and quality was analyzed with BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples were hybridized onto Agilent 44K microarrays with probes for the Human Genome (Agilent HPAG4112F), following the manufacturer's instructions, using the Agilent One-Color Microarray-Based Gene Expression Analysis Protocol Version 5.7 (Quick Amp Labeling Kit). After obtaining microarray images, fluorescence intensity was measured with the Agilent Feature Extraction software (version 10.5.1.1) and signal processing was performed according to the Agilent recommendations (GE1-v5_95_Feb07 Protocol). Processed signals were annotated and filtered using BRB Array Tools 4.2.1 (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differences in gene expression were assessed using Student's *t*-test implemented in BRB-tools, with a *p*-value cut-off of 0.05. Only genes with altered expression by at least 1.7-fold in relation to untreated cell lines were indicated as differentially ArrayExpress microarray database expressed. A hierarchical clustering method was applied to identify groups of differentially expressed genes (DEGs) between untreated and treated samples. DAVID software was used to analyze gene ontology and pathway enrichment (http://david.abcc.ncifcrf.gov) (Huang da et al., 2009). The Gene Expression Omnibus (GEO) acession number for the microarray data associated with this paper is GSE54319.

### 2.5. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA from MCF-7/AZ and SUM149 cell lines was extracted using the RNeasy Extraction kit (Qiagen), according to the manufacturer's instructions. Samples were subjected to treatment with DNase (Qiagen) during the extraction procedure. A list of probes is provided in the Supplementary Data. Assay relative quantifications between treated and untreated samples were determined by the ΔΔCt method using the internal standard human *GAPDH* (Hs.PT.51.19405051) to normalize for cDNA quantity.

### 2.6. Protein extraction and Western blot analysis

Protein extraction and Western blot were performed as previously described (Bernardes et al., 2013). For a more detailed please see the Supplementary Data.

### 2.7. Adhesion assay to ECM substrates

Cell adhesion was performed in 96-well plates coated with laminin 332 (Sigma–Aldrich, St. Louis, MO), fibronectin (Sigma–Aldrich), type-I or IV collagen (Sigma–Aldrich) (5 μg/ml) overnight at 4 °C. Afterwards, plates were washed three times with PBS and non-specific binding sites were blocked with 0.5% BSA (w/v) in PBS containing PenStrep (Invitrogen) for 2 h at 37 °C. After washing again, 100 μL of untreated or azurin (50 or 100 μM; 48 h) treated cells ($10^6$ cells/ml) were seeded in serum-free media for 30 min. Non-adherent cells were removed by washing plates three times with PBS, and the attached cells were fixed with acetone:methanol (1:1) for 10 min at 4 °C. Adhesion was determined