

## The anticancer effects induced by the bacterial protein azurin: evaluation of cytotoxicity, membrane destabilization and cell death in multiple cancer cell models

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### ABSTRACT

Azurin is a protein secreted by the bacterium *Pseudomonas aeruginosa* which has been studied as an anticancer agent. This exploratory work aims to contribute to the elucidation of the extent of cell proliferation inhibition, membrane disruption and cell death in multiple cancer cell lines (HeLa, cervix adenocarcinoma; AGS, gastric adenocarcinoma; and U2OS, osteosarcoma) upon treatment with azurin. In order to ascertain cell proliferation MTT assays were performed. To measure membrane disruption and cell death PI-FL3 flow cytometry was employed (with non-fixed and fixed cells, respectively). After 72 h exposure to treatment (100  $\mu$ M azurin), all cancer cell lines showed a dose dependent proliferation inhibition regardless of p53 status with IC50 roughly estimated to be around 140  $\mu$ M for HeLa, 75  $\mu$ M for AGS and 70  $\mu$ M for U2OS. Azurin contributed to the destabilization of the cell membrane upon long exposures (72 h) with 16.4 $\pm$ 6.4% (HeLa), 45.9 $\pm$ 5.0% (AGS), 19.9 $\pm$ 10.6% (U2OS) and 10.3%\* (BHK, non-tumorigenic fibroblast cell line) propidium iodide (PI) permeable populations. This destabilization was superior relative to the observed cell death, with HeLa, AGS, U2OS cells presenting 6.9 $\pm$ 3.2%, 14.9 $\pm$ 4.6%, 6.8 $\pm$ 3.9% hypoploid populations, respectively, (corresponding to dead cells with fragmented DNA) upon treatment with 100  $\mu$ M at 72 h of azurin WT against the 4.6 $\pm$ 0.8% hypoploid population in BHK cells. Thus, in this work it was observed that azurin has an anti-proliferative (cytostatic) effect in the tested cell lines with apparent membrane disruption in the population. Although present, azurin appears to have limited cytotoxic effects by itself and, given its diffuse mode of action, it seems more reasonable to use azurin as a co-adjuvant with a synergistic effect, in order to enhance the efficacy of other anti-cancer drugs and possibly to surpass multidrug resistance events, rather than as a standalone therapeutic.

**Key words:** Azurin, anti-cancer therapies, cell death, membrane destabilization.

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### 1 Introduction

Cancer, also commonly named neoplasm or malignant tumour, is a group of diseases characterized as the uncontrolled growth/multiplication of abnormal cells from any organ or tissue of the body with the ability to metastasize [1]. According to the Global Burden of Disease Study 2017 findings, cancer is the second most common human cause of death, both in the developed and developing countries, and a major health problem worldwide [2]. Conventional therapies mainly attempt to treat the disease by surgical resection, radiotherapy, and chemotherapy. However, tumour polymorphism and development of drug chemoresistance, as well as off target and treatment-related side effects, limit the efficacy of many therapeutic options as the disease progresses [3]. Significant advances have been made in the last two decades, with the development and clinical approval of targeted therapeutics such as receptor tyrosine kinase inhibitors (e.g., erlotinib in 2003) [4] and immunotherapy with personalized cancer vaccines (e.g., pembrolizumab in 2014) [5]. These compounds present much higher selectivity for cancer cells with minimum side effects compared with conventional treatment. Unfortunately, despite

the efforts, many malignancies remain impossible to treat with these approaches.

Cupredoxins such as rusticyanin, plastocyanin, azurin/azurin-like have been studied extensively for their electron transfer properties [[6], [7]]. More recently they emerged as a natural (*i.e.* not synthetically constructed) proteins with anti-cancer properties. The copper-containing redox protein azurin is a small 14 kDa protein with 128 amino acid residues naturally occurring in *P. aeruginosa*. This protein presents a characteristic single-domain signature consisting of a compact structurally rigid  $\beta$ -sandwich core, the immunoglobulin fold, formed by two main  $\beta$ -sheets made up of seven parallel and anti-parallel strands ( $\beta$ -barrel structure), which provides a large binding interface while being non-immunogenic [8], and also possesses an essentially neutral hydrophobic patch surrounding its copper site [[9], [10]] that provides a stable framework structure in the presence of disulphides enabling thermal stability retention even when its surface loops are replaced, thus opening azurin as an alternative protein scaffold.

Azurin can be readily overexpressed in *E. coli* and purified. However, it is difficult to ensure proper

endotoxin removal. Therefore, continuous efforts have been made in order to isolate specific peptide sequences with high potential in anti-cancer applications. These anticancer peptides (ACPs) candidates can be readily chemically synthesized, completely solving the endotoxin removal concern. The most well studied azurin derived peptides are p28 (amino acids 50–77) and p18, the minimal motif for the protein transduction domain (PTD) of azurin which encompass the first 18 amino acids of p28 [[8], [11]]. Truncation experiments reveal that penetration through the cell membrane is mediated mainly by the p28 region. Moreover, p28 (3 kDa) can promote payload internalization of 53 kDa cargo proteins in macrophages and melanoma cells [12].

Azurin preferentially enters a variety of human cancer cells (UISO-Mel2, melanoma; DU145, prostate cancer; SKOV-3, ovarian cancer; A549, lung cancer; MFC-7, breast cancer) while it is inefficiently internalized in their normal cell counterparts [[11], [12]]. The Yamada *et al.* (2005) studies suggest that azurin internalization is mediated, at least in part, by a receptor-mediated endocytic process [12] and the Taylor *et al.* (2009) assays with inhibitors points that membrane micro domains, caveolae-mediated endocytosis and the Golgi complex are of great importance in p28 and azurin cell penetration. [13].

Azurin is a versatile protein that interferes in several independent signalling pathways associated with cancer progression, such as the stabilization of p53 protein, the interference with the Eph-Ephrin receptor tyrosine kinase pathways [14], the modulation of cell membrane properties and invasion, and the extracellular suppression of tumour angiogenesis [15]. It has been suggested that this promiscuous behaviour happens due to its low binding affinity for its targets, thus allowing it to have the potential to become new anticancer drug not easily susceptible to induce cancer resistance [16].

Besides cell membrane penetration and interference in several independent signalling pathways associated with cancer progression, azurin and its derived peptides have been shown to inhibit proliferation or induce apoptosis in various cancer cell lines. Cancer sensitivity to azurin appears to be closely related with p53+ status; however there is a wide range of sensitivity even for p53 WT cancer lines. In vivo experiments in murine models also support the activity of these protein/peptides to produce an effective alternative to conventional chemotherapy treatments. In addition, recent studies successfully employed azurin to target solid tumours in murine models and following the safety profile of azurin and derived peptides two phase I clinical trials with p28 have been proposed and recently completed, with findings confirming anticancer activity and safety of the peptide in human cancer patients [[17], [18]].

Based on these anti-proliferative activities and preferential targeting/entry of azurin and derived peptides into cancer cells several therapeutic strategies have been designed. The use of chimeric proteins containing azurin or p28 in conjunction with other cytotoxic peptides for selective entrance into cancer cells [[19], [20]] or conjugates with increased radiotherapy sensitivity in tumours [21]. Also, the combined therapeutic effects of the azurin and bacteria with tumour-targeting ability, which may allow intratumour production of azurin, thus reducing dose to normal cells while concentrating the protein in the cancer tissue microenvironment [[15], [22], [23]]. Additionally, DNA recombinant vector plasmids expressing azurin [24] and nanoparticles complexed with p28 [25] vaccines strategies are being developed to induce immune responses against cancer tumours. Furthermore, combined application of azurin/p28 and broadly used chemotherapeutic agents (e.g. gefitinib, paclitaxel and doxorubicin) has been found to enhance tumour sensitivity [[26], [27], [28]] while minimising adverse effects.

The mechanisms of action of this protein are diffuse, involve increased uptake into cancer cells and metabolic modulation in multiple pathways, and for the most part are somewhat understood. But the relation between metabolic modulation, reduced proliferation and detection of apoptotic indicators (e.g. Bax/Bcl2), and actual cell death is very lacking. Thus raising the question: Do the previously observed metabolic modulation, inhibition of proliferation and increased detection of apoptotic indicators relate to actual percentage of cell death in the population? And if so, how much cell death is present upon treatment with azurin?

This work hopes to elucidate the relation between the previously observed metabolic modulation and inhibition of proliferation and the extent of cell death promoted by treatment with azurin in three cancer cell line models, HeLa (human cervix adenocarcinoma), AGS (human gastric adenocarcinoma), U2OS (human osteosarcoma).

## 2 Materials and Methods

### 2.1 Bacteria growth, over-expression, extraction and purification of WT azurin or mutated F114A protein

The continuous production of azurin was performed as described in Bernardes *et al.* (2013) [29].

#### 2.1.1 Bacteria and Growth Media

Succinctly, a previously cloned *Escherichia coli* SURE strain with the plasmid pWH844, containing the *azu* gene or the one containing the F114A mutation, from *Pseudomonas aeruginosa* PAO 1, which is responsible for the synthesis of azurin,

placed downstream of its T7 promoter was inoculated in a 250 mL Erlenmeyer flask containing 100 mL of Luria Broth medium (LB medium) and 100  $\mu$ L ampicillin at an 150  $\mu$ g/mL concentration. The SURE strain is proteases expression deficient, thus it is suitable for protein overexpression. This pre-inoculum was incubated overnight with agitation, at 250 rpm and 37 °C and cultured, in the next morning, at an initial optical density of 0.1 at 640 nm (OD<sub>640</sub>), in 3 L Erlenmeyer flasks containing 1 L Super Broth medium (SB medium; 20 g/L of yeast extract, 32 g/L of triptone and 5 g/L of NaCl) supplemented with 150  $\mu$ g/mL ampicillin with the same growing conditions. Upon reaching stable exponential growth, at an OD<sub>640</sub> of 0.6-0.8, IsoPropyl-21 $\beta$ -D-ThioGalactopyranoside (IPTG, inductor of azurin's promoter; Sigma Life Science) was added to the culture at a final concentration of 0.2 mM for Azurin WT and 0.5 mM for Azurin F114A, and the culture was left growing for an additional 4-5 h while maintaining the same conditions. After this time, cells were harvested by centrifugation (8000 rpm, 10 minutes, 4 °C; Beckman J2-MC Centrifuge); the resulting pellet was re-suspended in 15 mL of Start buffer (10 mM imidazole, 0.2 mM sodium phosphate, 0.5 M NaCl, pH 7.4). Cells were stored at -80 °C for further use.

### 2.1.2 Protein Purification

The cells were disrupted (mechanical lysis of cell walls and membranes) by sonication (Branson Sonifier Sound Enclosure 250) and the purification steps were performed by histidine affinity chromatography, using HisTrap™ HP columns (GE Healthcare), since the *azu* gene was cloned into a plasmid with a 6 histidine tail tag nucleotide sequence. Briefly, the disrupted cells were twice centrifuged (17600x g, 5 and 60 minutes, respectively, 4 °C; B. Braun Sigma-Aldrich 2K15), the pellets, consistent of cellular debris, were discarded after each cycle and the supernatant recovered. Then, the clarified extract was loaded into a 5 mL HisTrap HP column equilibrated with START buffer. Protein elution was achieved with a continuous imidazole gradient (from 20 to 500 mM) in the same buffer. After purification, the protein was immediately desalted and buffer exchanged to phosphate buffered saline (PBS; 137mM of NaCl, 2.7mM of KCl, 4.3mM of Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O and 1.47mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), in a HiPrep 26/10 Desalting column (GE Healthcare) in an ÄKTA purifier system (ÄKTA Start, Cytiva, USA), following the manufacturer's instructions. The collected protein was concentrated by centrifugation (5000 rpm, 4°C; Eppendorf Centrifuge 5804R) with Amicon Ultra Centrifugal Devices (Milipore), with a 10 kDa molecular mass cut-off. The final volume of purified protein was centrifuged in a 100 kDa cut-off filter, to remove eventual contaminants. Protein

concentration was assessed spectrophotometrically at 280 nm with the azurin specific peak at 292 nm. The purity of protein was analysed by sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE). Test spot assays were performed overnight at 37 °C (two spots with 10 $\mu$ L of azurin in a LB agarplate) to verify microbiological sterilization. Azurin was stored at 4°C until further use.

## 2.2 Cell cultures

Three human cancer cell lines and one normal baby hamster cell line was used in this work: HeLa (ATCC CCL-2, human cervix adenocarcinoma), AGS (ATCC CRL-1739, human gastric adenocarcinoma), U2OS (ATCC HTB-96, human osteosarcoma) and BHK-21 (ATCC CCL-10, hamster kidney fibroblast). All cell lines were maintained at 37 °C in 5% CO<sub>2</sub>. HeLa, U2OS, BHK-21 were grown in DMEM (GIBCO™, Paisley, UK) and AGS's was grown in (1:1) DMEM/F12 (GIBCO™) culture medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (GIBCO™), 2 mM L-glutamine (GIBCO™), 100U/mL penicillin and 100  $\mu$ g/mL streptomycin (PenStrep, GIBCO™) at 37 °C in humidified 95% air and 5% CO<sub>2</sub> (Binder CO<sub>2</sub> incubator C150). Cells were passage by chemical detaching with Trypsin 0.05% upon reaching ~80% confluence both for maintenance and for experiment initialization. 12.5  $\mu$ g/ml ciprofloxacin (Sigma-Aldrich) was added into the above mentioned culture media to avoid Mycoplasma contamination.

## 2.3 Cytotoxicity assays

### 2.3.1 MTT cell viability assay

MTT [3-(4,5 dimethylthiazol-2-yl)-2,5 tetrazolium bromide] assays were used to determine the proliferation rate of HeLa, AGS, U2OS cell lines after treatment with azurin WT and F114A. The assays were performed in 96-well plates (Corning Inc., NY, USA) (at least 3 replicates x3) with densities of 1.5  $\times$  10<sup>3</sup> HeLa cells/well, 1  $\times$  10<sup>3</sup> AGS cells/well, 1  $\times$  10<sup>3</sup> U2OS cells/well in 100  $\mu$ L culture medium. The cells were left to adhere and grow overnight at 37 °C in a humidified 95% air and 5% CO<sub>2</sub> incubator. In the next day, azurin treatments with set doses (from 25 to 200  $\mu$ M according to the specific experiment) were added from a stock concentrated solution (~650  $\mu$ M azurin in PBS); PBS and medium only controls were also set to ensure that the increased total volume and respective dilution of culture medium did not affect proliferation. The plates were placed in the incubator for 72 h at 37 °C. After, 10  $\mu$ L of MTT reagent (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C. Thereafter, the reaction was stopped by carefully removing the medium and addition of 100  $\mu$ L of spectrometric grade pure DMSO (Dimethyl sulfoxide; Sigma-Aldrich, St. Louis, MO, USA). MTT formazan

formed was spectrophotometrically read at 595 nm in a microplate reader (iMark™ microplate reader, Bio-Rad, Hercules, CA, USA).

### 2.3.2 PI Incorporation and Cell cycle analysis

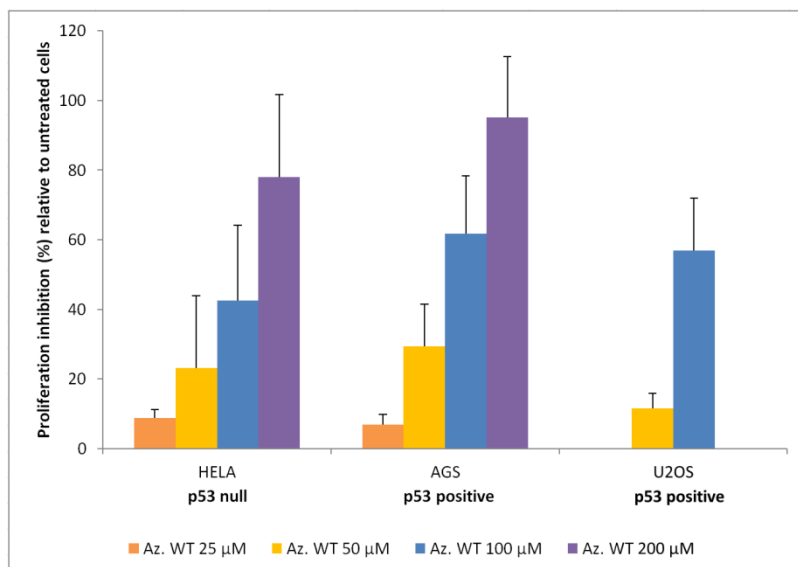
Propidium iodide (PI) incorporation assays by flow cytometry were used to determine cell membrane permeability and for quantitative determination of apoptosis through analysis of DNA fragmentation in HeLa, AGS, U2OS and BHK-21 cell lines. The cells were seeded in 24-well plates (Corning Inc.) with densities of  $2.5 \times 10^4$  HeLa cells/well,  $1.5 \times 10^4$  AGS cells/well,  $1.5 \times 10^4$  U2OS cells/well and  $1.5 \times 10^4$  BHK-21 cells/well in 1 mL. The cells were left to adhere and grow overnight at 37 °C in a humidified 95% air and 5% CO<sub>2</sub> incubator. In the next day, medium was changed and cells were treated with azurin WT or F114A at 100 μM from stock concentrated solutions (~650 μM azurin in PBS) added to the specific cell culture medium in order to achieve 500 μL of total volume. The plates were placed in the incubator for 72 h at 37 °C. After the incubation time the cells were centrifuged (Eppendorf 5804 R) (1200 rpm, 5 min). Half were then suspended in 400 μL of PBS in ice and immediately stained with 5 μg/mL PI (Sigma-Aldrich) and analysed by flow cytometry (Cytomics FC 500 Flow Cytometer, Beckman Coulter Inc., Brea, CA, EUA) for PI incorporation and the other half were fixed overnight in 700 μL of 70% ethanol at 4 °C. In the following day, cells were washed and centrifuged (Megafuge 2.0R, Controltecnica Instrumentacion Cientifica S.L., Spain) two times with PBS (1mL), incubated at least for 30 min with 1 mg/ml RNase A and 12.5 μg/ml PI at room temperature as previously

described in Gajate *et al.* (2000) [30], and then analysed for cell cycle with a Cytomics FC 500 (Brea, CA) flow cytometer. Quantitation of apoptotic cells was calculated as the percentage of cells in the sub-G1 region (hypodiploidy) in cell cycle analysis. All raw data was analysed using the Cyflogic version 1.2.1 software and compiled in a spreadsheet at Microsoft Office Excel 2010.

## 3 Results

### 3.1 Azurin inhibits proliferation in HeLa, AGS and U2OS cells

Cell viability through MTT assays decreased in a dose dependent manner, with cell arrest up to 80% for HeLa at 200 μM, 95% for AGS at 200 μM and  $57 \pm 15\%$  for U2OS at 100 μM, 72 h after treatment with azurin (**Figure 1**). Due to experimental limitations and azurin availability all further assays were performed at 100 μM, for which  $42 \pm 22\%$  and  $62 \pm 17\%$  proliferation inhibition was observed for HeLa and AGS, respectively. Values are presented as mean ± SD. Inhibition of proliferation was slightly higher for "p53 positive", with emphasis at 100 μM, although there was no statistical significance ( $p > 0.05$ ) between "p53 Null" status (HeLa) and "p53 positive" status (AGS and U2OS). All tested cell lines presented a positive dose response with a decrease in viability. This suggests that the azurin's cytotoxic/cytostatic must go beyond the activation of the p53 mediated cell arrest pathway, thus supporting the hypothesis that azurin presents a multi-targeted low specificity interaction with different metabolic pathways leading to cell viability loss.



**Figure 1** Azurin inhibits proliferation in HeLa, AGS and U2OS cells. Azurin (25, 50, 100 or 200 μM) decreases cell proliferation in a dose dependent manner.  $1.5 \times 10^3$  HeLa cells per well,  $1 \times 10^3$  AGS cells per well and  $1 \times 10^3$  U2OS cells per well were plated in 96-well plates and left to adhere overnight. In the next day, cells were treated with 25, 50, 100 or 200 μM of azurin, 100 μL of total volume. After 72 h, cell proliferation was determined by MTT assay. Results are expressed as the percentage of formazan crystals spectral absorbance at 595 nm of azurin treated cells relative to the control (untreated cells). Values are presented as mean ± SD. All bars have statistical significance related to untreated cells ( $p < 0.05$ ).

### 3.2 Azurin promotes membrane destabilization with pore formation in cancer cell lines

To evaluate the interaction and cell membrane disruption in azurin-treated cells, PI incorporation was measured by Flow cytometry in freshly harvested cells. PI is a red-fluorescent that is not permeant to live cells or cells with an intact cell membrane and it can be used to detect dead cells (PI binds to DNA). Only cells with loss of metabolic function or with abnormal pore/ micro-pore formation are positively detected to incorporate PI by Flow cytometry (FL3 wavelength). HeLa, AGS, U2OS and BHK cells presented 30.1±9.1%, 37.4±3.2%, 21.5±5.6% and 10.9±0.9% PI permeable populations, respectively, upon treatment with 100 µM azurin WT at 72 h (**Figure 2 a**). Statistical significance ( $p < 0.05$ ) in respect to the controls was achieved for AGS, U2OS and BHK, and  $p = 0,066$  for HeLa. Base control incorporation was ~12%, 17%, 9% and 1%, respectively.

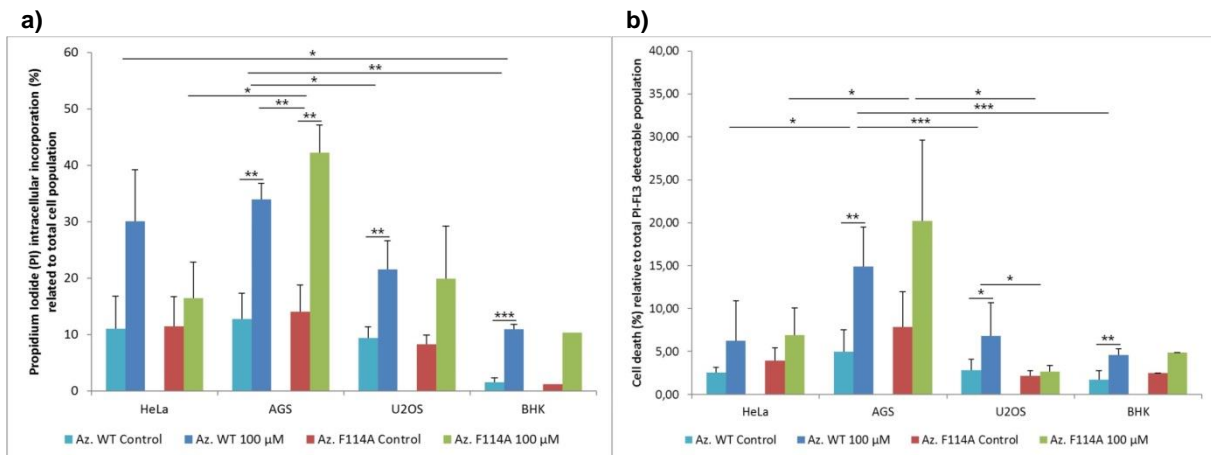
Moreover, in order to understand the role of the azurin hydrophobic patch in the formation of micro pores, the same cell lines were treated with the point mutation from the aromatic residue Phe114 to alanine (F114A; 100 µM, 72 h). Previously, Bernardes *et al.* (2018) used the azurin F114A mutant to study the role of the hydrophobic patch surrounding the copper site in the interaction of azurin WT with the lipid raft components ganglioside GM-1 and caveolin-1 in cell membrane and

caveolae-mediated endocytosis in cancer cells and its relation with induction of cell membrane disorder. The mutant produced half the penetration and, contrary of the effect of the WT, no induction of reduced membrane order was observed [26]. HeLa, AGS, U2OS and BHK cells presented 16.4±6.4%, 45.9±5.0%, 19.9±10.6% and 10.3%\* PI permeable populations, respectively.

Both azurin WT and the mutant produced an increase in membrane permeabilization relative to the controls. Also, BHK-21 cells (non-cancer hamster kidney line) were less susceptible than the cancer cell lines, with statistical significance observed relative to HeLa and AGS ( $p < 0.01$ ). However, there was no consistent preferential effect between azurin F114A and WT, across all cell lines.

### 3.3 Azurin increases the hypoploid population levels in cancer cells

In order to evaluate the cell death induced by azurin, flow cytometry assays with PI in treated cells fixed in ethanol were produced and the results are shown in **Figure 2 b**) and **Figure 3**. HeLa, AGS, U2OS and BHK cells presented 6.2±4.6%, 14.9±4.6%, 6.8±3.9% and 4.6±0.8% hypoploid populations, respectively, (corresponding to dead cells with fragmented DNA) upon treatment with 100 µM at 72 h of azurin WT. Statistical significance ( $p < 0.05$ ) in respect to the controls was achieved for AGS, U2OS and BHK.



**Figure 2** Effect of azurin WT and F114A in Propidium Iodide (PI) intracellular incorporation and in the cell death. **a)** Azurin WT and F114A at 100 µM differentially increased PI incorporation levels in HeLa, AGS, and U2OS versus BHK cells. After 72 h of incubation time at 37 °C, the cells were centrifuged (1200 rpm, 5 min), suspended in 400 µL of PBS in ice and immediately stained with 5 µg/mL PI and analysed by PI-FL3 flow cytometry. Results are presented as the percentage of PI positive relative to the total cell population for the given cell line; **b)** Azurin WT at 100 µM increases the hypoploid population levels (apoptotic or late-necrotic cells) in cancer cell lines (HeLa, AGS, and U2OS) more than in the non-cancer cell line (BHK), with emphasis in the Cav-1/p53<sup>+</sup> AGS primary gastric cancer line; Azurin F114A at 100 µM increases the hypoploid population levels in all cell lines similarly to azurin WT ( $p > 0.1$ ). Results are presented as the percentage of the hypoploid population (sub-G0/G1) in relation to the total population by PI-FL3 flow cytometry. In the experiments,  $2.5 \times 10^4$  HeLa cells per well,  $1.5 \times 10^4$  AGS cells per well,  $1.5 \times 10^4$  U2OS cells per well and  $1.5 \times 10^4$  BHK-21 cells per well were plated in 24-well plates with 1 mL medium and left to adhere overnight. In the next day, cells were treated with either azurin WT 100 µM or azurin F114A 100 µM, 500 µL of total volume. The dark blue bars represent cells treated with azurin WT, the green bars represent cells treated with azurin F114A and the light blue and red bars represent their respective controls (untreated cells). Values are presented as mean ± SD. The asterisks over each bar represent statistical significance related to untreated cells; the asterisks over a line connecting 2 bars represent statistical significance between those 2 conditions (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ). BHK treated with azurin F114A has no replicas thus is statistically invalid.

Also, previously Bernardes *et al.* (2018) proved a reduction in azurin penetration for short incubation times up to 2 h in cancer cells, when the protein's hydrophobic patch was disturbed [26]. To assess if said penetration reduction propagated into cell cycle abnormalities or increased cell death, the same procedure as stated in the previous paragraph was performed while using azurin F114A. HeLa, AGS, U2OS and BHK cells present 6.9±3.2%, 20.6±9.4%, 2.7±0.7% and 4.9%\* hypoploid populations, respectively, upon treatment with 100 µM at 72 h of Azurin F114A (**Figure 2 b**). Therefore, for long exposure times and for high dosage the cytotoxic effect of azurin F114A is statistically the same as the effect found with azurin WT ( $p > 0.1$  for all cell lines). These results are in line with the observations made regarding cell membrane destabilization (*cf.* section 3.2 and **Figure 2 a**). However, the extent of cell death was less than what would be expected given the relative higher proportion of destabilization of the cell membrane in the population observed across all cell lines.

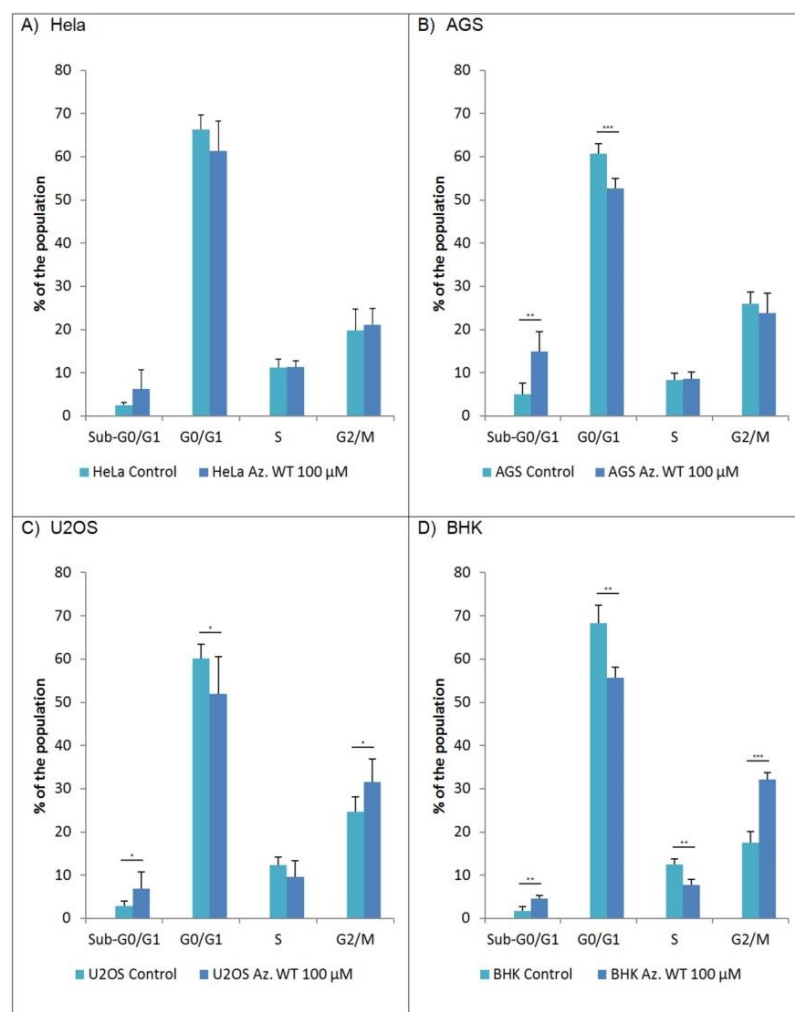
Regardless, the results point to a previously not known moderate cytotoxic effect of azurin in the AGS

cell line that might be further explored in gastric cancer therapeutics research.

#### 4 Discussion

##### Azurin is a small protein from *P. aeruginosa* shown to directly target cancer cells.

Cell death measurements using PI-Flow cytometry or equivalent have been previously assessed in breast cancer using azurin (in MCF-7, MDA-MB-231 and MDA-MB-157) [31] and p28 (in MCF-7 and MDD2) [13], osteosarcoma (U2OS; azurin) [32] and cervix cancer (HeLa; p28) [33]. In general the experiments were performed to evaluate the apoptosis of  $p53^+$  versus  $p53^-/p53^{mut}$  cancer cell lines. In the conditions that were set by the respective research groups  $p53^+$  cancer lines were the most sensitive with 20-60% while  $p53^{mut}/p53^-$  at most showed 10% cell death and more often than not there was no death at all. In regards to the extent of cell death, the present results contradict previous published data and raise some doubts on the real ability of azurin to promote apoptosis efficiently. Even taking into consideration the use of much higher



**Figure 3** Effect of azurin in the cell cycle. Azurin at 100 µM increases the hypoploid population levels (apoptotic or late-necrotic cells), in detriment of the main stage population (G0/G1), in (A) HeLa; (B) AGS; and (C) U2OS; versus (D) BHK cells.  $2.5 \times 10^4$  HeLa cells per well,  $1.5 \times 10^4$  AGS cells per well,  $1.5 \times 10^4$  U2OS cells per well and  $1.5 \times 10^4$  BHK-21 cells per well were plated in 24-well plates with 1 mL medium and left to adhere overnight. In the next day, cells were treated with azurin 100 µM, 500 µL of total volume. After 72 h, the cells were harvested and fixed in ethanol 70% at 4 °C. Results are presented as the percentage of the total population encompassed in each cell cycle phase (sub-G0/G1, G0/G1, S and G2/M) by PI-FL3 flow cytometry. The dark bars represent cells treated with azurin and light bars represent the controls (untreated cells). Values are presented as mean ± SD. The asterisks over each bar represent statistical significance related to untreated cells; the asterisks over a line connecting 2 bars represent statistical significance between those 2 conditions (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

azurin concentrations and longer treatment durations than in previous research, there was less cell death than expected for p53<sup>+</sup> cancer cells (AGS and U2OS) and more cell death than expected for the p53<sup>-</sup> cells (HeLa) (**Figure 2 b**). Note that HeLa was the only cancer line that did not achieve statistical significance ( $p < 0.05$ ) relative to the controls (untreated cells). For unknown reasons, in this work, the observed cell death in U2OS induced by azurin was far inferior (~7% vs. ~35% with 100  $\mu$ M, 72 h vs. 14.3  $\mu$ M, 48 h, respectively) than the one observed in the findings produced by Yang *et al.* (2005) [32]. Possible explanations for this disparity might be specific experimental setups and laboratory intrinsic variabilities. The experiments in this work were carried out in the presence of ciprofloxacin, a Mycoplasma inhibitor in order to avoid contamination, which could affect cell sensitivity to azurin in both ways (some cells might become very sensitive to certain drugs, while others become resistant).

#### **Consistency of azurin in inhibition of proliferation vs inductor of apoptosis**

According to Hanahan and Weinberg [34], cancer cells exhibit six important physiology changes: (1) self-sufficiency in signals of growth, (2) insensitivity to signals inhibiting growth, (3) resistance to apoptosis, (4) unlimited proliferative potential, (5) sustained angiogenesis and (6) metastasis. Regardless of the p53 status, all cancer lines demonstrated a significant dose dependent inhibition in proliferation measured through MTT assays (**Figure 1**). Therefore, it appears that the cytostatic effect of azurin is mostly independent of p53 status, but the cytotoxic effects leading to cell loss of function (cell death) are dependant. Thus, it is reasonable to consider that most other metabolic pathways deemed to be involved in the anti-cancer activity of azurin are more related with inhibition of proliferation without apoptosis (or any other cell death mechanism) than with direct cytotoxic action of the protein.

Prior findings support this notion that the properties of azurin involved in anti-cancer progression rely in mechanisms beside direct induction of cell death and reversion of resistance to apoptosis (3), such as interference with the Eph-Ephrin pathway (4) [35], tumour angiogenesis suppression (5) [36] and modulation of cell membrane and adhesion/invasion properties (2 and 6) [[29], [37], [38]]. Neither of which are directly related with total loss of cellular function, but rather cell arrest due to modulation of gene transcription, endogenous and exogenous inhibition of proliferation and morphological disturbances impairing metastatic phenotypes both at the membrane and cytoskeletal levels.

In this work, the azurin treatment achieved very low induction of cell death in cancer cell lines (**Figure 2 b**), in itself insufficient to be considered as an effective standalone drug to be used in cancer therapies in the future. However, the combined modest cell death induction with the much more accentuated anti-proliferative effect might prove to be effective in slowing, or even arresting (in especially azurin sensitive tumours), the progression of the disease.

#### **Loss of membrane integrity in cancer cell lines**

Previously, Bernardes *et al.* (2016 and 2018) demonstrated that azurin modulates membrane properties of lung cancer cells with altered morphological features, namely a reduced Young's modulus (E) and an increase in cell area, height and volume, analysed by Atomic Force Microscopy (AFM) imaging and Nano-indentation measurements [27] and increased membrane fluidity in colon (HT-29), cervix (HeLa) and breast (MCF-7) cancer lines, due to the azurin's interaction with the lipid raft components ganglioside GM-1 and caveolin-1 [26]. Regardless of how altered were the membrane properties, direct measurements of integrity remained undetermined.

The results hereby presented demonstrate the loss of membrane integrity with substantial increase in population permeability to propidium iodide (PI) for all cancer cell lines, whereas the non-cancer cell line (BHK), employed as a control, presented a smaller PI permeable population (**Figure 2 a**). Previous studies with MCF-7 and IUSO-Mel-2 cancer cell lines shown that short term (10 min) exposure to azurin does not lead to membrane pore formation even at very high concentrations (250  $\mu$ M azurin, LDH assay) [11]. Therefore, there must be an intrinsic mechanism through which azurin promotes cellular membrane instability. This pore/micro-pore formation seems to be the result of intracellular metabolic modulation related with membrane integrity rather than direct destabilization of the membrane due to membrane adsorption. If adsorption, owing to the high concentration treatment, were the main contributor to membrane destabilization and pore formation then all cell lines (cancer and non-cancer) would have been similarly affected. The intracellular metabolic modulation hypothesis is supported by some previous publications [[11], [27]].

Interestingly, the azurin mutant F114A, previously shown to have lower fluorescence resonance energy transfer (FRET) efficiency to the Caveolin Scaffolding Domain (17% vs. 35% for the WT) and not decrease membrane order [26], produced similar membrane permeabilization to PI in the population as the WT (**Figure 2 a**). This further suggests intracellular metabolic modulation as a mechanism of membrane destabilization that is, at least partially, independent

of direct interaction with lipid raft components and depletion of Caveolin. Notethat although the uptake rate of azurin F114A mutant in cancer cells is lower than it is for the WT counterpart, for long treatment durations the total uptake imbalance becomes much less accentuated [26], and thus, with longer exposures, the intracellular effects of the mutant should become similar to the WT ones.

#### **Comparison of cancer versus non-cancer cell lines**

In the current work, azurin shows preferential effect towards the promotion of cytoplasmic membrane disruption and cell death (**Figure 2**) in cancer cell lines (HeLa, AGS and U2OS) compared with the non-cancer cell line, BHK. This goes in accordance with the findings by Yamada *et al.* (2005 and 2009) where the researchers found a strong preference in internalization of azurin and p28 in diverse cancer lines in detriment of their non-cancerous counterparts [[11], [12]]. Additionally, there is evidence that azurin has the ability to induce apoptosis once inside normal cells. Upon microinjection with the protein (7  $\mu\text{M}$ , 0.5 s injection time and 100 hPa pressure) normal fibroblast and MCF-10F cells (non-cancer cell line) present significant nuclear condensation and fragmentation death and membrane disruption observed between the cancer and non-cancer cell lines. However, experiments in non-cancer cell lines with intracellular levels of azurin comparable with those found inside cancer lines upon azurin have never been made (*i.e.* are not in the literature).

Note that, the previous notion that caveolae-mediated endocytosis in Lipid rafts is central to the internalization of azurin [[11], [26]] might be incomplete at least for very long treatment durations. The AGS Cell line does not express Caveolin-1 (Cav-1) (beyond what is measurable through Western Blot and RT-PCR) or any Caveolin subunit [39], but it is the most sensitive line from the ones tested and the intracellular content Western Blot of treated AGS clearly shows that azurin is present intracellularly (result not shown). It is relevant to point that the aforementioned researchers tested for 1 h maximum exposures and at <20  $\mu\text{M}$  concentrations and not 100  $\mu\text{M}$  at 72 h, as is the case in the current work.

#### **Azurin is a natural protein with anti-cancer activity comparable and compatible with most alternative natural occurring molecules of bacterial origin.**

In general, anti-cancer drugs with high affinity to a specific target can either induce death (or indirect toxicity) in normal cells or chemo resistance in the highly metabolic/genetically instable cancer cells [40]. Thus, in the past decades, new approaches have been thought in order to attempt to surpass these hurdles. Many, as is the case of azurin, revolve around the use of natural occurring

molecules, proteins and peptides of bacterial origin, with cytotoxic properties, such as antibiotics (*e.g.* Actinomycin D from *Actinomyces antibioticus*; and Doxorubicin from *Streptomyces peuceitius* var. *caesius*), toxins (*e.g.* Botulinum neurotoxin type A from *C. botulinum*; Diphtheria toxin from *C. diphtheriae*; and Exotoxin A from *P. aeruginosa*), enzymes (*e.g.* Arginine deiminase from *Mycoplasma hominis* and *M. arginine*; and L-asparaginase from *Escherichia coli* and *Erwinia* sp.), and proteins/peptides involved in metabolism (*e.g.* Entap from *Enterococcus* sp.; and Pep27anal2 from *S. pneumoniae*) [41].

As previously stated, azurin has a diffuse mode of action leading to its cytotoxic and cytostatic properties in cancer cells. This promiscuity for its target receptors, as well as the preferential intake observed in cancer cells with apparently minor side effects, points to possible novel approaches to cancer therapies with reduced risk of resistance to therapy.

The results show that azurin has little toxicity towards the non-cancer cell line (**Figure 2 b**), while having a higher statistically significant toxicity towards the p53<sup>+</sup> lines, especially in AGS. In addition, all cancer cell lines display extensive proliferative inhibition, with natural tendency for increased inhibition in the lines where toxicity is higher. Therefore, it is arguable (given the unfortunate organizational inability to procure enough azurin to perform MTT assays for the non-cancer cell line) that azurin even at high doses<sup>1</sup> (100  $\mu\text{M}$ ) does not present major proliferative disruption in normal cells, while producing at least its anti-proliferative effects in its targets. This allows for avenues where azurin could be used in very high doses, assuming an otherwise compliance with safety, in order to inhibit tumour proliferation *in vivo*. Thus, potentially avoiding the common side effects present in the current clinically used cytostatic drugs, such as Doxorubicin (DOX), which also inhibits normal cell proliferation leading, for example, to alopecia or damage to most naturally proliferative epithelia [40]. The most highly effective and widely used in medicine antitumor active antibiotics, besides DOX: Actinomycin D (dactinomycin), Bleomycin (BLM) and Mitomycin C; have the same problems regarding side effects as DOX. Bernardes *et al* (2018) [26] demonstrated synergistic anti-proliferative effect between azurin and DOX in HT-29 colon cancer cells. For the same durations and concentrations used in this present work it was possible to archive the same loss of viability with a fifth of the concentration of DOX. Thus, azurin might be used to reduce traditional chemotherapeutic doses while

<sup>1</sup> The maximum dose in clinical trials using the derived peptide p28 has been ~1.4  $\mu\text{M}$  per kg bodyweight [17].



providing the same anti-tumour benefits, with the added reduction of the former's side effects.

Additionally, according to author of the present work, azurin and the derivate peptide p28 appear to possess very interesting similarities to the analogues of Pep27, a secreted peptide that initiates the cell death program in *S. pneumoniae* through signal transduction, especially Pep27anal2. While the anticancer cytotoxic activity of Pep27anal2 is reported to be caspase and cytochrome *c* independent, this 3.3 kDa peptide adopts a stable  $\alpha$ -helical conformation in solutions and presents increased hydrophobicity, which appears to play an important role in its membrane permeabilization as a cell-penetrating peptide (CPP) [42], just as azurin and p28 have been reported to be [[11], [12], [43]].

#### **Azurin as a targeting tumour targeting molecule**

As stated immediately above, azurin as a CPP has been demonstrated be able to deliver cargo to the interior of cancer cells. Previously, the cancer cells penetration ability of azurin/p28 containing complexes has been closely associated with the notion of the amphipathic characteristics associated with the protein and the presence of the hydrophobic patch surrounding the copper binding active centre [[9], [10]] and its interaction with caveolin-1 in lipid rafts [[11], [26]]. As stated before in this discussion, caveolae-mediated endocytosis in Lipid rafts might provide an incomplete picture in the internalization of azurin in cancer cells given the results obtained for the AGS cell line (**Figure 2**). These observations combined open prospects to use azurin or p28 as cancer targeting molecules even for cancer devoid of caveolin-1 expression.

#### **Azurin mode of action and its relevance in cancer therapy**

Azurin by itself seems to have cytostatic and some cytotoxic properties, although the direct cytotoxic effects are somewhat lacking, at least in accordance to the results hereby presented (**Figure 1** and **Figure 2**). Azurin appears to have a diffuse mode of action and given the degree of cytostatic/cytotoxic effects it seems more reasonable to be used as a co-adjuvant with other anti-cancer drugs, such as DOX or Paclitaxel (PTX) than stand alone as observed by Bernardes *et al.* (2016 and 2018) whom proposed the use of azurin in cancer therapeutics as a mean of dose reduction in widely used chemotherapeutic drugs in hopes of mitigating the severe side effects of said drugs [[26], [27]]. Also the low toxicity observed towards non-cancer cells allows azurin for use in therapeutic settings if economically viability and regulatory approval is achievable. In other words, evidence points that azurin might have tangible benefits in tumour progression *in vitro* and *in vivo* models and the clinical trials preformed to the moment using the

derived peptide p28 [[17], [18]], for all intents and purposes indicate safety in the use of the protein/peptide, thus there is good reason to contemplate their use as co-adjuvant or as a biotechnological platform in other to deliver chemotherapeutic constructs selectively to tumours.

## **5 Conclusions**

Azurin is a small protein from *P. aeruginosa* shown to directly target cancer cells, with extensive metabolic alterations in multiple pathways. Previously azurin has been deemed a cytostatic drug and this work corroborates said finding in all studied cell lines. Although the metabolic differential effects of azurin in cancer cells are somewhat understood its relation with total cell death are not. This study elucidates the extent of cell death promoted by azurin in three cancer cell lines (HeLa, AGS and U2OS). The results obtained in this work support previous research results about the metabolic actions that azurin elicits in cancer cells, however some findings contradict them, such as the amount of death in U2OS. These discrepancies may be related to specific experimental setups and laboratory intrinsic variabilities (e.g. specific azurin treatment regiment, azurin stability after transport from Lisbon to Madrid), which could have significantly affected the outcome of the experiments. The adding of ciprofloxacin to the culture media ensured the avoidance of Mycoplasma contamination in the current work. Membrane integrity was evaluated and it was confirmed that azurin disrupts cell membranes and furthermore disrupts differentially cancer lines membranes. By itself the protein seems to have very relevant cytostatic properties mediated through several metabolic pathways and some cytotoxic properties, although the direct cytotoxic effects are somewhat lacking, at least in accordance to the results obtained in the current work. However, a previously not known moderate cytotoxic effect of azurin was observed in the AGS cell line, which might be further explored in gastric cancer therapeutics research. Azurin appears to have a diffuse mode of action and given the degree of cytostatic/cytotoxic effects that were observed, it seems more reasonable to use this protein as a co-adjuvant with other anti-cancer drugs than stand alone. As a cell-penetrating protein, azurin can also be used as a tumour targeting molecule and since in this exploratory work it has shown to produce the most cytotoxic effect in a Cav-1- cell line, it is likely that the targeting mechanism is effective beyond the previous found association with caveolin-1 mediated vesicular endocytosis in lipid rafts. Future avenues of research in biotechnology pose azurin as an engineering enabler suitable for development for targeted pharmaceuticals such as transfection of tumour tropic Mesenchymal Stem

Cells, bioengineering of truncation products and bioactive nanoparticles.

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