Azurin interferes with lipid raft organization by decreasing the membrane protein caveolin – 1

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Abstract

Azurin, produced by *Pseudomonas aeruginosa,* acts as an anticancer agent and enters human cancer cells mediated by the amino acids 50-77 (peptide p28), via caveolae-mediated endocytic pathway, co-localized with caveolin-1. Azurin up-regulates genes associated with endosome formation, membrane organization and lipid transport and localization. Caveolae are involved in the cellular mechanisms deregulated in tumor cells and high caveolin-1 level is associated with several cancer metastases. These studies lead to the hypothesis that by being endocyted through caveolae to enter cells, azurin may delocalize lipid rafts and remove the membrane receptors located there, reducing the signaling through which they promote cancer progression.

In this work the breast cancer cell line SUM-149 and the lung cancer cell line A549 were studied. The similar effect in adhesion observed upon the treatment of these cells with MβCD and azurin suggests that treatment with azurin leads to an internalization of lipid rafts, which was confirmed by staining with CTxB. The level of caveolin-1 in cancer cells upon azurin treatment was also studied, showing an initial increment of caveolin-1 levels. However, at 24 hours of exposition, the total levels of caveolin-1 are decreased. Finally, it is shown by immunofluorescence that after 24h of azurin treatment, azurin and caveolin-1 still co-localize and it was shown by immunoprecipitation that these proteins interact by binding each other or forming a complex with an intermediary. The reduction of caveolin-1 level in tumor cells may contribute to a diminished aggressive tumor behavior.

Key-words: Azurin, caveolin-1, lipid rafts, breast cancer, lung cancer

Introduction

Azurin, a cupredoxin produced by *Pseudomonas aeruginosa,* can act as anticancer agent. Its entry in human cancer cells is mediated by the amino acids 50-77 (peptide p28) (Yamada *et al.,* 2005). p28 and azurin seem to penetrate the plasma membrane via caveolae-mediated endocytic pathway and reach late endosomes, lysosomes, and the Golgi associated with caveolae (Taylor *et al.,* 2009). p28 also preferentially penetrates human umbilical vein endothelial cells, co-localized with caveolin-1 (Mehta *et al.*, 2011). It was recently found that azurin up-regulates genes associated with vesicle-mediated transport, endosome formation, membrane organization, lipid transport and localization (Bernardes et al., 2014). Lipid rafts are involved in cellular mechanisms deregulated in tumor cells, as altered protein signaling and trafficking and enhanced cell migratory potential (Staubach & Hanisch, 2011), being potential targets in cancer cells. Signal transduction attenuation following caveolae disruption has been reported in the case of several signaling cascades. Lipid rafts are also involved in endocytosis, promoting

internalization of receptors and signaling molecules. Various classes of signaling molecules bind caveolin-1 through its caveolin scaffolding domain (Williams *et al.,* 2004). Also, high caveolin-1 level is associated with several tumor metastases (Ho *et al.* 2002).

These studies may lead to the hypothesis that the mechanism by which azurin blocks tumor progression is due to the disruption of lipid rafts. By using preferentially endocytosis through caveolae to enter cells, azurin may remove the membrane receptors there located, reducing the signaling through which they promote cancer progression. In this study, two cancer models were studied, the breast cancer cell line SUM-149 and the lung cancer cell line A549. It was observed a similar effect in adhesion upon the treatment of these cells with a cholesterol depleting agent and azurin, suggesting that azurin treatment leads to delocalization of lipid rafts. This result was confirmed by staining cells' lipid rafts with CTxB. The level of caveolin-1 in cancer cells upon azurin treatment was also studied, showing an initial increment of caveolin-1 levels. Though, at 24h of azurin exposition, the total levels of caveolin-1 are decreased. Finally, it is shown by immunofluorescence that after 24h of azurin treatment, azurin and caveolin-1 still co-localize and it was shown by immunoprecipitation that these proteins interact by binding each other. The reduction of the level of caveolin-1 in tumor cells may contribute to a diminished aggressive tumor behavior.

Materials and methods

Human cancer cell lines and cell cultures

Two human cancer cell models were used: the lung cancer cell line A549 and the breast cancer cell line SUM-149. The cell line A549 was maintained in F-12 (Gibco, Invitrogen Ltd); supplemented with 10% of fetal bovine serum (FBS) (Lonza), 100IU/mL penicillin and 100mg/mL streptomycin (Invitrogen). The cell line SUM-149 was maintained in in DMEM-F12 (1:1 v/v) medium, supplemented with 1µg/mL hydrocortisone, 5µg/mL insulin (Sigma-Aldrich-Aldrich), 5% of heat-inactivated FBS (Lonza), 50IU/mL penicillin and 50mg/mL streptomycin (PenStrep, Invitrogen). Both cell lines were grown at 37ºC in a humidified chamber containing 5% CO_2 (Binder CO_2 incubator C150).

Bacteria growth, over-expression, extraction and purification of azurin

This method was performed as described by (Bernardes *et al.*, 2013). Briefly, it was made a pre-inoculum with LB medium, ampicillin and an inoculum of *E. coli* SURE, cloned with the plasmid pWH844, containing the gene *azu*. This culture was grown over-night (O.N.). Then the culture was grown in SB medium, supplemented with ampicillin until reach an OD_{640} of 0.6-0.8, when the azurin expression was induced with IPTG. After 4-5h, the cells were recovered by centrifugation, ressuspended in Start buffer, and kept at -80ºC. To purify azurin, cells were disrupt by sonication and centrifuged. It was used a histidine affinity column to purify azurin, that was eluted with increased concentrations of imidazole. Next, the buffer was exchanged to PBS in ÄKTA system with a desalting column. The collected protein was concentrated, detoxified with a detoxing column and concentrated again. The concentration was estimated according to the absorbance at 280nm, using the Lambert-Beer equation. Azurin was stored at 4ºC until further use.

Adhesion assay to ECM components

This method was performed as described by (Bernardes et al., 2014). Briefly, both cell lines (A549 and SUM-149) were plated in flasks with or without collagen type-I and left to adhere O.N.. Then, cells washed with PBS, collected with trypsine and treated with metil-β-ciclodextrin (MβCD) 5mM in simple medium during 30 minutes. Cells untreated were the controls. Different components from the ECM (laminin-332, collagen type-I, and fibronectin) were coated in a 96-well plate and non-specific binding sites were blocked with BSA. Cells were plated in the coating plates and left to adhere to the ECM components during 30 minutes. After washed with PBS, the adherent cells were fixed with paraformaldehyde, stained with crystal violet and the dye was dissolved in ethanol. The absorbance was read at 570nm to quantify crystal violet staining. The analysis of the adhesion assay was made using control absorbance as 100% of staining, meaning 100% of adhesion.

Confocal microscopy

Cholera Toxin Subunit B (CTxB)

A549 cells and SUM-149 cells were seeded on a round glass coverslip, with or without collagen type-I (1mg/mL, Millipore), in 24-well plates with $5x10^4$ cells and left to adhere in a CO_2 incubator at 37ºC. The following day, they were treated with 100μM of azurin in complete medium. Untreated cells were the control condition. After 24h, cells were treated with CTxB (Invitrogen, Alexa Fluor 594 conjugate) (1μg/mL) during 10 minutes. Afterwards, coverslips were rinsed with PBS three times. For fixation, cells were immersed in 4% formaldehyde for 20 minutes at room temperature. After washed three times in PBS, cells in coverslips were mounted with Vectashield with DAPI (Vector Inc., Burlingame, CA, USA) and observed in confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany; model no. DMI6000), with a 63 X water (1.2-numerical-aperture) apochromatic objective.

Immunocytochemistry

This method was performed as described by (Bernardes et al., 2013). Briefly, A549 cells and SUM-149 cells were seeded on a round glass coverslip in 24-well plates and left to adhere O.N.. Then, cells were treated with azurin 100μM in complete medium. Untreated cells were the control condition. After 24h, cells were rinsed with PBS and fixed with formaldehyde. After wash in PBS, cells were immersed in ammonium chloride and washed in PBS again. To permeabilize, the cells were immersed in Triton X-100. For immunostaining, cells were blocked with BSA. Then cells were incubated with primary antibody (1:400 anti-caveolin-1 and 1:600 anti-azurin), washed in PBS and incubated in 1:500 secondary antibody (Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-goat, Invitrogen). After washed in PBS, cells in coverslips were mounted with Vectashield with DAPI and observed in confocal microscope, with a 63 X water (1.2-numerical-aperture) apochromatic objective.

Protein extraction and Western Blot

This method was performed as described by (Bernardes et al., 2013). Cells of cell line A549 and of the cell line SUM-149 were plated in plastic or in the collagen-I matrix, and left to adhere O.N.. Then, cells were treated with azurin (50μM or 100μM), during the intended time (30 minutes, 2h, 8h, 24h or 48h). Cells were washed with PBS, lysed in catenin lysis buffer with phosphatases inhibitor and

proteases inhibitor mixture. After lyses, the cells were scratched, collected, vortexed, centrifuged and quantified by Bradford method. 20µg of total protein per sample were denatured and separated by electrophoresis in a SDS-PAGE. Gels were transferred onto nitrocellulose membranes, using Trans-Blot Turbo Transfer System. After blocking the non-specific binding sites with non-fat dry milk, the membranes were incubated in an agitator O.N. at 4ºC with different primary antibodies (anti-actin 1:1000 and anti-caveolin-1 1:500). The membranes were washed with PBS-tween-20 and probed with secondary antibody, conjugated with horseradish peroxidase (anti-goat for actin; anti-rabbit for caveolin-1, both diluted 1:2000). After washed, the membranes were developed by adding ECL substrates and capture the chemiluminescence by Fusion Solo equipment. The band intensity was measured using ImageJ and results are present as the ratio between the signal intensities in azurin treated samples to untreated cells. The protein levels were normalized by the respective actin level.

Co-immunoprecipitation

Cells of cell lines A549 and SUM-149 were plated with $7{,}5x10^5$ cells in 6-well plate respectively and left to adhere and grow O.N. at 37ºC. Then, cells were treated with 100µM of azurin, during the intended time (24h for SUM-149 cell line and 48h for A549 cell line). Cells untreated were the control condition. The wells with the cells, treated or not with azurin, were then washed twice with PBS, lysed in 100µL of catenin lysis buffer (1% Triton X-100, 1% Nonidet-P40 in deionized PBS) with 1:100 phosphatases inhibitor (Cocktail 3, Sigma-Aldrich) and 1:7 proteases inhibitor (Roche Diagnostics GmbH, Germany) for 10 minutes at 4ºC. After that time, the cells were scratched, collected, vortexed three times (10 seconds each), centrifuged (14000rpm, 4ºC, 10 min; B.Braun Sigma-Aldrich 2K15) and quantified by Bradford method (BioRad Protein Assay). Afterwards, 250µL of lysate were incubated with 10µL of primary antibody anti-caveolin-1 or anti-azurin in an agitator O.N. at 4ºC. The next day, 100µL of beads (Protein G Agarose, Thermo Scientific) were incubated with the mixture of lysate and antibodies, in an agitator during 2h at room temperature. After that time, 500µL of IP buffer (Thermo Scientific) were added, in order to precipitate the mixture, and then it was centrifuged (2500xg during 3 minutes), 10 times. At every time, the supernatant was discarded. To elute the proteins from the beads, the pellet was incubated twice with 50µL of Elution Buffer (Thermo Scientific), each time during 5 minutes, and then it was centrifuged (2500xg during 2 minutes) and the supernatant was recovered. To neutralize the supernatant, 10µL of Neutralization Buffer (Thermo Scientific) were added. To the pellet, that contains the beads, 60µL of sample buffer were added and to the supernatant with the Neutralization Buffer it was added 30µL of sample buffer. 20µL per sample were denatured at 95ºC during 5 minutes, and then separated by electrophoresis in a SDS-PAGE. WB was performed as previously described.

Statistical analysis

For *in vitro* experiments, at least one independent replicate was performed (n=1 to 4 sample/experiment). All *p*-values were calculated using Student's t-test (two-tailed distribution, two-sample equal variance). Values of $p<0.05$ were considered statistically significant (*: $p<0.05$).

Results

MβCD and azurin have similar impact on cell adhesion to ECM components

Previous work from our group has shown that azurin decreases the adhesion of the breast cancer cell line SUM-149 and the lung cancer cell line A549 to some ECM proteins, (Bernardes *et al*., 2014; Bernardes *et al.*, in preparation) in a dose-dependent manner. To study the hypothesis that azurin is endocyted through caveolae, having an impact on the cell lipid rafts organization it was used methyl-β-cyclodextrin (MβCD) to deplete cholesterol of the membrane, disrupting the lipid rafts, to compare with results obtained after azurin treatment. Cells of both cell lines were treated with 5mM MβCD during 30 minutes and were left to adhere to the different proteins of the ECM during another 30 minutes. BSA and plastic were used as control conditions.

The adhesion of the cell line A549 to laminin-332 is reduced by 30% and 40% when the cells are grown in plastic and collagen-I matrix, respectively (Figure 1). Although the adhesion to collagen-I and fibronectin is practically not altered when treated with MβCD when the cells are grown in plastic, there is a decrease in the adhesion to these ECM components when the cells are grown in collagen-I matrix (80% and 40%, respectively). On the cell line SUM-149, when the cells grown in plastic are treated with MβCD, the adhesion to laminin-332 and collagen-I reduced 70% and the adhesion to fibronectin diminishes 50%. When the same cell line is grown in collagen-I matrix, the adhesion to laminin-332, collagen-I and fibronectin is decreased 65%, 60% and 75%, respectively.

Comparing the adhesion of the cells of the cell line A549 grown in plastic treated with MβCD, with the obtained when the same cell line is treated with azurin (Figure 1), it is possible to see that the loss in adhesion to laminin-332 is similar when the cells are treated with MβCD or with azurin 100μM (30%). In adhesion to plastic, both treatments with MβCD or with azurin 50μM lead to a decrease of 10% in adhesion. In BSA, both treatments seem to lead to an increase of adhesion. In the other matrixes there is no difference in adhesion when cells are exposed to MβCD, although the adhesion when treated with azurin is decreased. In the adhesion of the cells of the cell line SUM-149, the treatment with MβCD have a higher impact than the treatment of the cells of the same cell line with both doses of azurin tested. The adhesion to laminin-332 of the cells treated with azurin 100μM decreases 30%, but the adhesion of the cells treated with MβCD to the same component is reduced 70%. Moreover, the adhesion to collagen-I of the cells treated with azurin 50μM is diminished 20%, whereas the effect of the treatment with MβCD is a reduction on the adhesion of 60%. In the adhesion to fibronectin, exposition to azurin 100μM leads to a decrease of adhesion of 20% and the treatment with MβCD cause a reduction of adhesion 40%, when cells grow in plastic. Nevertheless, overall it is interesting to note that the same effects are observed when cells are treated with azurin as when cells are treated with a chemical agent that depletes cholesterol. These results suggest that a possible mechanism by which azurin acts is by reducing the lipid rafts and, therefore, alter the lipid membrane composition, affecting cell adhesion to ECM components.

Azurin leads to an internalization of lipid rafts of the cells

The effect of azurin in the lipid rafts of the cell was then studied, using the Cholera Toxin

subunit B (CTxB), a marker that binds to the glycosphingolipid GM1, present in the lipid rafts. To study the impact of azurin in the lipid rafts, namely caveolae, cells of the cell lines A549 and SUM-149 were seeded on a glass coverslip, with or without collagen type-I, treated with azurin 100μM during 24h, marked with CTxB and observed in a confocal microscope (Figure 2). The nuclei were stained with DAPI (blue) and the lipid rafts were marked with CTxB (green). It is possible to observe that the cellular membrane of the untreated cells of both cell lines is specifically marked. When the cells are treated with azurin, there is internalization of the lipid rafts. This effect seems to be more pronounced when cells are in a collagen-I matrix.

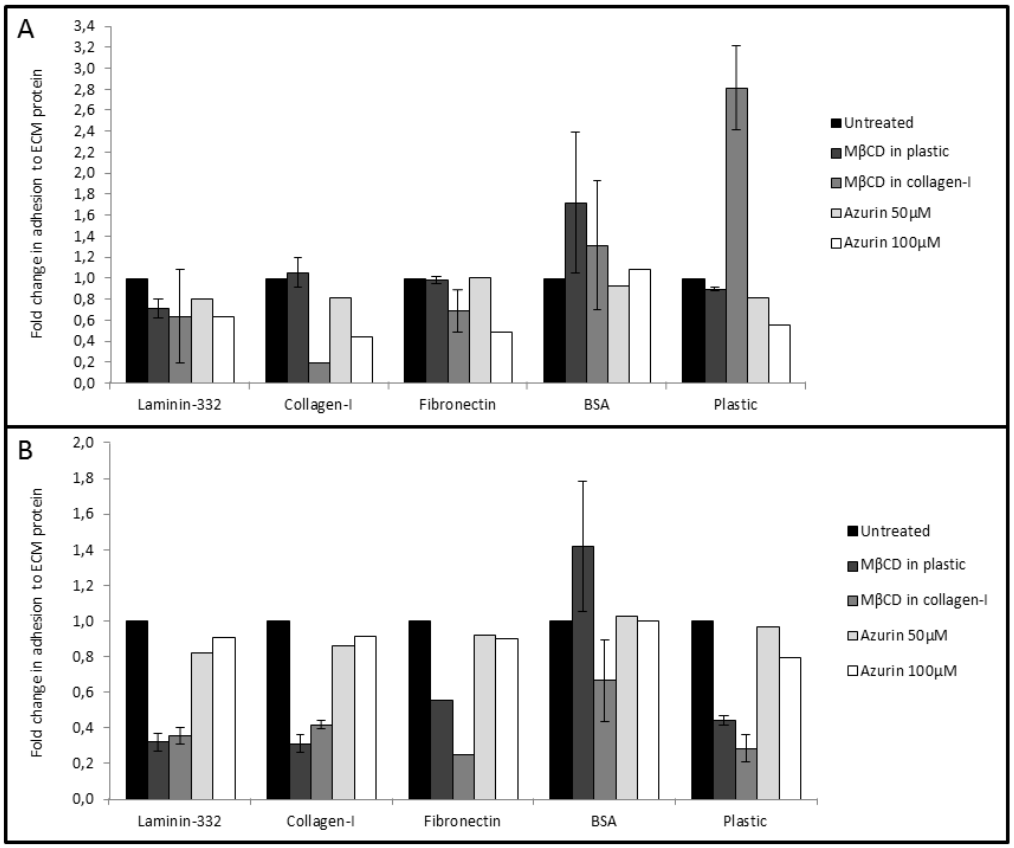

Figure 1. Comparison of the effect of 5mM methyl-β-cyclodextrin (MβCD) treatment and azurin 50 μM and 100 μM on cell adhesion of the lung cancer cell line A549 (A) and the breast cancer cell line SUM-149 (B). In the case of treatment with MβCD, A549 lung cancer cell line and SUM-149 breast cancer cell line were grown in plastic or on top of a collagen type-I matrix (1mg/mL), treated with 5mM MβCD during 30 minutes and let to adhere during 30 minutes in different ECM components. In the case of azurin exposition, cells of A549 lung cancer cell line were grown in plastic and exposed to azurin during 48h and cells of SUM-149 breast cancer cell line were grown in plastic and treated with azurin during 24h.Adapted from (Bernardes *et al*., 2014; Bernardes *et al.*, in preparation)

Azurin leads to a decrease in caveolin-1 protein levels after an initial increase

Previous results have already suggested that azurin may exert its anti-cancer effects by disruption of caveolae, removing from the cell membrane selective receptors that may be over activated (Bernardes et al., 2014). In order to assess the influence of azurin in caveolar rafts, the variation of caveolin-1, a scaffold protein of caveolae, along treatment was measured. Cells of both cell lines were left to adhere in plastic and collagen-I coated wells, and then were exposed to azurin, 50μM and 100μM, for diverse time-points. Afterwards, the cells were lysed, the total protein was quantified and it was made a WB, to verify the variation of caveolin-1.

Actin, a constitutive protein of both cell lines, was used as a control. Both cell lines were exposed to the referred concentrations of azurin protein during 30 minutes, 2h, 8h and 24h. The cell line A549 was also exposed to azurin during 48h. In the cell line A549 it is possible to see that in plastic, until 8h of exposure, the caveolin-1 levels are higher than untreated cells (Figure 3, A). In collagen-I there is a fluctuation in caveolin-1 levels during at least the first 8h of treatment with azurin, (Figure 3, B). There is an increase at 30 minutes of treatment, in which the exposure to azurin 100μM leads to a rise of 160% and 195% in plastic and collagen-I respectively. At 2h of exposition with the same azurin concentration, the content of caveolin-1 alters to 140% and 60% in plastic and collagen-I respectively. At 8h of treatment with azurin 100μM in plastic, there is an increase of 260% and in collagen-I there is an increase of 175%. Afterwards, the caveolin-1 levels diminish to 60% at both time points, 24h and 48h, when cells are grown in plastic and treated with azurin 100μM. When the cells are grown in collagen-I and treated with azurin 100μM, the caveolin-1 content is reduced to 80% and 40% at 24h and 48h, respectively. In the cell line SUM-149, the treatment with azurin leads to a variation in the caveolin-1 content of the cell (Figure 3, C and D). After 30 minutes of treatment with azurin 100μM, the caveolin-1 levels rise to 150% and 210% in plastic and collagen-I, respectively. Afterwards, the caveolin-1 levels diminish to 60%, 80% and 70% at the time points 2h, 8h and 24h, respectively, when the cells are in plastic. When the cells are in collagen-I matrix, the caveolin-1 level decrease to 65%, 70% and 40% at the time points 2h, 8h and 24h, respectively.

Azurin binds to caveolin-1

It is known that azurin enters in cancer cells co-localized with caveolin-1 (Mehta *et al.*, 2011). In this work we also show that the levels of caveolin-1 in cells treated with azurin are reduced over time (Figure 3). Therefore, it

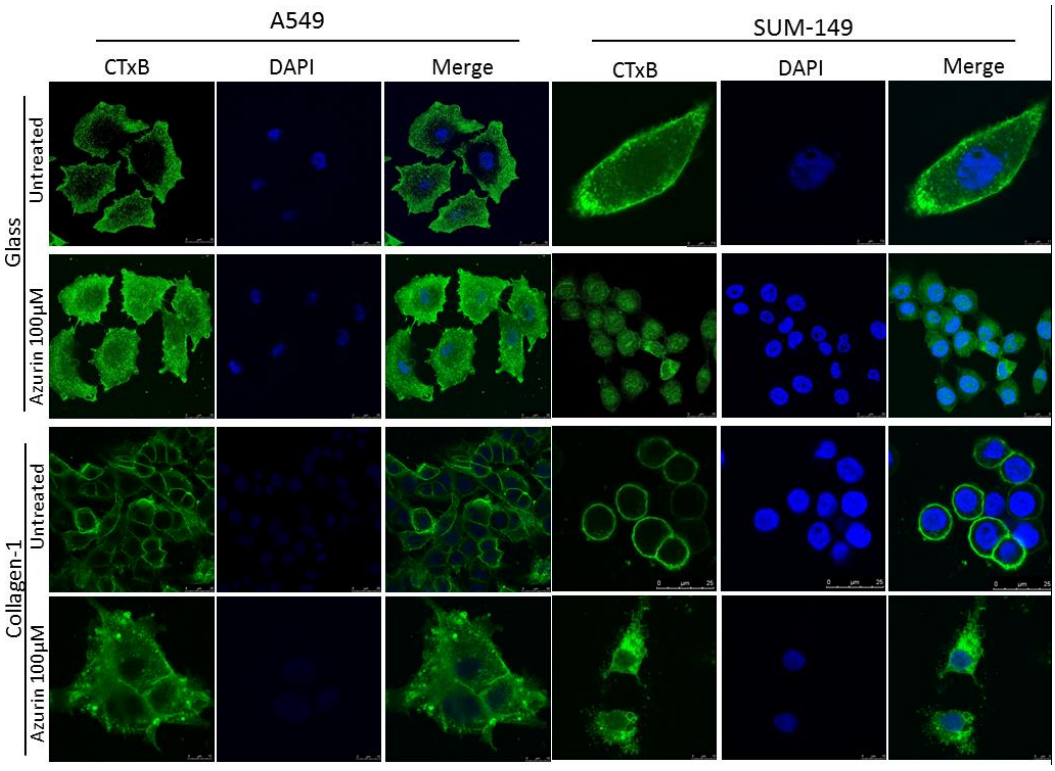

Figure 2. The effects of azurin in the cell's lipid raft organization. Cells of the cell line A549 (left panel) and of the cell line SUM-149 (right panel) were grown in plastic (upper part) and in collagen-I (lower part) and treated with azurin 100μM. The glycosphingolipid GM1 of lipid rafts is marked with CTxB (green) and the nuclei of the cells is stained with DAPI (blue).

would be interesting to verify if these proteins form a complex or bind each other, for periods longer than the initial entry process. To do so, it was performed a co-immunoprecipitation assay, in which the cell line A549 was treated with 100μM of azurin during 48h and incubated with a primary antibody anti-azurin, and SUM-149 cell line was treated with 100μM of azurin for 24h and incubated with a primary antibody anti-caveolin-1. Afterwards, the mixture was incubated with beads of Protein G Agarose, which were precipitated. The antibodies were eluted from the beads and the precipitate was analyzed by Western Blot (WB). The lysate of the cells and the beads were also analyzed as controls. As shown on WB against azurin, this protein binds to caveolin-1 in both cell lines (Figure 4). WB in the upper panel are controls. The WB represented on lower panel shows a signal in samples correspondent to the lysate of cells treated with azurin presenting a complex between azurin and caveolin-1.

To confirm this result, the localization of caveolin-1 and azurin in the cell after 24h of treatment was studied by immunocytochemistry. A549 cells and SUM-149 cells were treated with 100μM of azurin during 24h. Then cells were fixed, permeabilized and incubated with primary antibodies anti-caveolin-1 and anti-azurin. Afterwards, cells were incubated in secondary antibody and DAPI and were observed in confocal microscope.

It is possible to see that there is a delocalization of caveolin-1 from the cell membrane of some cells treated with azurin (Figure 5). It is shown that in some cells of both cell lines treated with azurin 100μM azurin and caveolin-1 co-localize.

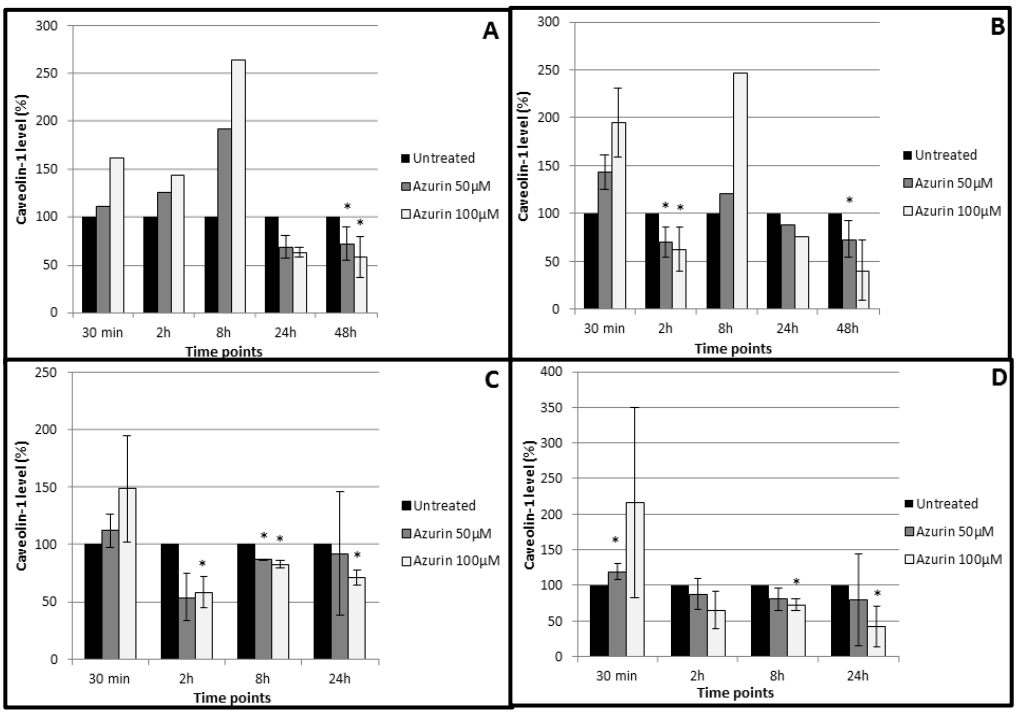

Figure 3. Influence of azurin in caveolin-1 level at several time points. A - A549 in plastic; B - A549 in collagen-1 matrix; C - SUM-149 in plastic; D - SUM-149 in collagen-1 matrix. The band intensity was measured using ImageJ and results are present as the ratio between the signal intensities in azurin treated samples to untreated cells. The protein levels were normalized by the respective actin level (*: $p<0.05$).

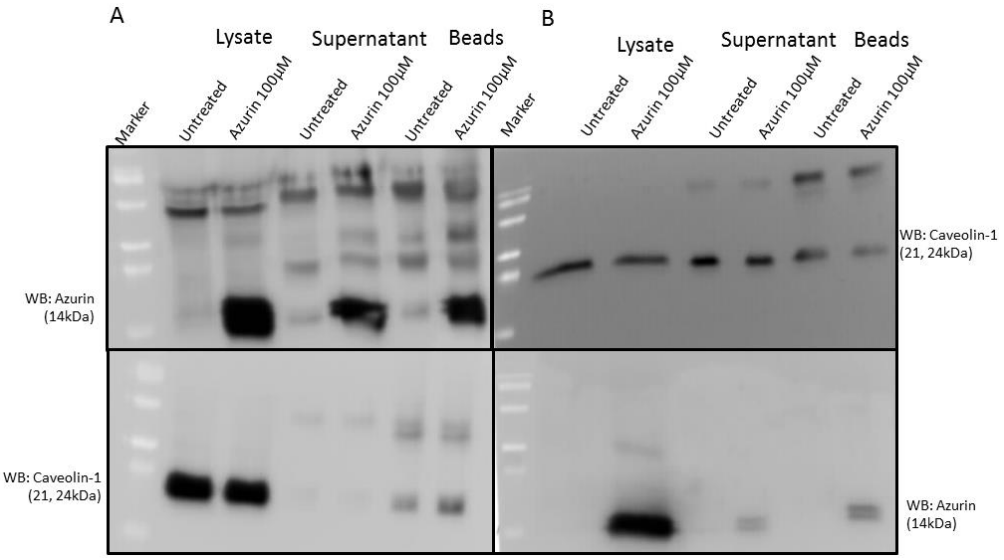

Figure 4. Azurin forms a complex with caveolin-1, probably to enter the cell. A - A549 treated with azurin 100µM during 48h, co-immunoprecipitation with antibody anti-azurin; B - SUM-149 treated with azurin 100µM during 24h, co-immunoprecipitation with antibody anti-caveolin-1.

Discussion

It is proposed that the azurin endocytosis through caveolae leads to an internalization of tumor inducers localized there reducing the signaling through which they promote cancer progression (Bernardes et al., 2014). Also, if lipid rafts are disrupted, these tumor markers do not go to the membrane and the signaling through which they promote cancer progression is reduced. To study this hypothesis, cholesterol was depleted from cell membranes to disrupt the lipid rafts (Figure 1). Adhesion is mediated by cadherins and integrins, which form focal adhesions that contact with ECM ligands (White

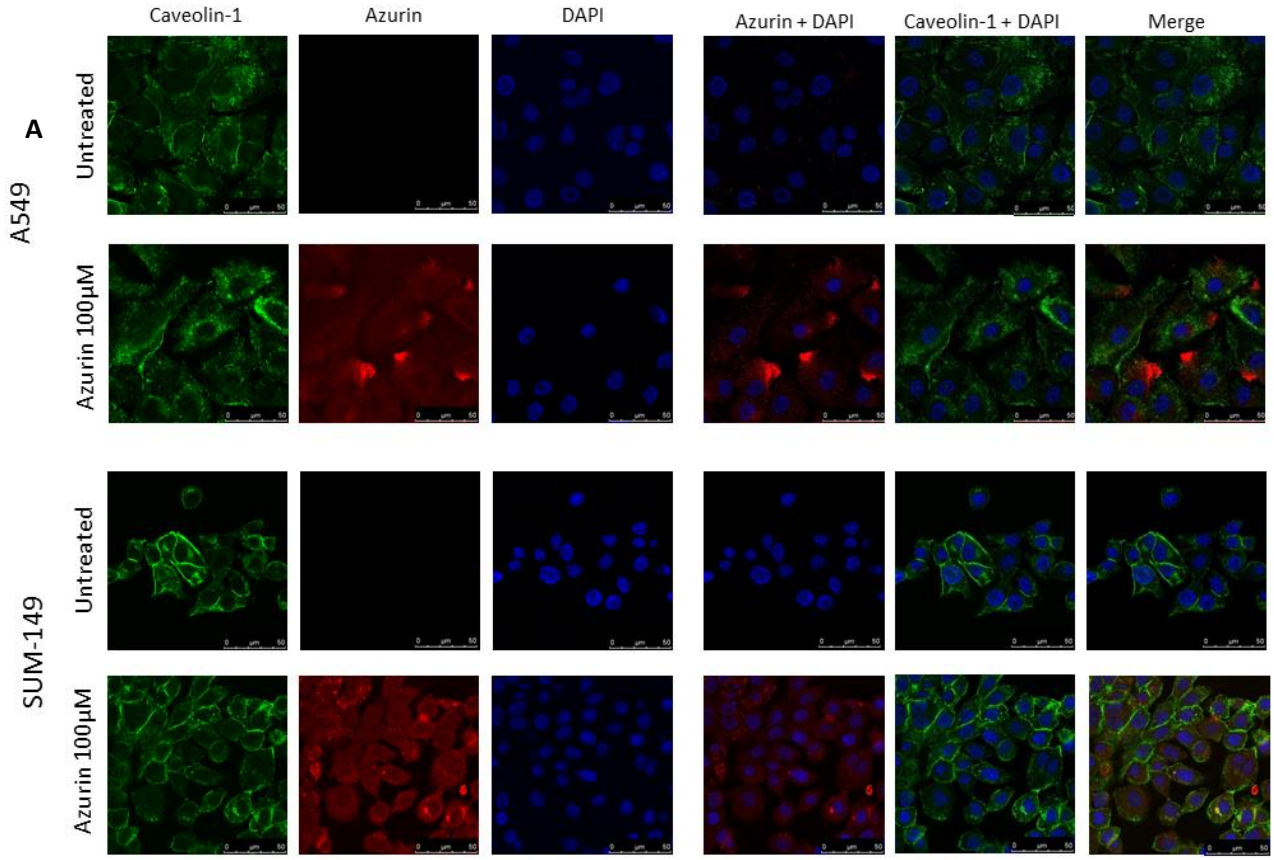

Figure 5. Azurin and caveolin-1 co-localize within the cells. A - A549 lung cancer cell line treated with azurin 100µM during 48h (upper part) and SUM-149 breast cancer cell line treated with azurin 100µM during 24h (lower part). Caveolin-1 is marked with Alexa Fluor 488 anti-rabbit (green), azurin is marked with Alexa Fluor 594 (red) and the nuclei were stained with DAPI (blue).

& Muller, 2007). It was showed that azurin leads to a decrease in some integrin receptors in breast cancer models (Bernardes et al., 2014). In this work two cancer models were treated with azurin, a breast cancer cell line and a lung cancer cell line. Despite some differences in response to the treatment, the overall response of both cell lines to the treatment was similar, meaning that azurin may have a broad effect on tumor cells. In the cell line A549, the impact of treatment with MβCD is higher when the cells are grown in collagen-I matrix than in plastic, leading to a decrease in the adhesion to the ECM components. The adhesion of the cells to the ECM components changes with their growth conditions (Shekhar *et al.*, 2003) and although the main component of lung ECM is fibronectin (Alitalo *et al.*, 1981), the collagen-I matrix gets closer to the natural environment of these cells than plastic. That may be a reason why the results of adhesion in collagen-I are more pronounced. On the other hand, in the cell line SUM-149 the diminution of cell adhesion to ECM proteins is more accentuated when treated with MβCD, with almost no differences on growth conditions (plastic or collagen-I), except in adhesion to fibronectin (Figure 1).

Comparing the adhesion of the cells of the cell line A549 grown in plastic treated with MβCD, with the obtained when the same cell line is treated with azurin 100μM (Figure 1), it is possible to see that the loss in adhesion to laminin-332 is similar. In the other matrix there is no difference in adhesion when cells are exposed to MβCD, although the adhesion when treated with azurin is decreased. However, in the adhesion of the cell line SUM-149, the treatment with MβCD has a higher impact than the treatment of the cells of the same cell line with azurin, which may be due to the concentration of MβCD used to treat the cells. Nevertheless, it is interesting to see that exposure to azurin and MβCD lead to a decrease in the adhesion of both cell lines. The reduction in adhesion observed upon the treatment of these cells with an agent that depletes the cholesterol, leading to a disruption of the lipid rafts, confirms that these structures of the cell membrane are necessary for the cells to adhere, having the motifs needed for that. Therefore, azurin may have the same impact on the cell membrane and caveolae, by inducing endocytosis through these structures.

Endocytosis is a mechanism that can lead to long-term signaling attenuation by committing receptors to degradation (Fiore *et al.*, 2003). In mammals, it was demonstrated that receptor tyrosine kinases are monoubiquitinated at multiple sites (Dikic *et al.*, 2003), a post-translational modification that can promote receptor endocytosis and targets receptors for lysosomal degradation (Haglund & Dikic, 2012).

To confirm the endocytic effect of azurin in cells' lipid rafts, the localization these motifs was then studied by confocal microscopy, using CTxB. It was possible to observe that the cell membrane of untreated cells of both cell lines is specifically marked (Figure 2). When the cells are treated with azurin, there is internalization of the lipid rafts, which confirms the hypothesis that azurin delocalizes lipid rafts, and possibly receptors, to endosomes and possible degradation. Having a role in signal attenuation, endocytosis also has influence in the factors that determine the tumor behavior of cells. It has already been suggested that azurin exerts an anti-cancer effect by entering the cell, a process that disrupt caveolae (Bernardes et al., 2014). As caveolin-1 is a scaffold protein of caveolae, the influence of azurin in caveolar rafts was studied, by measuring the variation of caveolin-1 along treatment. It was possible to see an

increase in caveolin-1 level at initial time points, but in the following time points the levels of the same protein are generally reduced in both cell lines (Figure 3).

It was already described the preferential entry of peptide p28 (amino acids 50 to 77 of azurin) on human breast cancer cell lines through a caveolin-mediated pathway (Yamada *et al.*, 2009). Moreover, it has been demonstrated that p28 preferentially penetrates human umbilical vein endothelial cells, co-localized with caveolin-1 (Mehta *et al.*, 2011). For that reason, probably the initial increment of caveolin-1 levels is due to a production and delocalization of this protein to the cell membrane, in order to increase the content of caveolae and, therefore, to augment endocytosis of azurin. After that time, caveolin-1 may be targeted to lysosomes and degraded. Consequently, the overall level of caveolin-1 in the cell is diminished at certain time of exposition and remains low.

Despite some studies show an anti-tumor activity of caveolin-1, this protein is also reportedly a promoter of more aggressive traits in tumor cells, such as metastasis (Ho *et al.*, 2002), promotion of anchorage-independent survival by preventing anoikis (Fiucci *et al.*, 2002) and increased multi-drug resistance, being associated with poor patient prognosis. Therefore, the reduction of the level of this protein in tumor cells may contribute to a diminished aggressive tumor behavior. Remarkably, there is a relationship between caveolin-1 and multidrug resistance (MDR). MDR is cellular resistance to drugs (Gottesman, 1993), in which cells employ mechanisms to survive the cytotoxic effect of drugs utilized in chemotherapy. MDR phenotype is associated with up-regulation of lipids that constitute caveolae, especially cholesterol (Lavie & Liscovitch, 2001). It was already shown that MDR cancer cells express very high caveolin-1 levels and exhibit a high surface density of caveolae (Yang *et al.,*1998; Lavie *et al.*, 1998; Demeule *et al.*, 2000). Also, it was demonstrated that caveolin-1 expression is correlated with drug resistance and a poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine-based chemotherapy (Ho *et al*., 2008). Therefore, having already demonstrated that azurin treatment reduces caveolin-1 levels in cells, it would be interesting to study the effect of azurin treatment in combination with several drugs, in order to see if the efficacy of these drugs would increase. Although it is known that azurin is endocyted by cancer cells through caveolae, it is not yet clear how this mechanism is triggered. It was already shown that peptides p18 and p28 account for the entry of azurin into human cancer cells, but not by binding to cell membrane glycosaminoglycans (Taylor *et al.*, 2009). In addition, studies from our group have revealed that azurin anisotropy is independent of lipid content (low *vs* high cholesterol) in artificial mammalian membrane systems (POPC/PS/Chol) (unpublished). An interaction between azurin and a membrane protein could lead to the endocytic process. There is evidence proving that azurin enters into cancer cells co-localized with caveolin-1 (Mehta *et al.*, 2011). However, until now it was not shown if the co-localization remains after azurin endocytosis. Also, it was not yet clear if these proteins directly interact and bind each other or form some complex. In this study, it is shown that after 24h of azurin exposure, azurin and caveolin-1 still co-localize (Figure 3). It was also possible to see that these proteins interact by binding each other or forming a complex with an intermediary (Figure 4). In fact, caveolin-1

interacts with many signaling molecules (Razani *et al.*, 2002). Caveolin-1 harbors the called Caveolin Scaffolding Domain (CSD), a 20 amino acid cytosolic domain derived from the N-terminal region of the protein (Couet *et al.*, 1997; Okamoto *et al.*, 1998). Caveolin-protein interactions are proposed to occur between the CSD and an aromatic-rich caveolin binding motif (CBM) on the binding partner (Couet *et al.*, 1997; Okamoto *et al.*, 1998). Interestingly, azurin harbors several aromatic aminoacids (fenilalanine and histidine), which are close when the protein is in its natural scaffold (Fialho *et al.*, 2008). The peptide p28, responsible for the preferential entry of azurin in cancer cells and shown to enter in cancer cells co-localized with caveolin-1 (Yamada *et al.*, 2009; Mehta *et al.*, 2011), has a tyrosine, which is an aromatic residue. It is possible that these regions have a role on the interaction. However, structural and bioinformatic analyses argue against such direct interactions, not only because in the majority of signaling proteins the CBM is inaccessible, but also findings suggest that interfaces between caveolin and targets may be more structurally diverse than presently appreciated (Byrne *et al.*, 2012; Collins *et al.*, 2012). To better study this interaction between azurin and caveolin-1, it would be interesting to directly mutate some aminoacids in the hydrophobic patch, including the tryptophan, and study the interaction of the mutated azurin with cancer cells. By Western Blot it would be possible to see if the mutant form of azurin would still enter in cancer cells and if the process of entry is or not different.

Conclusion and future perspectives

In this work, in the two cancer models treated with azurin it was observed a similar effect, showing that azurin has a broad effect on tumor cells. The reduction in adhesion observed upon the treatment of these cells with an agent that depletes the cholesterol and azurin confirms the hypothesis that these motifs of the cell membrane are necessary for the cells to adhere. Therefore, azurin has the same impact on the cell membrane and caveolae, by inducing endocytosis through these structures, leading to internalization of the lipid rafts. Having a role in signal attenuation, endocytosis also has influence in the factors that determine the tumor behavior of cells. The level of caveolin-1 in cancer cells along azurin treatment was studied, showing an initial increase in caveolin-1 level, but afterwards the levels of the same protein are generally reduced in both cell lines. Probably the initial increment of caveolin-1 levels is due to a production and delocalization of this protein to the cell membrane, in order to augment endocytosis of azurin. After that time, caveolin-1 may be targeted to lysosomes and degraded. Consequently, the overall level of caveolin-1 in the cell remains low. Caveolin-1 is reportedly a promoter of more aggressive traits in tumor cells, being associated with poor patient prognosis. Therefore, the reduction of the level of this protein in tumor cells may contribute to a diminished aggressive tumor behavior.

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