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

Catarina Benavente Perdigão

Thesis to obtain the Master of Science Degree in

Biotechnology

Supervisors:

Doctor Arsénio do Carmo Sales Mendes Fialho

Doctor Nuno Filipe Santos Bernardes

Examination Committee

Chairperson: Doctor Isabel Maria de Sá Correia Leite de Almeida

Supervisor: Doctor Arsénio do Carmo Sales Mendes Fialho

Member of the Committee: Doctor Miguel Nobre Parreira Cacho Teixeira

October, 2014

“What we know is a drop, what we don't know is an ocean.”

Isaac Newton

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor Arsénio Fialho, who gave me this opportunity and helped me and guided me throughout my thesis.

I want to thank Nuno Bernardes for all the teaching, help and support during lab work. Thank you, Nuno, for your patience and good mood.

I also want to thank all BSRG for the help, especially to Sofia Abreu, Dalila Mil-Homens and Daniela Ferreira.

In addition, I would like to thank Fábio Fernandes, who gave me support with the confocal microscopy and shared ideas and advices about the work.

To my friends, those who are close to me every day, and the one who is far away but always there, I want to thank. Clara and Sara, all those chocolate croissants and laughs made the joy of some days.

Last but definitely not the least, I would like to thank André for being always there for me and all my family for the support, specially my sister Joana, Zé and my mother Bábá.

Thank you!

Obrigada!

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 led 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 metil-β-ciclodextrin and azurin suggests that treatment with azurin leads to an internalization of lipid rafts, which was confirmed by staining with cholera toxin subunit B. The level of caveolin-1 in cancer cells upon azurin treatment was also studied, showing an initial increment of caveolin-1 levels, possibly due to its recruitment to the cell membrane. 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

Resumo

A azurina é uma proteína produzida por *Pseudomonas aeruginosa* que actua como agente anticancerígeno e entra nas células cancerígenas humanas através dos aminoácidos 50-77 (péptido p28), por uma via endocítica mediada pelas *caveolae*, co-localizada com a caveolina-1. A azurina causa um aumento da expressão de genes associados à formação de endossomas, à organização membranar e ao transporte e localização de lípidos membranares. As *caveolae* estão envolvidas em mecanismos celulares desregulados nas células tumorais, sendo que elevados níveis de caveolina-1 estão associados à formação de metástases. O trabalho realizado no âmbito desta dissertação coloca a hipótese de que, recorrendo a endocitose preferencial através das caveolae para entrar nas células, a azurina pode deslocalizar as jangadas lipídicas e remover os receptores membranares aí localizados, reduzindo a sinalização através da qual é promovida a progressão do cancro.

Neste estudo, dois modelos de cancro foram tratados com azurina, a linha celular de cancro da mama SUM-149 e a linha celular de cancro de pulmão A549. O efeito semelhante na adesão observado tanto com o tratamento das células com metil-β-ciclodextrina como com azurina sugere que o tratamento com azurina leva a uma internalização das jangadas lipídicas, o que foi confirmado através da marcação com CTxB (sub-unidade B da toxina da cólera). O nível de caveolina-1 nas células cancerígenas aquando do tratamento com azurina também foi estudado, mostrando um aumento inicial dos níveis de caveolina-1, possivelmente devido à sua incorporação na membrana celular. No entanto, após 24h de exposição à azurina, os níveis de proteína totais de caveolina-1 estão diminuídos, quando comparados com as células não tratadas. Finalmente verifica-se, por imunofluorescência, que após 24h de tratamento com azurina, a azurina e a caveolina-1 ainda estão co-localizadas e é possível observar, por imunoprecipitação, que estas proteínas interagem, ligando-se uma à outra ou através de um intermediário. A diminuição dos níveis de caveolina-1 nas células tumorais pode contribuir para uma diminuição do comportamento agressivo tumoral.

Palavras-chave: Azurina, caveolina-1, jangadas lipídicas, cancro de mama, cancro de pulmão

Table of Contents

Index of Figures

Index of Tables

List of Abbreviations

BSA bovine serum albumine
CBM caveolin binding motif
cdk cyclin-dependent kinase
CSD caveolin scaffolding domain
CTD C-terminal domain
CTxB cholera toxin subunit B
cyt cytochrome
DBD DNA-binding domain
ECM extracellular matrix
EGFR epidermal growth factor receptor
ER endoplasmic reticulum
FBS Fetal bovine serum
GPI glycosylphosphatidylinisotol
HUVEC human umbilical vein endothelial cells
IPTG isopropyl-beta-D-thiogalactopyranoside
MβCD metil-β-ciclodextrin
MAP mitogen-activated protein
MMP matrix metalloproteinases
NTD N-terminal domain
OD_{640} optical density of 640nm
PBS phosphate buffered saline
PI3K phosphoinositide 3-kinase
RTK receptor tyrosine kinases
sP-cad soluble form of P-cadherin
WB western blot

1. Introduction

A number of bacterial proteins or peptides have been described to exert an anticancer activity towards diverse cancer cell models. Bacterial protein azurin, a cupredoxin produced by *Pseudomonas aeruginosa,* is the case study of this work. Azurin can act as an anticancer agent and demonstrates the ability to bind multiple targets in mammalian cells, both extra- and intracellular. It combines antiangiogenic and tumor cell cytotoxic effects. Azurin entry in human cancer cells is mediated by the amino acids 50-77 of the protein (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 (HUVEC), co-localized with caveolin-1, inhibiting HUVEC motility and migration (Mehta *et al.*, 2011).

It was recently found that two invasive breast cancer cell lines treated with azurin present an up-regulation in genes associated with apoptosis and with the entry mechanism of azurin (Bernardes *et al.*, 2014). By entering the cells through caveolae, azurin up-regulates genes associated with vesicle-mediated transport, endosome formation and membrane organization, common for both cell lines (Bernardes *et al.*, 2014). A class of genes up-regulated in MCF-7/AZ.Pcad related to mock control is the lipid transport and localization, suggesting that P-cadherin may be regulating the biosynthesis or localization of particular lipid molecules or receptors (Bernardes *et al.*, 2014).

Lipid rafts are actively involved in the numerous cellular mechanisms deregulated in tumor cells, as modified cell fate, altered protein signaling and trafficking, and enhanced cell migratory potential (Staubach & Hanisch, 2011), being recognized as potential targets in cancer cells. Signal transduction attenuation following lipid raft and caveolae disruption has been reported in the case of several signaling cascades. Lipid rafts are also actively involved in endocytosis, promoting internalization of receptors and signaling molecules. Various classes of signaling molecules, including G-protein subunits, receptor and non-receptor tyrosine kinases, endothelial nitric oxide synthase, and small GTPases, bind caveolin-1 through its caveolin scaffolding domain (Williams *et al.,* 2004; Staubach & Hanisch, 2011). In addition, elevated caveolin-1 level is associated with lung, breast, prostate, and their lymph node metastases, strengthening the possibility that caveolin-1 may act as an oncogene (Ho *et al.* 2002).

All these studies may lead to the hypothesis that the mechanism by which azurin acts to block 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. Therefore, it is intended to study the endocytic route of azurin, and the consequences at the function and signaling of important cancer therapeutic targets present in the membrane of cancer cells. To evaluate the importance of these regions for azurin entry, we intend to study the influence of azurin treatment in lipid rafts, specifically in caveolae. Also, it is aimed to increase the knowledge regarding the

impact of azurin in caveolin-1 content of cancer cells, and also to study the interaction between both proteins.

2. Literature Overview

2.1. Bacterial protein azurin

Cancer therapy often leads to the problem of tumor cell resistance and the inability to eliminate micrometastases, leading to a need of new therapies. Bacteria were discovered as anticancer agents in late-nineteenth-century. However, besides the regression of tumor patients after being infected with bacteria, there was also a high mortality associated to bacterial infections at that time (Chakrabarty, 2003). Currently, the problems with systemic infections after bacteria delivery are being overcome either by using engineered attenuated bacteria with low infection capabilities or bacterial products, which are capable of targeting and specifically kill tumor cells (Bernardes *et al.*, 2010). Specific targeting of cancer cells would then allow the use of more cytotoxic products without undesired toxicity to normal tissues.

A number of bacterial proteins or peptides have been described to exert an anticancer activity towards diverse cancer cell models. Although these proteins, or peptides, are found in unrelated bacteria specimens, they seem to possess the ability to conduct cancer cells to death or to interfere with signaling pathways that drive tumor progression (Bernardes *et al*., 2010).

Pseudomonas aeruginosa is a gram-negative opportunistic pathogenic, rod-shaped bacterium, which produces azurin protein. Azurin is a single peptide chain with 128 amino acids and its blue color is given by the copper ion present in its structure (Pozdnyakova *et. al.,* 2001). This protein is a member of a group of copper-containing redox proteins called cupredoxins, produced by different aerobic bacteria as agents of electron transfer (Fialho *et. al.*, 2012). It is the simplest of all copper proteins so far discovered, having only one copper atom (type-1 copper site) (Van Pouderoyen *et al.* 1997). As shown on Figure 1, this protein is composed of one α-helix and eight β-sheets, forming a β-barrel motif. Azurin holds two potential redox centers: the T1 blue-copper ion coordinated directly to amino acid residues, and a disulfide bridge present at the opposite end of the molecule (Farver *et al*., 1982).

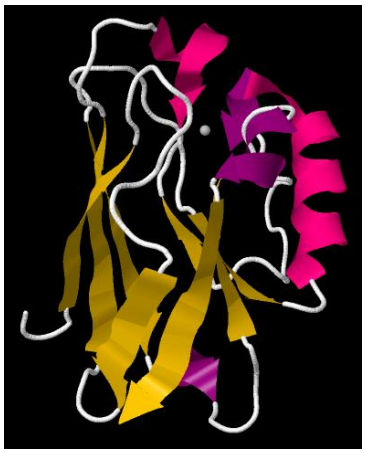

Figure 1. The three-dimensional structure of azurin from *Pseudomonas aeruginosa*.("RCSB PDB," n.d.)

Azurin is a secondary metabolite derived from bacterial species, is an electron carrier protein during respiration (Hoitink & Canters, 1992) and leads to apoptosis in cancer cells (Punj *et al.*, 2004). It transports an electron between cytochrome (cyt) c-551 and cyt oxidase in bacteria respiration process (Parr *et al.*, 1977; Silvestrini *et al.*, 1982).

2.1.1. Family of cupredoxins and azurin-like proteins

Azurin is also produced by other bacteria besides *Pseudomonas aeruginosa*. Azurins are exclusive of some members of the gamma and beta subdivisions of the Proteobacteria and belong to the cupredoxin family, as it can be seen in Figure 2 (De Rienzo *et al.*, 2000).

The family of cupredoxins is characterized by small (10-14kDa) water soluble proteins which contain at least one copper ion bound to a copper site (their active site). Some cupredoxins have a combination of four or more copper ions per molecule, of which one or more are bound to type-1 site, whose function is to shuttle electrons and catalyze di-oxygen reduction to water (Farver & Pecht, 1991). This family has a characteristic single domain: a structurally rigid β-sandwich core (immunoglobulin fold) formed by two main β-sheets made up of seven or more parallel and antiparallel strands (Greek key β-barrel structure) (De Rienzo *et al.*, 2000).

One of the azurin-like proteins is termed Laz and is uniquely found in *Neisseria* species, namely gonococci/meningococci. It is produced by the meningitis-causing bacterium *Neisseria meningitides* (Kawula *et al.*, 1987). Laz is surface-exposed (unlike the other azurins) and has a 39 amino acid epitope called H.8 in its N-terminal region. Beyond this epitope, the protein is highly homologous to the *P. aeruginosa* azurin (Hong *et al.* 2006). This epitope is responsible for entry in gliobastoma cells, an ability that azurin does not possess, because of the blood-brain barrier which restricts the uptake of various compounds including drugs into the brain (Fialho *et al.,* 2012). This epitope has already been cloned in the N-terminal part of *P. aeruginosa* azurin, allowing its entry and cytotoxic activity in glioblastoma cells (Fialho *et al.*, 2012). It may as well be useful to allow entry of other drugs in the brain (Bernardes *et al.*, 2010).

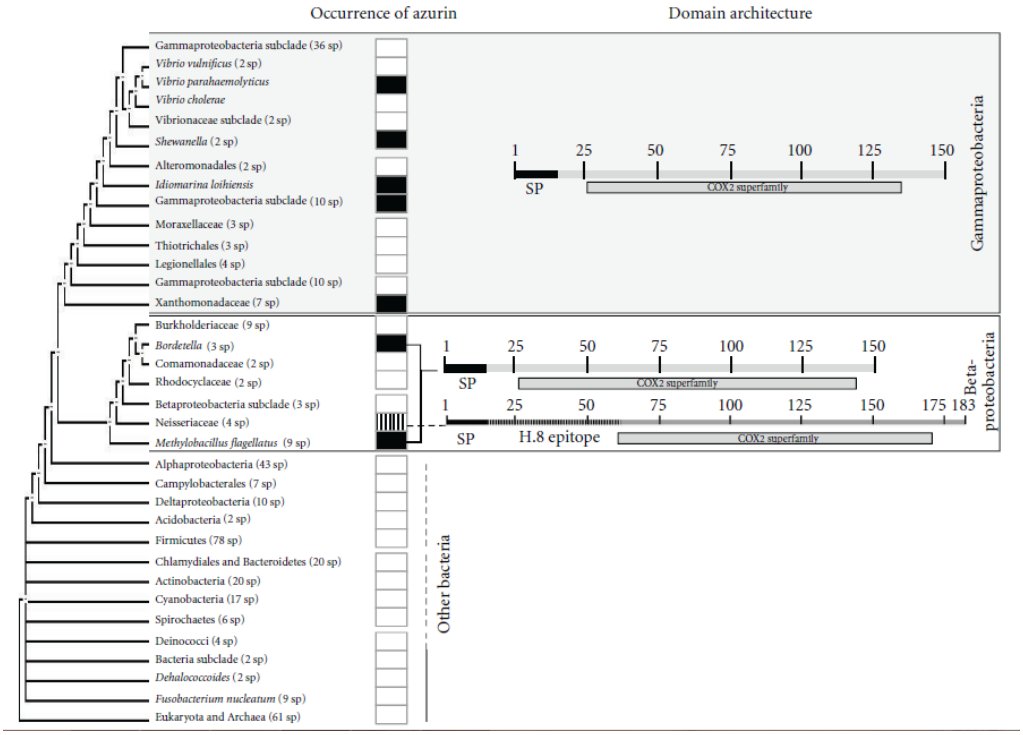

Figure 2. Occurrence of azurin-like proteins across a phylogenetic tree. Black and w hite boxes indicate presence and absence of azurin, respectively. The box w ith vertical bars shows the existence of an azurin-like protein Laz in *Neisseria* species. Shown on the right are the schematic representations of the conserved core domain presented in azurin-like proteins (Fialho *et al.*, 2012)

2.1.2. The anti-cancer activity of azurin

Cells communicate with each other and with the extracellular matrix (ECM) via junctions and receptors, hormones and other soluble factors, and through a complex network of signals generated by cell-ECM and cell-cell adhesion and junctional molecules (Bissell & Hines, 2011). Typically, cells in healthy tissues only divide if they receive growth stimulatory signals, or growth factors, from other cells. These are detected in the ECM by growth factor receptors. However, tumor cells are independent on exogenous growth factors, due to endogenous production of their own mitogenic factors, which they secrete into the ECM (Leber & Efferth, 2009). Moreover, tumor cells may over-express the receptor tyrosine kinases (RTKs) on the surface or produce structurally altered RTKs that activate the signal transduction pathways even in the absence of mitogenic factors. Additionally, several mechanisms and proteins which detect and repair damage to the DNA or the metabolism in a normal cell are mutated in cancer cells (Gupta & Massagué, 2006).

Azurin can act as an anticancer agent, combining antiangiogenic and tumor cell cytotoxic effects. There are several patents covering the use of azurin and Laz in cancer therapies, and azurin has shown significant activity (Fialho *et al.*, 2012). Two main effects of azurin's action to

cancer cells have been described: one acts through p53 and the other by direct action through transmembrane proteins, such as the Eph receptors family or P-cadherin (Figure 3). It has been demonstrated that azurin forms a complex with p53, which leads to its stabilization and increase of its intracellular level in cytosolic, mitochondrial, and nuclear fractions. As p53 is an inducer of apoptosis at several levels, its presence leads to apoptosis in cancer cells (Yamada *et al.*, 2002). Azurin also binds to several Eph receptor tyrosine kinases, a family of extracellular receptor proteins up-regulated in many tumors. This binding interferes in EphB phosphorylation, resulting in inhibition of cell signaling and cancer growth (Chaudhari *et al.,* 2007). It was recently discovered another consequence of azurin treatments in breast cancer cells, related with P-cadherin and FAK/Src signaling. It was found that azurin decreases breast cancer cells motility and invasion, by reducing the amount of P-cadherin in the cell (Bernardes *et al.*, 2013).

2.1.2.1. Azurin and p53 interaction

p53 is a 393-residue tumor suppressor protein that can be divided into three domains: the N-terminal domain (NTD, composed by a transcription activation domain and a proline-rich domain); a DNA-binding domain (DBD); and an helical tetramerization domain which together with a sequence-unspecific DNA-binding domain forms the C-terminal domain (CTD) (Okorokov & Orlova, 2009). It has a role in multiple central cellular processes, including transcription, DNA repair, cell cycle control and apoptosis, among others. This protein can transcriptionally transactivate genes involved in cell cycle arrest and interact with proteins modulating apoptosis (Vogelstein *et al.*, 2000). Cell cycle arrest induced by p53 is primarily mediated by up-regulation of p21. The cyclin-dependent kinase complexes, cdk2-cyclin A and cdk2-cyclin B, phosphorylate p53, stimulating its DNA binding to p21 preferentially (Harris, 1996). MDM2 binds to the 18-23 residues of the p53 transactivation domain, changing its conformation from unstructured to α-helical, resulting on the inhibition of the p53 transcriptional activity, its nuclear export and the stimulation of its degradation. In another words, the inhibition of the p53-MDM2 interaction stabilizes p53 and rescues its function (Gabellieri *et al.*, 2011).

It has been demonstrated that azurin can directly interact and stabilize this tumor suppressor, in an exothermic reaction involving four azurin molecules per p53 monomer. The dissociation constant for each site is 33 ± 12 nM (pH 7.5, 25ºC) (Apiyo & Wittung-Stafshede, 2005). Possibly, azurin has the capability to bind to the various domains of p53, in multiple possible configurations. However, the regions of both proteins involved in the binding are not totally identified. It was suggested that azurin binds to the trans-activation domain of p53, positioning the copper near the tryptophans (Apiyo & Wittung-Stafshede, 2005). Azurin may also bind the DBD of p53, increasing its intracellular levels and it is hypothesized that the hydrophobic patch of azurin surrounding this residues would be important for interaction with p53 (Yamada *et al.*, 2002). It is known that azurin does not bind to the MDM2-binding site (Yamada *et al.*, 2009).

The azurin peptide p28 exhibit preferential penetration and an antiproliferative effect on human breast cancer cells mediated by p53, since there is an increase in p53 in response to p28. Possibly, p28 binds within amino acids 80 to 276 of the p53 DBD, interfering with the interaction between p53 and the ubiquitin ligases, which results in a decrease in the ubiquitination and degradation of p53 (Yamada *et al*., 2009). It subsequently upregulates p21 and p27 and inactivates the CDK2-cyclin A complex, thereby causing a cell cycle arrest in breast cancer cells (Yamada *et al*., 2009). The induction of apoptosis occurs in melanoma cells harboring a functional p53, but much less efficiently in p53-null mutant melanoma cells (Yamada *et al.*, 2002).

The intracellular trafficking of azurin to the nucleus is p53-dependent. The complex formation may account for the transport of azurin to the nucleus where p53 may be stabilized and may induce a higher level of synthesis of Bax and other pro-apoptotic proteins. Higher levels of Bax may trigger apoptosis in the cancer cells by lowering the mitochondrial membrane permeability, thus enhancing the release of mitochondrial cytochrome *c* to the cytosol, therefore initiating the onset of apoptosis (as shown in Figure 3)(Yamada *et al.*, 2002).

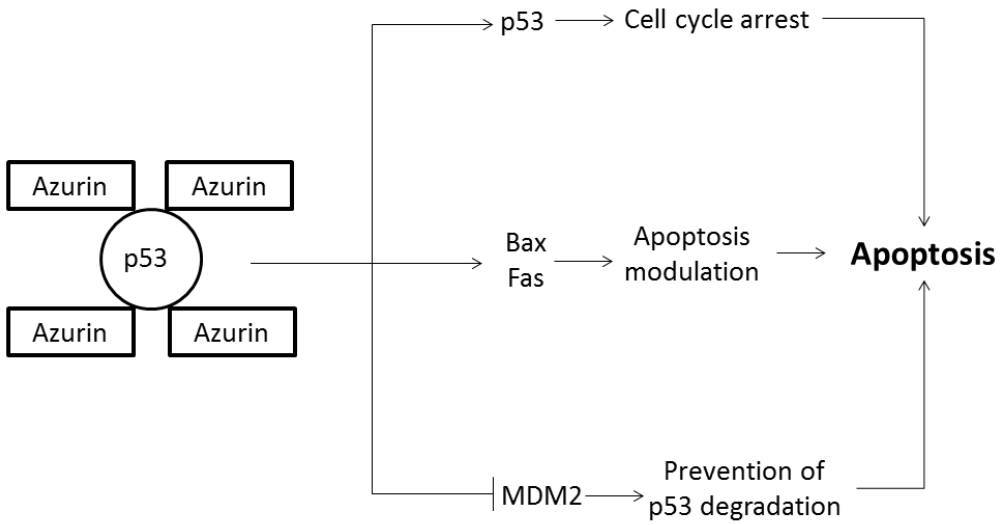

Figure 3. Intracellular level of azurin action: azurin penetrates in cancer cells, and four proteins bind per 1 molecule of tumor suppressor protein p53, stabilizing it and leading to apoptosis.

2.1.2.2. Azurin and Eph receptor interaction

Besides its interaction with p53, azurin also targets a cell proliferation pathway mediated by the EphB2 tyrosine kinase. Eph/ephrin interaction induces various cellular signaling processes, like proliferation, migration, invasion and angiogenesis (Blits-Huizinga *et al*., 2004). These receptors and ligands, as well as their signaling, are known to be involved in cancer progression and are up-regulated in several tumors (Nakada *et al*., 2004).

Azurin has structural similarities to the ligand ephrinB2, which binds its related receptor tyrosine kinase EphB2 to initiate cell signaling (Chaudhari *et al.* 2012). This competitive binding to EphB2, overexpressed in several types of cancer, prevents the tumor progression caused by the binding of the natural ligand ephrinB2. It has been demonstrated that azurin binds to the EphB2-Fc receptor with high affinity, inhibiting the ephrinB2-mediated autophosphorlyation of the EphB2 tyrosine residue, thus interfering in upstream cell signaling and contributing to cancer cell growth inhibition (Chaudhari *et al.* 2012). The region of azurin responsible for the interaction is a G-H loop (amino acids 88-113) and this is similar to the loop in the ephrinB2 ligand that mediates the recognition to the receptor (Bernardes *et al.,* 2013).

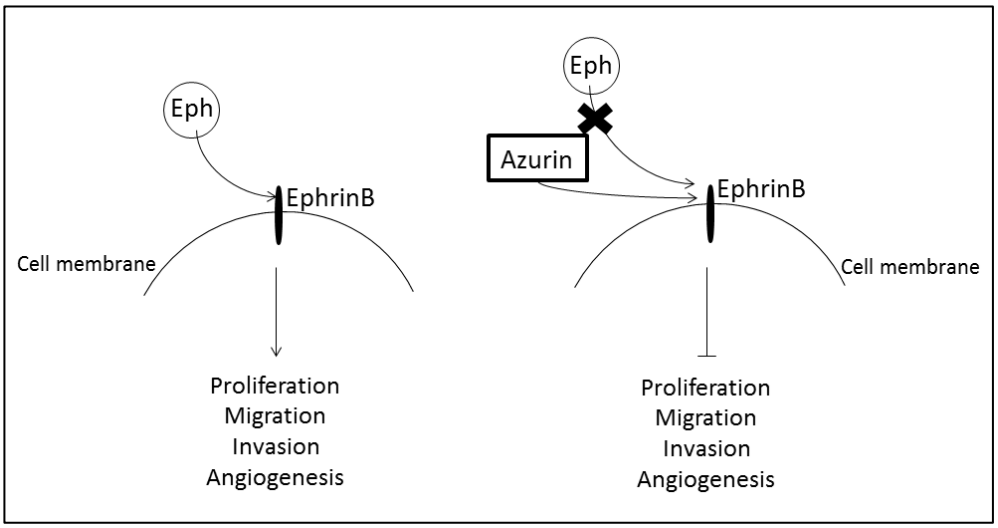

Figure 4. Extracellular level of azurin action. Azurin prevents Eph/EphrinB complex by binding to the receptor EphrinB.

2.1.2.3. Azurin and Cadherins

Cadherins are cell-cell adhesion glycoproteins that form calcium-dependent intercellular junctions, which are achieved by the establishment of homophilic interactions between two cadherin molecules of adjacent cells to form a homodimer (Gumbiner & McCrea, 1993; Paredes *et al*., 2012). However, during tumor progression, these molecules are frequently altered.

E-cadherin forms adherens junctions, which are crucial for the initiation and maintenance of a homeostatic intercellular space and cell-to-cell interaction. E-cadherin also confines signaling molecules and polarity cues spatially and serves as docking sites for vesicles (Nelson, 2003). P-cadherin also contributes to cell-to-cell adhesion, but its expression is restricted to specific areas of epithelial tissues, as proliferating regions, co-localizing partially with E-cadherin expression (Hirai *et al.*, 1989). Changes in the expression or function of adhesion proteins have been implicated in all steps of tumor progression, including detachment of tumor cells from the primary site and every steps of metastatic process (Paredes *et al.,* 2012).

E-cadherin is a tumor suppressor silenced in many cancers, leading to the aberrant activation of some signaling pathways and interaction with other molecules. One example of E-cadherin associated signaling regards the regulation of the cytoskeletal network, which organization is modulated by the activity of the members of the Rho family of small GTPases (Rho, Rac and Cdc42) with direct consequences in actin filaments, that involve filipodia, lamellipodia and contractile forces to move the body of a migrating cell (Paredes *et al.,* 2012). Also, E-cadherin has been shown to co-localize with several receptor tyrosine kinases (RTKs) to basolateral areas of polarized epithelial cells and to form multicomponent complexes with them (Pece *et al.,* 2000). Additionally, it has been reported that matrix metalloproteinases (MMP) MMP2 and MMP9 correlate with tumor progression and that there is an involvement of E-cadherin in the regulation of MMP activity (Weaver, 2006). Furthermore MMP activity is known to inactivate E-cadherin by cleavage of its extracellular domain. Proteolytic ectodomain fragments of E-cadherin have been proposed to promote cancer cell invasion by interfering with E-cadherin function in cells containing intact E-cadherin/catenin complexes (Noe *et al.,* 2000).

P-cadherin expression has a relevant role in the prognosis of invasive breast cancer that maintains E-cadherin expression, thus can be classified as a biomarker of poor prognosis in E-cadherin positive breast carcinomas. It was demonstrated that overexpression of P-cadherin is associated with cytoplasmic accumulation of one of the catenins, p120ctn, and cadherin switching in pancreatic ductal adenocarcinoma cells (as shown in Figure 5) (Taniuchi *et al.,* 2005). Furthermore, P-cadherin–dependent activation of cell motility is associated with inhibition of RhoA and activation of Rho GTPases, Rac1 and Cdc42, through accumulation of p120ctn in cytoplasm and cadherin switching. The activation of Rho GTPases alters the actin cytoskeleton polymerization and promotes cell migration and motility (Taniuchi *et al.,* 2005).

P-cadherin overexpression in wild-type E-cadherin breast cancer cells leads to increased cell invasion, motility and migration. The presence of P-cadherin induces the secretion of pro-invasive factors, such as MMPs, which then lead to P-cadherin ectodomain cleavage. The

formed soluble P-cadherin fragment (called sP-cad) is released to the extracellular media and is responsible for the *in vitro* invasion of wild-type E- and P-cadherin expressing cells even in non-invasive cells (Ribeiro *et al.* 2010).

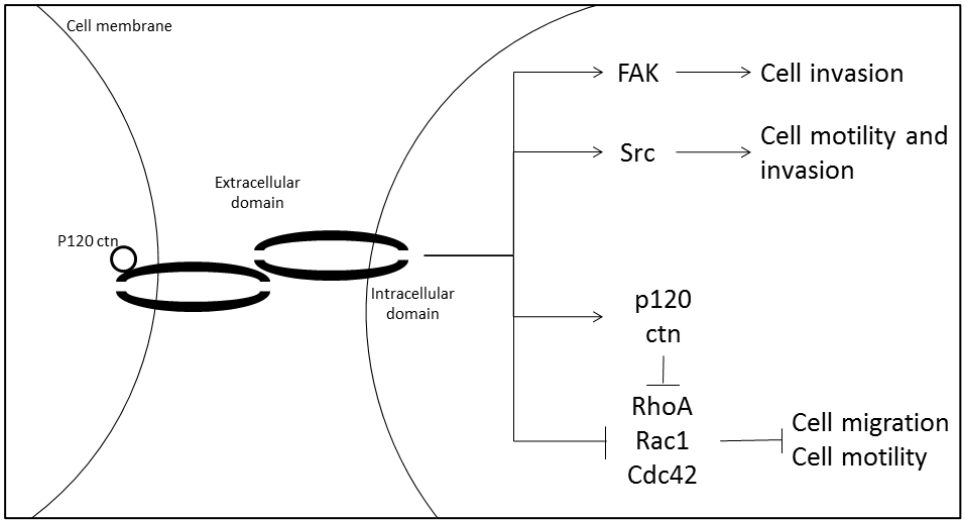

Figure 5. P-cadherin adhesive structure and interactions. Stable cell-to-cell contacts are formed through lateral clustering of P-cadherin molecule via their extracellular domains. Intracellularly, catenins (ctn) bind to the cytoplasmic tail of P-cadherin. p120-catenin binds the cadherin tail at the juxtamembrane domain. P-cadherinis associated w ith an augment of cell invasion, motility and migration through FAK, Src and p120 catenin.

A recent study has shown that the invasive phenotype of P-cadherin-overexpressing breast cancer cells was significantly reduced by azurin, as well as the levels of sP-cad were diminished, with no effects on E-cadherin levels (Bernardes *et al.* 2013). The decrease in P-cadherin caused by azurin was revealed to be parallel to a decrease in the phosphorylation level of FAK and Src without any alteration in total FAK and Src protein levels (Bernardes *et al.* 2013). FAK is necessary to promote breast cancer cell invasion. On the other hand Src, when activated, can facilitate motility and invasion through reorganization of the actin cytoskeleton and disruption of normal cell-cell and cell-matrix adhesion.

Azurin was shown to decrease the invasion of two P-cadherin expressing breast cancer cell models, which was associated with a decrease in the total P-cadherin (Bernardes *et al.*, 2013). In these cell lines, it had been previously demonstrated the pro-invasive role played by P-cadherin. Interestingly in these models azurin interfered solely with P-cadherin protein expression but not E-cadherin.

Azurin present the ability to target both P-cadherin and integrin expression at the membrane level, as well as signaling pathways associated with them. In fact, azurin showed the power to reduce the mammosphere forming efficiency of these cells in anchorage-independent growth conditions (Bernardes *et al.*, 2014).

2.1.3. Azurin entry in host cells

Azurin enters preferentially in human cancer cells compared to normal cells, mediated by the amino acids 50-77 of the protein (p28), that form an amphipathic α-helix with both a hydrophobic amino acids (50-66) and hydrophilic amino acids (67-77)(Yamada *et al.*, 2005). The peptide p28 was further refined to amino acids 50-67. This is the minimal fragment responsible for the preferential entry of azurin into human cancer cells and it is called p18 (Taylor *et al.,* 2009). p18, p28 and azurin seem to penetrate the plasma membrane and reach late endosomes, lysosomes, and the Golgi associated with caveolae. The lipid rafts were disrupted, through the depletion of cholesterol, which significantly inhibited the penetration of p18 and p28, suggesting that they penetrate the plasma membrane via caveolae-mediated endocytic pathway (Taylor *et al.,* 2009). Although it is known that this process is not dependent on membrane bound glycosaminoglycans neither on clathrins, it is possible that N-glycosylated proteins may have a role at least in the initial steps of recognition. The referred protein and peptides all bind to cancer cells with high affinity and high capacity relative to other potential anticancer peptides (Taylor *et al.,* 2009), suggesting that this protein/receptor complex localizes in caveolae. In addition to caveolar- mediated entry, it is suggested that p28 and p18 penetrate the plasma membrane via a non clathrin-caveolae–mediated process (Taylor *et al.,* 2009).

p28 also preferentially penetrates human umbilical vein endothelial cells (HUVEC), co-localized with caveolin-1 and VEGFR-2, and inhibits VEGF-induced migration, capillary tube formation and neoangiogenesis in xenograft models. The antiangiogenic effect of p28 in HUVEC is associated with an inhibition of VEGFR-2 kinase activity, by decreasing the downstream phosphorylation of focal adhesion (FAK) and Akt. These proteins precede cellular repositioning of the cytoskeleton, inhibiting HUVEC motility and migration (Mehta *et al.*, 2011).

It was recently found that two invasive breast cancer cell lines treated with azurin present an up-regulation of genes associated with apoptosis and with the entry mechanism of azurin (Bernardes *et al.*, 2014). By entering the cells through caveolae, azurin up-regulates genes associated with vesicle-mediated transport, endosome formation and membrane organization, common for both cell lines (Bernardes *et al.*, 2014).

In MCF-7/AZ.Pcad cell line, genes associated with a more aggressive phenotype were altered, as well as genes associated to cell and biological adhesion (Bernardes *et al.*, 2014). Overexpression of P-cadherin resulted in changes regarding entry in apoptosis, with negative regulators of apoptosis being up-regulated. In opposite, treating cells with azurin results in a down-regulation of these genes and up-regulation of those associated with apoptosis progression.

2.2. Lipid Rafts in tumor progression

Numerous cellular mechanisms are deregulated in tumor cells, including modified cell fate, altered protein signaling and trafficking, and enhanced cell migratory potential (Leber & Efferth, 2009). Although these events are subject to regulation by multiple elements, evidence has suggested that specialized cell membrane domains termed lipid rafts are actively involved in each of these processes (Staubach & Hanisch, 2011). Lipid rafts are being recognized as potential targets in cancer cells (Razani *et al.*, 2000). Signal transduction attenuation following lipid raft and caveolae disruption has been reported in the case of several signaling cascades. Lipid rafts are also actively involved in endocytosis, promoting internalization of receptors and signaling molecules.

2.2.1. Lipid rafts

Cell membranes contain a variety of lipid species. The lipid composition of rafts differs from the surrounding membrane, being rich in sphingolipids and cholesterol. Lipid rafts are liquid-ordered domains that are more tightly packed than the surrounding non-raft phase of the bilayer, due to the saturated hydrocarbon chains in raft lipids (Rajendran & Simons, 2004; Staubach & Hanisch, 2011).

The structure and function of lipid raft domains depend on their lipid and protein compositions. Two types of lipid rafts can be distinguished: planar lipid rafts, also known as non-caveolar, and caveolae. Planar rafts are non-invaginated microdomains lacking specific morphological features. Caveolae, on the other hand, are tube-like invaginations of the plasma membrane characterized by specific scaffolding proteins, the caveolins. Non-caveolar lipid rafts and caveolae have different structural protein markers and different proteins associated with them, as shown on Table 1, but their lipid composition and the mechanisms of protein targeting to them are very similar. Functionally, lipid rafts can be regarded as sorting platforms for targeted transport of transmembrane and glycosylphosphatidylinisotol (GPI)-anchored proteins, Src-family tyrosine kinases (e.g. Fyn and Lyn), palmitoylated and myristoylated proteins such as flotillins; cholesterol-binding proteins, such as caveolins and phospholipid-binding proteins such as annexins (Simons & Toomre, 2000; Rajendran & Simons, 2004). One important protein, which exhibits scaffolding functions in caveolar raft formation, is caveolin-1 that plays a key role in caveolae-mediated endocytosis and transport. In caveolin-independent rafts, reggies/flotillins are an important prerequisite for raft formation in the so-called reggie microdomains (Otto & Nichols, 2011). Coexpressed in caveolar rafts, flotillins can interact with caveolin-1, but may also serve as a functional substitute in caveolin-1-deficient cells (Volonté *et al.*, 1999).

Flotillins provide platforms for the assembly of signaling molecules, like caveolins do. These proteins interact with the adaptor protein CAP (Baumann *et al*., 2000), the Src family kinase Fyn (Stuermer *et al*., 2001) and with small Rho-family GTPases and regulate the

dynamics of the actin cytoskeleton (Langhorst *et al*., 2008). In the regulation of cell adhesion, reggie 1/flotillin-2 becomes phosphorylated by Src kinase upon stimulation of cells with EGF and enhances the cell spreading on a substrate involving actin polymerization and myosin contraction (Stuermer, 2009).

Lipid rafts are bilayer structures with a variable composition, which may play a role in recruitment of various proteins. Accordingly, proteins can be targeted to rafts in many dynamically-regulated ways, including attachment of GPI anchors or via lipid modifications such as prenylation and palmitoylation (Babina *et al.,* 2011). They are dynamic, therefore both proteins and lipids can move in and out of raft domains with different partitioning kinetics. Clustered rafts can sequester specific sets of signaling and other proteins and could serve as platforms to execute functions in membrane trafficking, signaling and polarization (Rajendran *et al.,* 2004).

Lipid rafts organize signaling molecules into functional complexes, and the central organizing proteins are those that provide a scaffolding domain. A switch between raft and non-raft localization of signaling components may represent an important regulatory mechanism, which is disturbed at some point in cancer (Staubach & Hanisch, 2011).

Table 1 . Lipid and protein contents of caveolae and non-caveolar lipid rafts. Adapted from (Babina *et al.,* 2011).

	Lipids	Protein Markers	Receptor Proteins	Signaling Proteins
Non-caveolar lipid rafts	Cholesterol, glycosphingolipid, sphingomyelin, ganglioside GM1, ganglioside GM3	Flotilin-1, -2	Fas, EGFR, HER2, IGF-IR, CD44, ER	Ras, Src, Erk2, Shc
Caveolae	Cholesterol, glycosphingolipid, sphingomyelin, ganglioside GM1	Caveolin-1, -2	Fas, EGFR, HER2, IGF-IR, CD44, ER, uPAR, MMP-1, -2, -9	Ras, Src, eNOS, PI3 kinase, phspholipase C

Endocytosis may represent a mechanism to attenuate anti-proliferative signals received from plasma membrane receptors (Polo *et al.*, 2004). For instance, the regulation of E-cadherin trafficking is a major alternative mechanism of dynamically modulating E-cadherin levels and activity. Receptor and non-receptor tyrosine kinases can promote E-cadherin internalization by endocytosis. The deregulation of E-cadherin adhesive function in tumor progression and metastasis might also be achieved by subversion of mechanisms regulating its trafficking. The action of active RTKs, which are frequently overexpressed in cancer and promote E-cadherin internalization, might trigger a growth-promoting loop comprising both enhanced positive and attenuated negative signaling (Polo *et al.,* 2004).

Kinases also play a significant role in regulating cell adhesion and migration. The Src family of kinases integrates signal transduction from many receptor tyrosine kinases, including EGFR, IGF-1R and HER2 to multiple downstream targets including PI3-kinase, Ras and focal adhesion kinase (Babina *et al.,* 2011). Src family of kinases activation has been linked to lipid

rafts in breast cancer cells (Hitosugi *et al.*, 2007). Lipid rafts and caveolin-1 have also been shown to be crucial for the formation of invadopodia, membrane protrusions that penetrate the surrounding matrix. Invadopodia cluster together proteins involved in actin cytoskeleton organization, signaling, cell-extracellular matrix (ECM) adhesion and membrane remodeling. Lipid rafts have been reported to be concentrated at the leading edge of invadopodia in a panel of breast cancer cell lines, and disruption of lipid rafts may suppress invadopodia formation. Invasive potential has also been linked with the raft-affiliated proteins caveolin-1 and membrane type 1 matrix metalloproteinase (MMP14). In fact caveolin-1 and MMP14 have been shown to co-associate and to be co-trafficked in invasive breast cancer cell lines (Yamaguchi *et al.,* 2009). Lipid rafts and caveolin-1 are important for invadopodia function in cancer cells.

2.2.2. Caveolae and Caveolins

Caveolae are defined as pits of 60–80-nm diameter in the plasma membrane, and present a characteristic flask shape and no obvious coat (Stan, 2005). These structures have been implicated in endocytosis, transcytosis, calcium signaling and numerous other signal transduction events. They have also been exploited by pathogens for endocytic entry (Couet *et al.*, 2001; Razani *et al.*, 2002). Caveolae and their scaffolding proteins, caveolins, have also been linked to disease (Razani & Lisanti, 2001).

Caveolins are integral membrane proteins that constitute the major protein component of caveolae. The caveolin family consists of three isoforms in mammals: caveolin-1, -2, and -3. Caveolin-1 is co-expressed with caveolin-2 in a variety of tissues, whereas caveolin-3 expression is restricted to muscle tissues (Williams *et al.,* 2004). Caveolin-1 and caveolin-3 form homo-oligomers, essential for caveolae biogenesis. It has been already demonstrated that double knock-out mice for caveolins-1 and -3 completely lack caveolae (Park *et al.*, 2002). Caveolin-2 forms hetero-oligomers with caveolin-1 and requires caveolin-1 presence for stability. Thus, caveolin-1 knock-out mice also lacks caveolin-2 (Murata *et al.,* 2007).

Caveolin-1 is a 21 to 24 kDa membrane protein and is an essential constituent of the coat structure of caveolae (Figure 7). The caveolin-1 sequence harbors a central hydrophobic domain (residues 102-134) that inserts into the inner leaflet of the plasma membrane, and both carboxy- and amino-termini face the cytoplasm (Figure 6). Adjacent to the hydrophobic domain, it is the “Caveolin Scaffolding Domain” (CSD, residues 82–101), in the amino-terminal region. This domain is required for homo- and hetero-oligomerization, as well as for interaction with signaling proteins (Okamoto *et al.*, 1998). In the carboxy-terminal region, caveolin-1 contains three palmitoylated cysteine residues that are important for oligomerization, but not localization to caveolae (Dietzen *et al.*, 1995).

Two variants of caveolin-1 are described and have different functions: 1α (residues 1-174) and 1β (residues 34-174)(Fang *et al.*, 2006). They are generated either from alternative transcripts or by alternative initiation from the same transcript (Kogo *et al.*, 2004). Interestingly,

caveolin-1 gene is localized to a suspected tumor suppressor locus that is deleted in human cancers, including mammary carcinomas. Besides the plasma-membrane caveolae, this protein also localizes to the Golgi apparatus and trans-Golgi-derived transport vesicles (Li *et al.*, 2001), endoplasmic reticulum (ER), endosomes, mitochondria and associated with the nucleus (Li *et al.*, 2001).

Caveolin family members also have other roles in cells besides caveolae formation, like vesicle trafficking, endocytosis, cholesterol homeostasis, as well as regulation of signal transduction, gene expression and protein turnover (Razani *et al.*, 2002). Accordingly to the "caveolae signaling hypothesis" (Lisanti *et al.*, 1994), caveolae serve as signaling platforms by compartmentalizing and concentrating signaling molecules. Various classes of signaling molecules bind caveolin-1 via an interaction that involves the CSD and a scaffolding domain binding motif in the respective target protein. These proteins include G-protein subunits, receptor and non-receptor tyrosine kinases, endothelial nitric oxide synthase, and small GTPases, among others (Razani *et al.*, 2002). Caveolin-1 also appears to inhibit the downstream activation and signaling of many proteins, including c-Src, H-Ras and mitogen-activated protein (MAP) kinases (Williams *et al.*, 2004; Staubach & Hanisch, 2011), and suppresses some oncogenes, like H-ras, c-myc, among others (Razani *et al*., 2002).

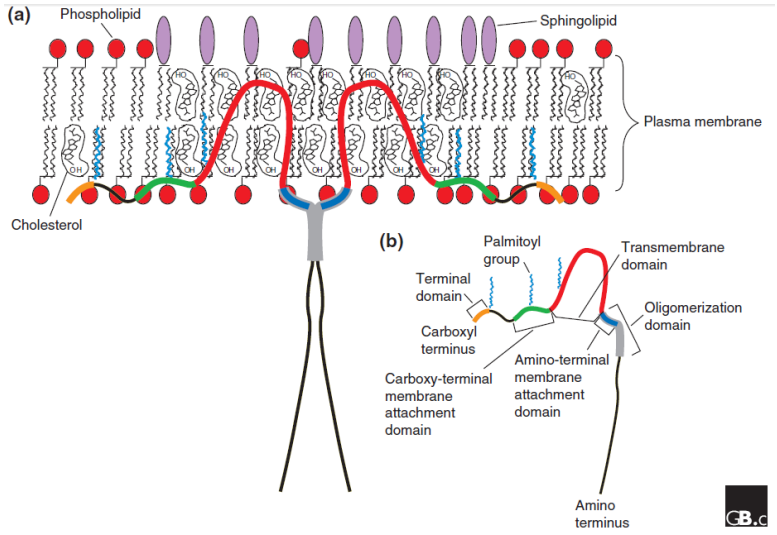

Figure 6. Primary structure and topology of caveolin-1. (a) The predicted membrane topology of caveolin-1. Two caveolin-1 monomers form a dimer. Both the amino- and carboxy-terminal domains are oriented towards the cytosolic face of the plasma membrane, with a hairpin loop structure inserted within the membrane bilayer. (b) The domains present in caveolin-1. (Williams *et al.,* 2004)

A large number of signaling pathways have been shown to be regulated by caveolin-1. Therefore, this protein is important and has potential involvement in many pathologies. One example is cancer, where caveolin-1 plays a highly ambiguous role that depends on a variety of factors (Quest *et al.*, 2013). Initially, caveolin-1 was proposed to behave as a tumor suppressor,

since its presence was related with inhibition of signaling pathways that favored cell proliferation and viability and revert characteristics associated with cell transformation, inhibit tumor growth and promotion of cell death. However, at later stages of tumor progression, caveolin-1 has been shown to promote tumor cell migration and multi-drug resistance (Quest *et al.*, 2013).

Caveolin-1 expression is greatly reduced in some oncogenically transformed and human cancer cells. Furthermore, this protein has been implicated in cell death, either by sensitizing to or directly inducing apoptosis, in a variety of possible mechanisms. Strong links between caveolin-1 and the MAPK/ERK pathway were established in oncogenically transformed fibroblasts, where caveolin-1 expression is lost or diminished (Koleske *et al.*, 1995) and upon its re-expression cell transformation is reversed. Moreover, in human laryngeal carcinoma cell lines, caveolin-1 interaction with the EGFR is associated with reduced MAPK/ERK phosphorylation and increased apoptotic cell death (Gu *et al.*, 2007).

On the other hand, caveolin-1 is also a protein that promotes more aggressive traits in tumor cells, such as metastasis. For instance, higher expression of caveolin-1 induces filopodia formation in lung adenocarcinoma with enhanced metastasis (Ho *et al.*, 2002). Caveolin-1 may also signal through PI3K–Akt pathway, and improve metastasis in cancer. Akt is a serine/threonine kinase that is a critical regulator for cell survival and proliferation, especially in human malignant cancers (Zhuang *et al.*, 2002). Activated Akt phosphorylates pro-apoptotic proteins, thereby inactivating them. Akt activation also up-regulates anti-apoptotic genes (Patra, 2008). Elevated caveolin-1 level is associated with lung, breast, prostate, and their lymph node metastases, strengthening the possibility that caveolin-1 may also act as an oncogene (Ho *et al.*, 2002). As an example, caveolin-1 expression is increased in multi-drug resistant MCF7 breast cancer cell and promotes anchorage-independent survival by preventing anoikis (Fiucci *et al.*, 2002).

Caveolin-1 also participates as an important mediator of drug-resistance in cancer cells. Although chemotherapy is one of the most frequently used treatments against cancer, tumor cells often become resistant, avoiding drug-induced cell death (Wu *et al.*, 2008). In patients, it is frequently observed an up-regulation of caveolin-1 levels in advanced stages of lung, prostate, breast, pancreas and renal cancer (Quest *et al.*, 2013). In these cases, caveolin-1 is associated with poor patient prognosis. In addition, there is strong evidence that caveolin-1 presence is associated with the development of drug-resistance. For instance, in lung cancer patients, caveolin-1 expression is inversely correlated with responses to gemcitabine, since the absence of this protein expression is associated with increased responsiveness to therapy and patient survival (Ho *et al.*, 2008). The main transporter involved in the development of drug-resistance is P-glycoprotein, which belongs to the ABC transmembrane transporter family. In an energy-dependent fashion, P-glycoprotein transport drugs to the cell exterior, thereby reducing the effective intracellular concentration (Higgins, 1995). Remarkably, a tight association has been noted between caveolin-1 and P-glycoprotein, since both proteins co-distribute in the same detergent-resistant membrane fractions, and coimmunoprecipitate in several cell types (Quest

et al., 2013). This interaction seems to be mediated by the presence of a caveolin-1-binding motif in P-glycoprotein (Demeule *et al.*, 2000).

In a conclusion, caveolin-1 presents paradoxical roles in cancer development and progression. Inactivation of caveolin-1 appears to be necessary for cell transformation and tumor induction, whereas its re-expression facilitates tumor progression and metastasis (Staubach & Hanisch, 2011; Quest *et al.*, 2013). A possible explanation for these discrepancies is that caveolin-1 functions as a tumor suppressor in systems where negative signaling events downstream of caveolin-1 prevail. Alternatively, caveolin-1-mediated positive signaling is likely to be important in those cases where presence of the protein is associated with more aggressive tumor behavior (Quest *et al.*, 2013).

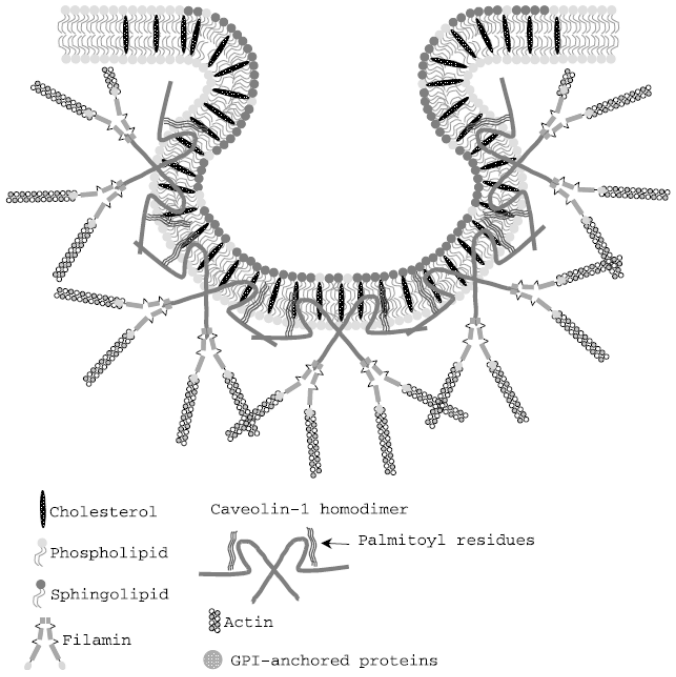

Figure 7. Schematic representation of the constitutive components present in caveolae. Caveolae are flask-shaped invaginations coated by caveolin-1, which is essential for caveolae formation because of its ability to form homo- and hetero-oligomers (Quest *et al.*, 2004).

3. Materials and methods

3.1. Human cancer cell lines and cell cultures

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

3.2. 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 in a flask of 250mL with 100mL of LB medium, ampicillin in a concentration of 150ug/mL and an inoculum of *Escherichia coli* SURE, cloned with the plasmid pWH844, containing the gene *azu*, responsible for the synthesis of azurin, from *Pseudomonas aeruginosa* PAO 1. This culture was grown over-night, at 37ºC, in an agitator at 250rpm.

Then the culture was grown in 3L flasks containing 1L of SB medium (20g/L of yeast extract, 32 g/L of triptone and 5g/L of NaCl) supplemented with 150ug/mL of ampicillin. The volume of the pre-inoculum was calculated in a way that the initial culture had an optical density at 640 nm (OD_{640}) of 0.1. The growing conditions were the same of the pre-inoculum: 30ºC in an agitator of 250rpm. When the culture reached an OD_{640} of 0.6-0.8, the azurin expression was induced with 0.2mM of IPTG (inductor of azurin's promoter), during 4-5h, at the same agitation and temperature. After this time, the cells were recovered by centrifugation (8000 rpm, 10 min, 4ºC; Beckman J2-MC Centrifuge), ressuspended in 15mL of Start buffer (10mM of imidazole, 0.2mM of sodium phosphate and 0.5M of NaCl at a pH of 7.4), and stored at -80ºC until azurin's purification.

In order to purify azurin, cells were disrupt by sonication (Branson Sonifier Sound Enclosure) and centrifuged (17600g, 4ºC, 5min; B. Braun Sigma-Aldrich 2K15), after which the pellet was discard. The supernatant was again centrifuged in the same conditions for 1h. It was used an histidine affinity column (HisTrapTM FF, GE Healthcare) to purify azurin, that was eluted with increased concentrations of imidazole (20-500 mM). Azurin is eluted in concentrations of 100-200 mM of imidazole. Next, the buffer rich in imidazole was exchanged to PBS in ÄKTA system (ÄKTA Prime, Amersham Biosciences) with a desalting column (HiPrepTM 26/10 Desalting, GE Healthcare). The collected protein was concentrated by

centrifugation (5000 rpm, 4 ºC; Eppendorf Centrifuge 5804R) in a 3 kDa cut-off column (Amicon Ultra Centrifugal Filter, Ultracel 3K, Milipore). In order to remove endotoxins from *E. coli*, the sample was detoxified by passing through a detoxing column (Detoxi-GelTM Endotoxin Removing Column, Thermo Scientific) and concentrated again. The final volume of around 2mL of purified protein was centrifuged in a 100kDa cut-off filter, to remove eventual contaminants.

The concentration was estimated according to the absorbance at 280 nm, using the Lambert-Beer equation, where $\varepsilon(280)=9.1 \times 10^3\ M^{-1}.cm^{-1}$ (van Amsterdam *et al.,* 2002). Azurin was stored at 4ºC until further use.

3.3. Adhesion assay to ECM components

Lung cancer cell line A549 and breast cancer cell line SUM-149 were plated in flasks with or without collagen type-I (1mg/mL, Millipore) and left to adhere overnight. Then, cells were washed twice with sterile PBS, collected with trypsine, ressuspended in complete medium supplemented with 10% FBS and washed twice with PBS and ressuspended in simple medium. Afterwards, cells were treated with metil-β-ciclodextrin (MβCD) in a concentration of 5mM in simple medium supplemented with 10% FBS during 30 minutes. The control condition was the cells untreated. Different components from the ECM [laminin-332 (Sigma-Aldrich), collagen type-I (Millipore), and fibronectin (Sigma-Aldrich)] at a concentration of 5µg/mL, diluted in sterile PBS, were coated in a 96-well plates (2h at 37ºC); and BSA 0.5% and plastic were used as controls.

Before addition of cells, the plates were washed three times with sterile PBS and non-specific binding sites were blocked with 0.5% BSA during 2 hours at 37 ºC. Cells (100 µL at the density of 10^6 cells/mL) were plated in the 96-well coating plates and left to adhere to the different ECM components during 30 minutes at room temperature. Then they were washed three times with PBS to remove non-adherent cells, and the adherent cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet (0.25mM crystal violet, 20% ethanol, 56.3mM ammonium oxalate) during 10 minutes at room temperature. After washed excessive dye twice with PBS, the dye was dissolved in 200µL of 100% ethanol. The absorbance was read at 570 nm to quantify crystal violet staining. The analysis of the adhesion assay was made using control absorbance as 100% of staining, meaning 100% of adhesion.

3.4. Confocal microscopy

3.4.1. 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, cells were treated with 100 µM of azurin in complete medium. Untreated cells were the control condition. After 24 hours, cells in coverslips 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 in coverslips 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.

3.4.2. Immunocytochemistry

A549 cells and SUM-149 cells were seeded on a round glass coverslip in 24-well plates with $5x10^4$ cells and left to adhere in a CO_2 incubator at 30ºC. The following day, cells were treated with 100 µM of azurin in complete medium. Untreated cells were the control condition. After 24 hours, coverslips were rinsed with PBS three times. For fixation, cells in coverslips were immersed in 4% formaldehyde for 20 minutes at room temperature. After wash three times in PBS, cells in coverslips were immersed in ammonium chloride (50 mM in PBS) for 10 minutes at room temperature and then the cells were washed three times in PBS again. To permeabilize, the cells in coverslips were immersed in 0.2% Triton X-100 (Sigma) for 5 minutes at room temperature. For immunostaining, cells in coverslips were blocked with 5% BSA in PBS at room temperature during 30 minutes. BSA excess was then removed and cells were incubated with primary antibody (1:400 anti-caveolin-1 and 1:600 anti-azurin) during 1-2 hours, in the dark at room temperature, washed three times in PBS and incubated in 1:500 secondary antibody (Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-goat, Invitrogen) during 1 hour, 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.

3.5. Protein extraction and Western Blot

Collagen type-I (1mg/mL, Millipore) was used to coat 6-well plate (200 µL/well). After 2 hours at 37 ºC, cells of the lung cancer cell line A549 and of the breast cancer cell line SUM-149 were plated with $7,5x10^5$ cells in plastic or 10^6 cells in the collagen matrix, and left to adhere and grow over night at 37ºC. Then, cells were treated with 50µM or 100µM of azurin, during the intended time (30 minutes, 2h, 8h, 24h or 48h).

Cells, treated or not with azurin, were washed twice with PBS, lysed in 100 µL 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 mixture (Roche Diagnostics GmbH, Germany) for 10 minutes at 4ºC. After that time, the cells were scratched. The lysates were 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). 20 µg of total protein per sample were denatured at 95ºC during 5 minutes, and then separated by electrophoresis in a SDS-PAGE.

Gels were transferred onto nitrocellulose membranes (RTA Transfer Kit, BioRad), using Trans-Blot Turbo Transfer System (BioRad), following manufacturer's instructions. After blocking the non-specific binding sites for 1 hour with 5% (w/v) non-fat dry milk in PBS-tween-20 (0.5% v/v), the membranes were incubated in an agitator overnight at 4 ºC with different primary antibodies (anti-actin [sc-1616, Santa Cruz Biotecnology] diluted 1:1000 in 5% non-fat milk; anti-caveolin-1 diluted 1:500 in 5% non-fat milk). The membranes were washed three times with PBS-tween-20 (0.5% v/v) for 5 minutes and probed with the appropriated secondary antibody, conjugated with horseradish peroxidase [anti-goat (sc-2354, Santa Cruz Biotecnology) for actin, diluted 1:2000 in 0.5% PBS tween-20 and anti-rabbit (sc-2354, Santa Cruz Biotecnology) for caveolin-1] at room temperature for 1 hour, in an agitator.

After washed, the membranes were developed by adding ECL substrates (Pierce) and capture the chemiluminescence by Fusion Solo (Vilber Lourmat) 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.

3.6. Co-immunoprecipitation

Cells of lung cancer cell line A549 and of the breast cancer cell line SUM-149 were plated with $7,5x10^5$ cells in 6-well plate respectively and left to adhere and grow over night 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 cancer cells, treated or not with azurin, were then washed twice with PBS, lysed in 100 µL 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. The lysates were collected, vortexed three times (10 seconds each) and 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 overnight 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 2 hours 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. Western Blot was performed as previously described.

3.7. Statistical analysis

For *in vitro* experiments, at least one independent replicate were performed (n=1 to 4 sample/experiment). Experiment performed once was considered preliminary results. 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 (*: $p<0.05$).

4. Results

4.1. Methyl-β-cyclodextrin and azurin have similar impact on cell adhesion to ECM components

It is proposed in the Introduction section that the azurin endocytosis through caveolae may lead to an internalization and degradation of tumor inducers localized there, like integrins and cadherins, reducing the signaling through which they promote cancer progression (Bernardes *et al.*, 2014). Adhesion is mediated by transmembrane glycoproteins, some of which located in lipid rafts, that are responsible for cell-cell and cell-matrix adhesion. Previous work from our group has shown that azurin interferes with the adhesion of the breast cancer cell line SUM-149 and the lung cancer cell line A549 to some ECM proteins, particularly laminin-332, fibronectin and collagen-I, decreasing the binding of these proteins to the ECM (Bernardes *et al*., 2014; Bernardes *et al.*, in preparation). The adhesion of both cell lines to the referred matrixes decreases when treated with azurin in a dose-dependent manner.

To study the hypothesis that azurin is endocyted through caveolae to enter cells, having an impact on the cell lipid rafts organization, especially on the caveolae, 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 tested with 5mM MβCD during 30 minutes. After that time, the cells were left to adhere to the different proteins of the ECM during 30 minutes. Untreated cells were used as control. Also, BSA and plastic were used as control conditions.

As showed on Figure 8, the adhesion of the cells of the lung cancer cell line A549 to laminin-332 is reduced by 30% and 40% when the cells are grown in plastic and collagen-I matrix, respectively. Although the adhesion to collagen-I and fibronectin is practically not altered with treatment 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 breast cancer cell line SUM-149, the adhesion of the cells to the three ECM components tested is diminished. When the cells are grown in plastic and treated with MβCD, the adhesion to laminin-332 and collagen-I is reduced 70% and the adhesion to fibronectin diminishes 50%. When the cells of the same cell line are grown in collagen-I matrix, the adhesion to laminin-332, collagen-I and fibronectin is decreased 65%, 60% and 75%, respectively.

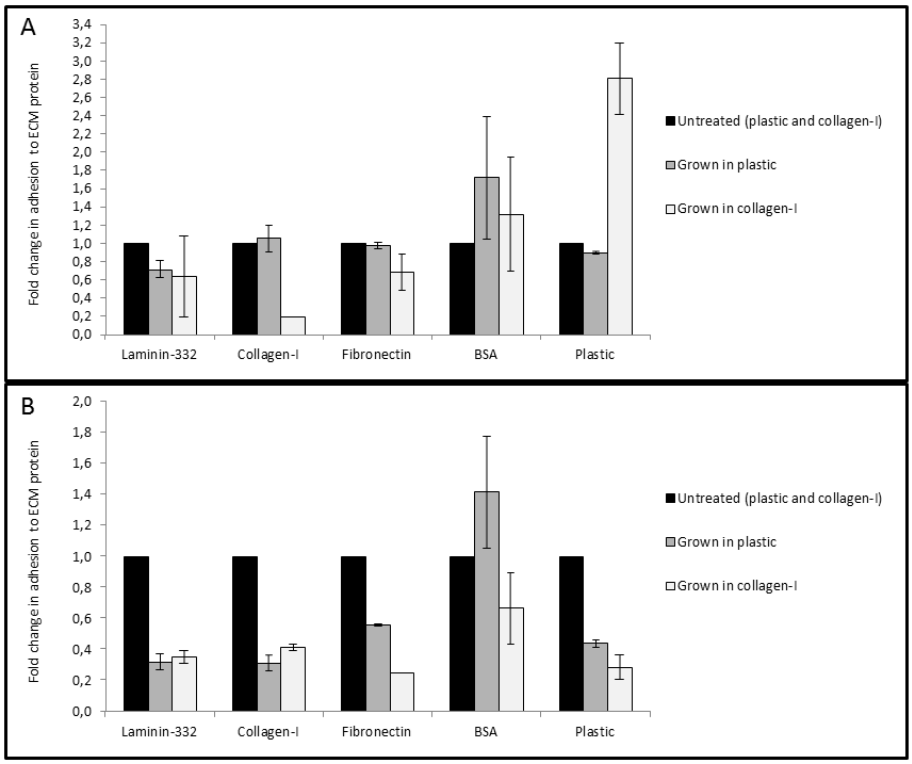

Figure 8. The effect of methyl-β-cyclodextrin (MβCD) on cell adhesion of the lung cancer cell line A549 (A) and the breast cancer cell line SUM-149 (B), grown in plastic or on top of a collagen type-I matrix (1mg/mL). A549 lung cancer cell line and SUM-149 breast cancer cell line were treated with 5mM MβCD during 30 minutes and let to adhere during 30 minutes in different ECM components.

Comparing the adhesion of the cells of the lung cancer cell line A549 grown in plastic treated with MβCD, with the obtained when the same cell line is treated with azurin (Figure 9 and Appendix A), it is possible to see that the loss in adhesion to laminin-332 is the same when the cells are treated with MβCD 5mM or with azurin 100μM (30%). The same is observed in adhesion to plastic: both treatments with MβCD 5mM 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 5mM, although the adhesion when treated with azurin is decreased.

In the adhesion of the cells of the breast cancer cell line SUM-149, the treatment with MβCD 5mM seems to 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%, the adhesion of the cells 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 5mM is a reduction on the adhesion of 60%. There is also a difference between the effects in adhesion to fibronectin: the treatment with azurin (100μM) leads to a decrease of adhesion of 20% and the treatment with MβCD 5mM cause a reduction of adhesion 40%, when the cells are grown in plastic.

However, 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

(Figure 9). 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.

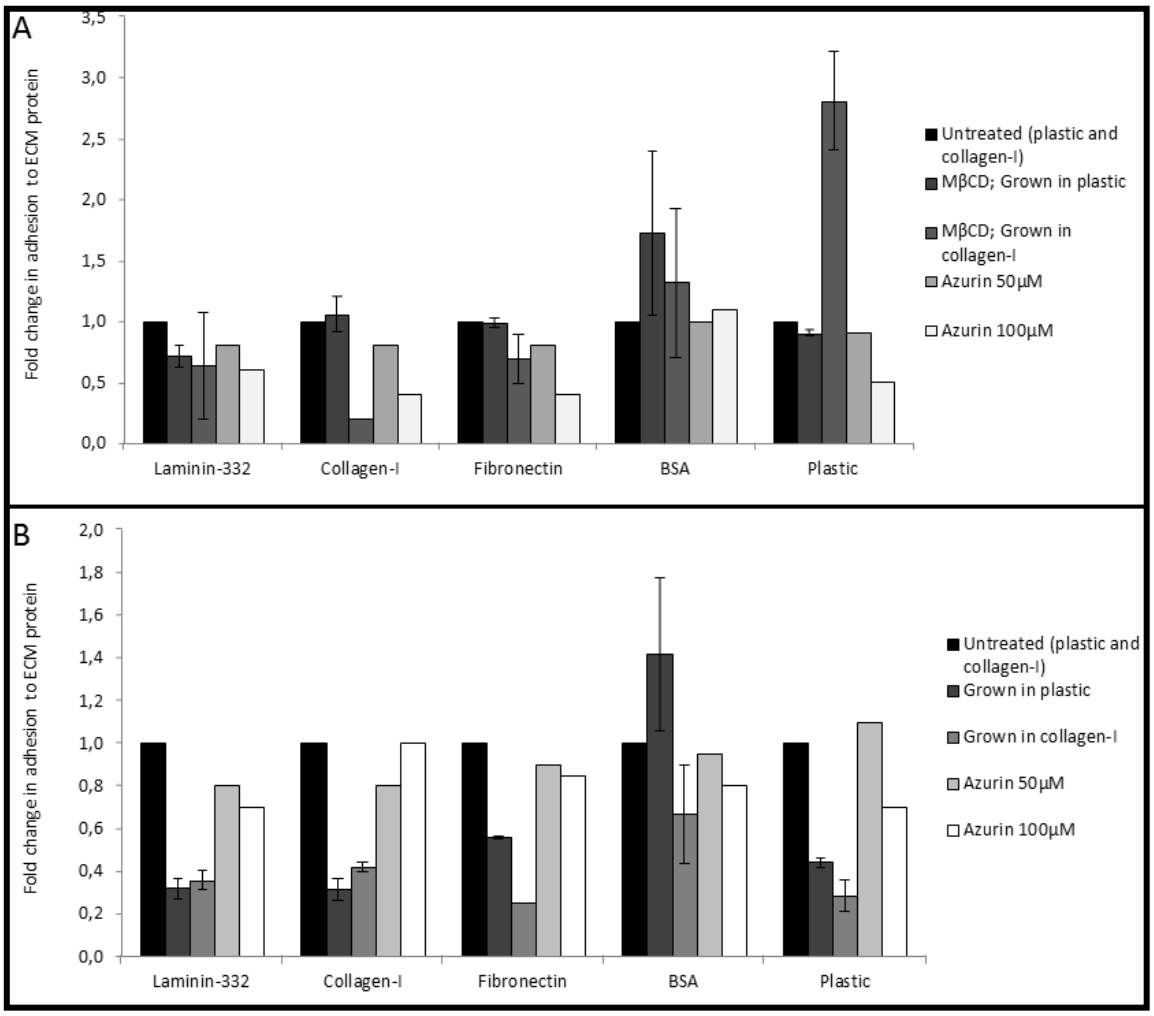

Figure 9. Comparison of the effect of 5mM methyl-β-cyclodextrin (MβCD) treatment and azurin 50 µM and 100 µM on cell adhesion of the lung cancer cell line A549 (A) and the breast cancer cell line SUM-149 (B). In the case of treatment with MβCD, A549 lung cancer cell line and SUM-149 breast cancer cell 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).

4.2. Azurin leads to an internalization of lipid rafts of the cells

It is suggested that lipid rafts are actively involved in cellular mechanisms deregulated in tumor cells, such as altered protein signaling and trafficking and enhanced cell migratory potential(Staubach & Hanisch, 2011). Therefore, the effect of azurin in the lipid rafts of the cell was studied, using the Cholera Toxin subunit B (CTxB). CTxB is a marker that binds to the

glycosphingolipid GM1, present in the lipid rafts. Despite the fact that GM1 is present in several types of lipid rafts, some studies on internalization of CTxB have generally focused on clathrin-independent mechanisms (Orlandi & Fishman, 1998; Nichols *et al.*, 2001).

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 round glass coverslip, with or without collagen type-I, treated with azurin 100µM during 24 hours and marked with CTxB (1µg/mL). Untreated cells were the control condition.

Afterwards, the cells were observed in a confocal microscope (Figure 10). The nuclei were stained with DAPI (represented in blue) and the lipid rafts, marked with CTxB, are colored in 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 and possible compartmentalization of the lipid rafts. This effect seems to be more pronounced when cells are in a collagen-I matrix.

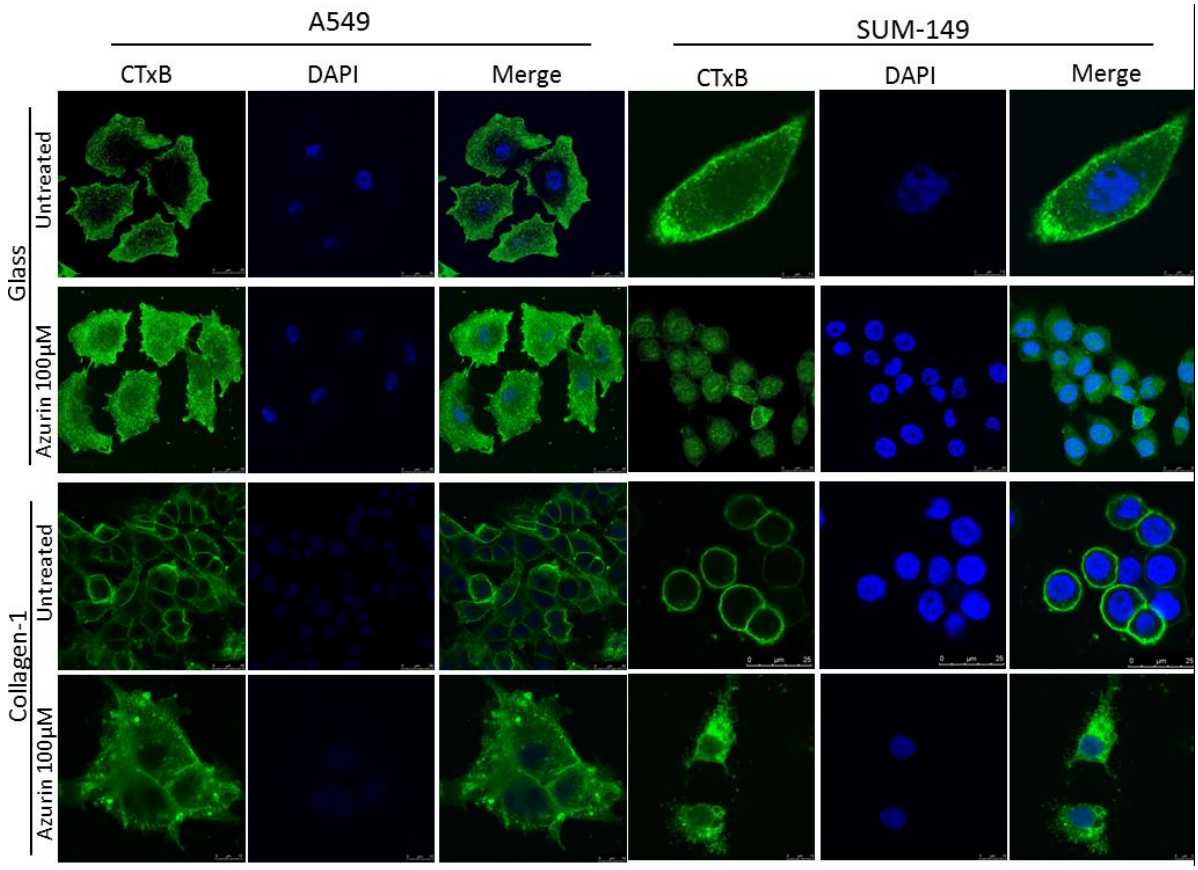

Figure 10. The effects of azurin in the cell's lipid raft organization. Cells of the cell line A549 (left panel) and of the cell line SUM-149 (right panel) were grown in plastic (upper part) and in collagen-I (lower part) and treated with azurin 100µM. The glycosphingolipid GM1 of lipid rafts is marked with CTxB (green) and the nuclei of the cells is stained with DAPI (blue).

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

Previous results have already suggested that the mechanism by which azurin exerts its anti-cancer effects depends on its route of cancer cell entry, disrupting caveolae and removing from the cell membrane selective receptors that may be over activated (Bernardes *et al.,* 2014). As is described in literature, caveolin-1 is a scaffold protein of caveolae. In order to assess the influence of azurin in caveolar rafts, the variation of caveolin-1 along treatment was measured.

Cells of the lung cancer cell line A549 and the breast cancer cell line SUM-149 were left to adhere overnight in plastic and collagen-I coated wells, and then were exposed to diverse concentrations of azurin, for different times. Afterwards, the cells were lysed, the total protein was quantified and it was made a Western Blot, whose purpose was to verify the presence and variation of caveolin-1. Actin is a constitutive protein of both cell lines therefore it was used as a control. Cancer cells were exposed to concentrations of 50μM and 100μM and untreated cells were the controls. 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 lung cancer cell line A549 it is possible to see that there is a fluctuation in caveolin-1 levels during at least the first 8 hours of treatment with azurin, in both growth conditions (plastic and collagen-I) (Figure 11, A and B). There is an increase at 30 minutes of treatment, in which the treatment with azurin 100μM leads to a rise of 160% and 195% in plastic and collagen-I respectively. At two hours of exposition with the same azurin concentration (100μM), the content of caveolin-1 alters to 140% and 60% in plastic and collagen-I respectively. At 8h of treatment with azurin 100μM there is another increase of caveolin-1 levels. In plastic, the treatment leads to an increase of 260% and in collagen-I there is an increase of 250%. 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 breast cancer cell line SUM-149, the treatment with azurin leads to a variation in the caveolin-1 content of the cell (Figure 11, 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.

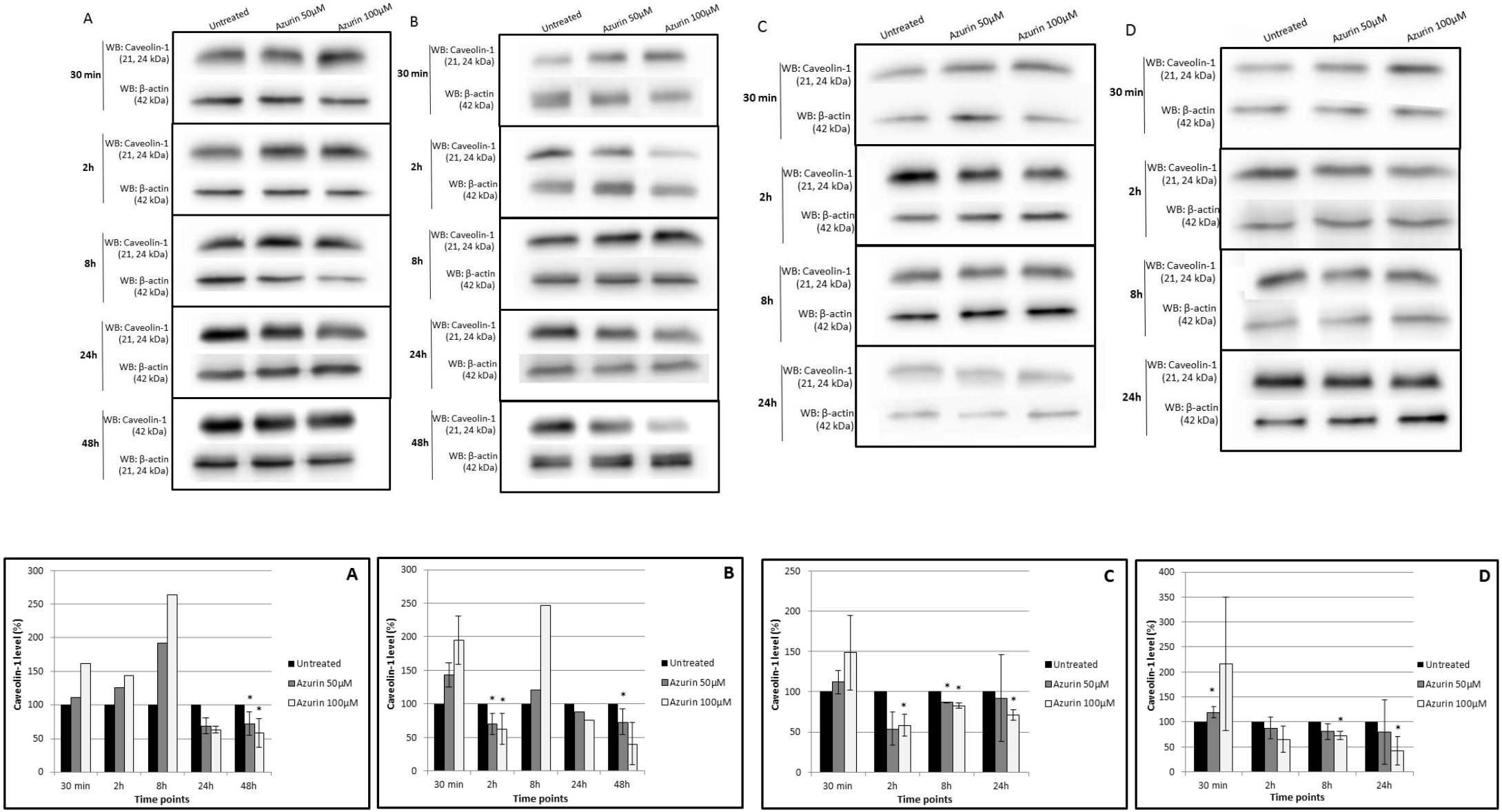

Figure 11. Influence of azurin in caveolin-1 level at several time points. A - A549 in plastic; B - A549 in collagen-1 matrix; C - SUM-149 in plastic; D - SUM-149 in collagen-1 matrix. The band intensity was measured using ImageJ and results are present as the ratio between the signal intensities in azurin treated samples to untreated cells. The protein levels were normalized by the respective actin level (*: $p<0.05$).

4.4. 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 11). Therefore, it 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 to allow the antibody to bind to the beads. After the precipitation of the beads and the elution of the antibodies from the beads, the precipitate was analyzed by Western Blot. Untreated cells are condition controls. The lysate of the cells and the beads were also analyzed by Western Blot as controls.

As shown on Western Blot against azurin, this protein binds to caveolin-1 in both cell lines (Figure 12). Western Blots in the upper panel are controls. The Western Blots represented on lower panel show a signal in samples correspondent to the lysate of cells treated with azurin presenting a binding (or a complex) between both azurin and caveolin-1.

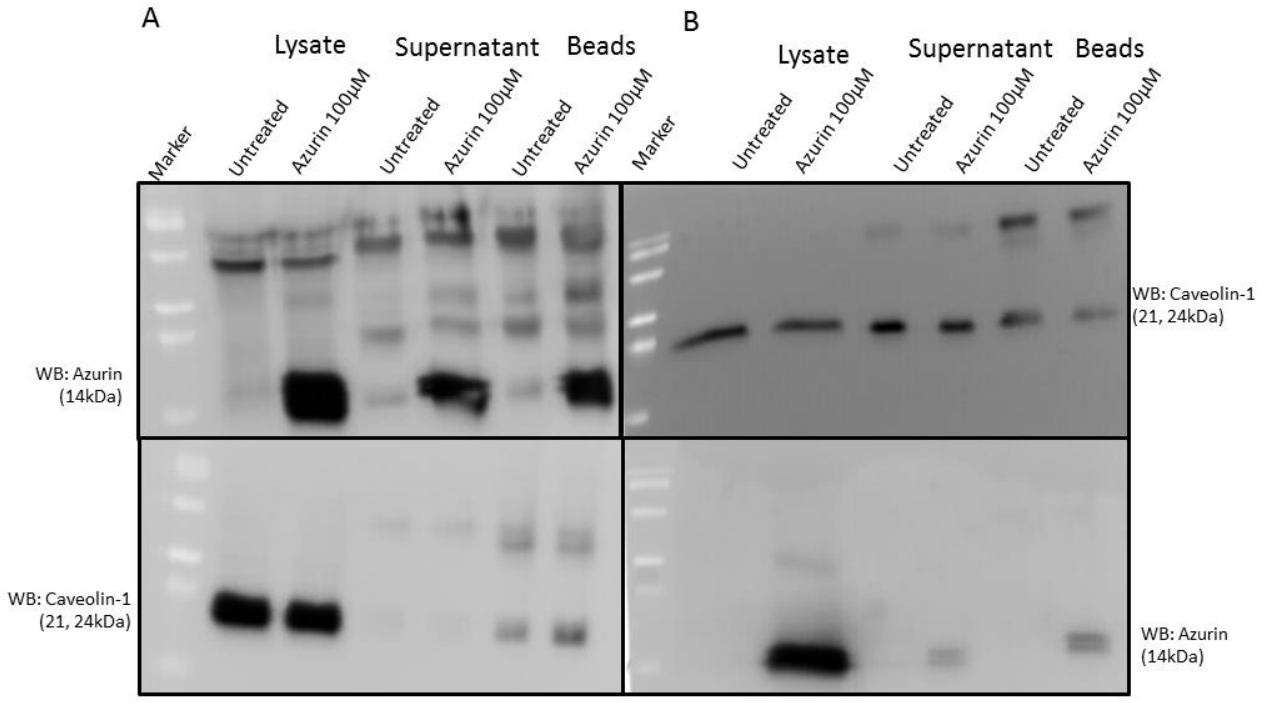

Figure 12. Azurin forms a complex with caveolin-1, probably to enter in the cell. A - A549 treated during 48h, co-immunoprecipitation using anti-body anti-azurin; B - SUM-149 treated during 24h, co-immunoprecipitation using anti-body anti-caveolin-1.

To confirm this result, the localization of caveolin-1 and azurin in the cell after 24h of treatment was studied by immunocytochemistry and confocal microscopy.

A549 cells and SUM-149 cells were seeded on a round glass coverslip and left to adhere. The following day, cells were treated with 100µM of azurin in complete medium. Untreated cells were the control condition. After 24 hours, cells were fixed, permeabilized and incubated with primary antibody 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 13 A). It is shown that in some cells of both cell lines treated with azurin 100µM azurin and caveolin-1 co-localize (Figure 13 B).

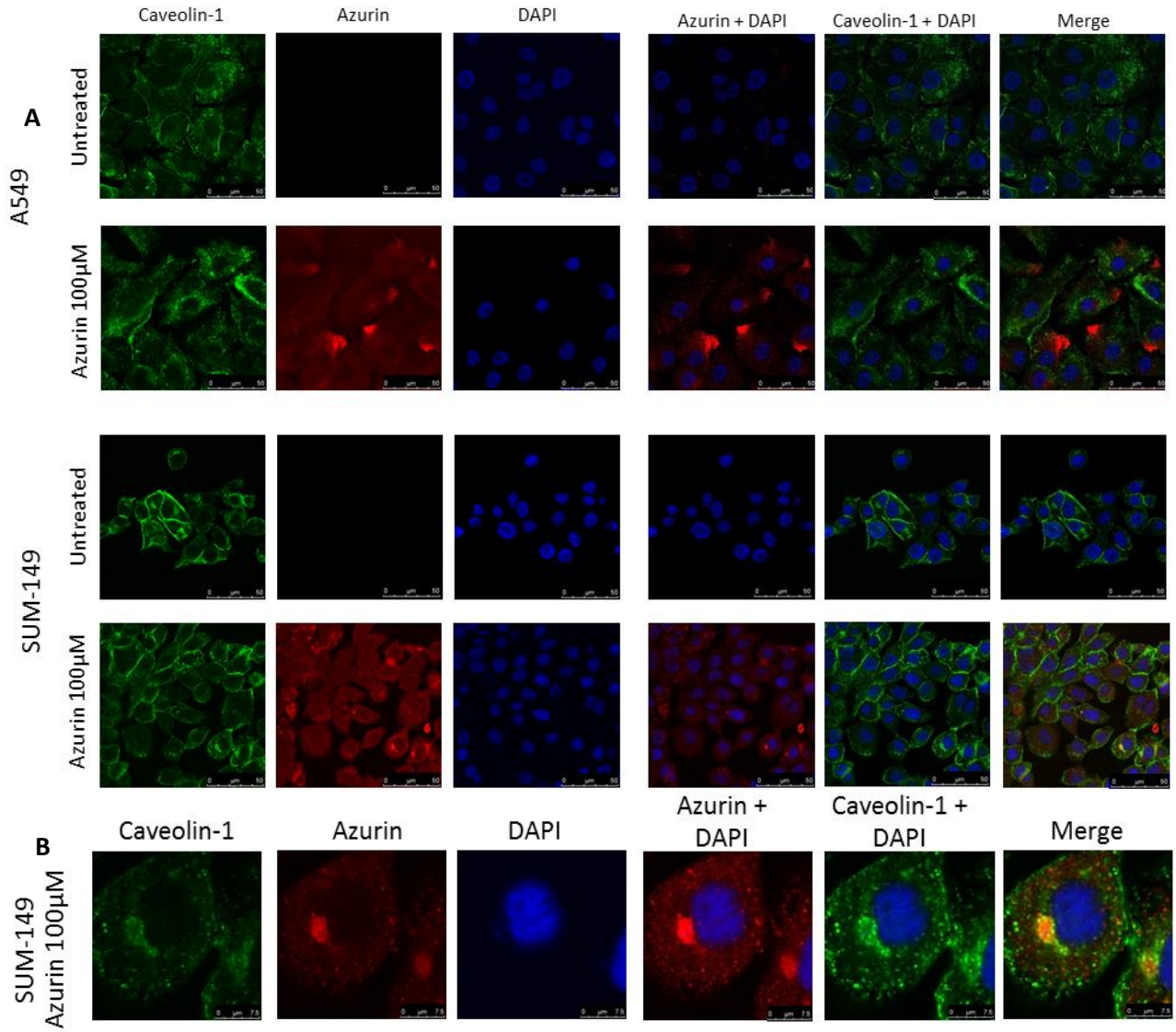

Figure 13. Azurin and caveolin-1 co-localize within the cells. A - A549 lung cancer cell line treated with azurin 100µM during 48h (upper part) and SUM-149 breast cancer cell line treated with azurin 100µM during 24h (lower part). B - Detail of the co-localization of azurin and caveolin-1 within the cells of breast cancer cell line SUM-149. 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).

5. Discussion

It is proposed that the azurin endocytosis through caveolae leads to an internalization and degradation of tumor inducers localized there, as integrins, reducing the signaling through which they promote cancer progression (Bernardes *et al.*, 2014). Also, if lipid rafts are disrupted, these inducers and tumor markers do not go to the membrane. Therefore, the signaling through which they promote cancer progression is reduced. To study the hypothesis that azurin removes the membrane receptors located in lipid rafts, MβCD (a chemical agent that depletes cholesterol from cell membranes) was used, disrupting the lipid rafts (Figure 8). The impact of MβCD was then compared to the previous studied impact of azurin on the adhesion of the cells (Figure 9).

Adhesion is mediated by cadherins and integrins which are transmembrane glycoproteins. These transmembrane glycoprotein receptors mediate cell-cell and cell-matrix adhesion, forming focal adhesions that contact with ECM ligands by the long extracellular domain (i.e. fibronectin, laminin and collagen)(White & Muller, 2007). We have showed before that azurin leads to a decrease in some integrin receptors in breast cancer models (Bernardes *et al.*, 2014)

In this work two different 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 behavior of the cells on their extracellular environment as well as their adhesion to the ECM components changes with their growth conditions (Shekhar *et al.*, 2003). 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 is a reason why the results of adhesion in collagen-I are more pronounced. On the other hand, in the breast cancer cell line SUM-149 the diminution of cell adhesion to ECM components is more accentuated when treated with MβCD, with almost no differences on growth conditions (plastic or collagen-I), except in adhesion to fibronectin (Figure 8).

Comparing the adhesion of the cells of the lung cancer cell line A549 grown in plastic treated with MβCD, with the obtained when the same cell line is treated with azurin (Figure 9), 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. 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. However, in the adhesion of the cells of the breast cancer cell line SUM-149, the treatment with MβCD 5mM has a higher impact than the treatment of the cells of the same cell line with both doses of azurin tested. This difference may be due to the concentration of MβCD used to treat the cells.

Nevertheless, it is interesting to see that both treatments (with azurin and with 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 the hypothesis that these structures of the cell membrane are necessary for the cells to adhere, having the motifs needed for that. Therefore, azurin protein 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, as the ubiquitination of endocytic proteins and of surface receptors is a sorting signal in the endocytic route (Fiore *et al.*, 2003). In mammals, it was demonstrated that receptor tyrosine kinases (RTKs), such as the epidermal growth factor receptor (EGFR), are monoubiquitinated by the E3 ligase Cbl at multiple sites (Dikic *et al.*, 2003). This post-translational modification can promote receptor endocytosis and targets receptors for lysosomal degradation, thereby ensuring termination of receptor signaling (Haglund & Dikic, 2012).

To confirm the endocytic effect of azurin in the lipid rafts of the cell, the localization of lipid rafts was then studied by confocal microscopy, using CTxB, a marker that binds to the glycosphingolipid GM1. It was possible to observe that the cellular membrane of the untreated cells of both cell lines is specifically marked (Figure 10). When the cells are treated with azurin, there is internalization and possible compartmentalization of the lipid rafts in endosomes, which confirms the hypothesis that azurin delocalize 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 and removes from the cell membrane selective receptors that may be over activated (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 time point 30 minutes, but in the following time points the levels of the same protein are generally reduced in both cell lines, adherent to either plastic or collagen-I (Figure 11). 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 also preferentially penetrates human umbilical vein endothelial cells (HUVEC), 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 two hours of treatment 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. Multidrug resistance is cellular resistance to multiple and divergent drugs (Gottesman, 1993), in which cells employ mechanisms to survive the cytotoxic effect of drugs utilized in chemotherapy. For instance, P-glycoprotein and ATP-dependent drug efflux pumps mediate drug resistance by actively extruding drugs from the cells (Gottesman, 1993). Multidrug resistance phenotype is associated with upregulation of lipids that constitute caveolae, especially cholesterol (Lavie & Liscovitch, 2001). It was already shown that multidrug resistant 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 preferential 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 other protein, namely, a membrane protein, could lead to the endocityc 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 13). It was also possible to see that these proteins interact by binding each other or forming a complex with an intermediary (Figure 12).

In fact, caveolin-1 interacts with many signaling molecules including Src family tyrosine kinases, PI3K, heterotrimeric G proteins, integrins, EGFR, among others (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 a single tryptophan residue (Gilardi *et al.*, 1994) on its hydrophobic patch. In addition, azurin harbors, on its CD loop and GH loop, 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 Blotting 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.

6. Conclusion and future perspectives

It is proposed that the azurin endocytosis through caveolae leads to an internalization and degradation 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 inducers and tumor markers do not go to the membrane. Therefore, the signaling through which they promote cancer progression is reduced.

In this work two different 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 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 (MβCD) confirms the hypothesis that these motifs of the cell membrane are necessary for the cells to adhere, having the motifs needed for that. Therefore, azurin has the same impact on the cell membrane and caveolae, by inducing endocytosis through these structures. When the cells are treated with azurin, there is internalization and possible compartmentalization of the lipid rafts in endosomes, which confirms the hypothesis that azurin delocalize 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. The level of caveolin-1 in cancer cells along azurin treatment was studied, showing an increase in caveolin-1 level at time point 30 minutes, but in the following time points 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 increase the content of caveolae and, therefore, to augment endocytosis of azurin. After that time, caveolin-1 is targeted to lysosomes and is degraded. Consequently, the overall level of caveolin-1 in the cell is diminished at two hours of treatment and 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.

7. References

Alitalo, K., Keski-Oja, J., & Vaheri, A. (1981). Extracellular matrix proteins characterize human tumor cell lines. *Int. J. Cancer*, *27*, 755–761.

Apiyo, D., & Wittung-Stafshede, P. (2005). Unique complex between bacterial azurin and tumor-suppressor protein p53. *Biochemical and biophysical research communications*, *332*(4), 965–8. doi:10.1016/j.bbrc.2005.05.038

Babina, I. S., Donatello, S., Nabi, I. R., & Hopkins, A. M. (2011). Lipid rafts as master regulators of breast cancer cell function. In *Breast Cancer – Carcinogenesis, Cell Growth and Signalling Pathways* (pp. 401–428).

Baumann, C., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., … Saltiel, a R. (2000). CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature*, *407*(6801), 202–7. doi:10.1038/35025089

Bernardes, N., Chakrabarty, A. M., & Fialho, A. M. (2013). Engineering of bacterial strains and their products for cancer therapy. *Applied microbiology and biotechnology*, *97*(12), 5189–99. doi:10.1007/s00253-013-4926-6

Bernardes, N., Ribeiro, A. S., Abreu, S., Mota, B., Matos, R. G., Arraiano, C. M., … Fialho, A. M. (2013). The bacterial protein azurin impairs invasion and FAK/Src signaling in P-cadherin-overexpressing breast cancer cell models. *PloS one*, *8*(7). doi:10.1371/journal.pone.0069023

Bernardes, N., Ribeiro, A. S., Abreu, S., Vieira, A. F., Carreto, L., Santos, M., … Fialho, A. M. (2014). High-throughput molecular profiling of a P-cadherin overexpressing breast cancer model reveals new targets for the anti-cancer bacterial protein azurin. *The international journal of biochemistry & cell biology*, *50*, 1–9. doi:10.1016/j.biocel.2014.01.023

Bernardes, N., Seruca, R., Chakrabarty, A. M., & Fialho, A. M. (2010). Microbial-based therapy of cancer: current progress and future prospects. *Bioengineered bugs*, *1*(3), 178–90. doi:10.4161/bbug.1.3.10903

Bissell, M. J., & Hines, W. C. (2011). Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature medicine*, *17*(3), 320–9. doi:10.1038/nm.2328

Blits-Huizinga, C. T., Nelersa, C. M., Malhotra, A., & Liebl, D. J. (2004). Ephrins and their receptors: binding versus biology. *IUBMB life*, *56*(5), 257–65. doi:10.1080/15216540412331270076

Byrne, D. P., Dart, C., & Rigden, D. J. (2012). Evaluating caveolin interactions: do proteins interact with the caveolin scaffolding domain through a widespread aromatic residue-rich motif? *PloS one*, *7*(9). doi:10.1371/journal.pone.0044879

Chakrabarty, A. M. (2003). Microorganisms and Cancer : Quest for a Therapy. *J. Bacteriol.*, *185*(9), 2683–2686. doi:10.1128/JB.185.9.2683

Chaudhari, A., Mahfouz, M., Fialho, A. M., Yamada, T., A.T., G., Zhu, Y., … Chakrabarty, A. M. (2007). Cupredoxin-Cancer Interrelationship: azurin binding with EphB2, interference in EphB2 tyrosine phosphorylation, and inhibition of cancer growth. *Biochemistry*, *46*, 1799–1819.

Chaudhari, A., Mahfouz, M., Fialho, A., Yamada, T., Granja, A., Zhu, U., … Chakrabarty, A. (2012). Cupredoxin−Cancer Interrelationship: Azurin Binding with EphB2, Interference in EphB2 Tyrosine Phosphorylation, and Inhibition of Cancer Growth. *Seminars in Cell & Developmental Biology*, *23*(1), 51–57.

Collins, B. M., Davis, M. J., Hancock, J. F., & Parton, R. G. (2012). Structure-based reassessment of the Caveolin Signaling Model: Do caveolae regulate signaling through caveolin-protein interactions? *Dev Cell*, *23*(1), 11–20. doi:10.1016/j.devcel.2012.06.012.Structure-based

Couet, J., Belanger, M. M., Roussel, E., & Drolet, M. C. (2001). Cell biology of caveolae and caveolin. *Advanced drug delivery reviews*, *49*(3), 223–35. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11551396

Couet, J., Li, S., Okamoto, T., Ikezu, T., & Lisanti, M. P. (1997). Identification of Peptide and Protein Ligands for the Caveolin-scaffolding Domain: Implications for the interaction of caveolin with caveolae-associated proteins. *Journal of Biological Chemistry*, *272*(10), 6525–6533. doi:10.1074/jbc.272.10.6525

De Rienzo, F., Gabdoulline, R. R., Menziani, M. C., & Wade, R. C. (2000, August). Blue copper proteins: a comparative analysis of their molecular interaction properties. *Protein science : a publication of the Protein Society*. doi:10.1110/ps.9.8.1439

Demeule, M., Jodoin, J., Gingras, D., & Béliveau, R. (2000). P-glycoprotein is localized in caveolae in resistant cells and in brain capillaries. *FEBS letters*, *466*(2-3), 219–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10682831

Dietzen, J., Hastings, W., & Lublin, D. (1995). Caveolin is palmitoylated on multiple cysteine residues. Palmitoylation is not necessary for localization of caveolin to caveolae. *The Journal of Biological Chemistry*, *270*(12), 6838–6842.

Dikic, I., Szymkiewicz, I., & Soubeyran, P. (2003). Cbl signaling networks in the regulation of cell function. *Cellular and molecular life sciences : CMLS*, *60*(9), 1805–27. doi:10.1007/s00018-003-3029-4

Fang, P.-K., Solomon, K. R., Zhuang, L., Qi, M., McKee, M., Freeman, M. R., & Yelick, P. C. (2006). Caveolin-1α and −1β Perform Nonredundant Roles in Early Vertebrate Development. *The American Journal of Pathology*, *169*(6), 2209–2222. doi:10.2353/ajpath.2006.060562

Farver, O., Blatt, Y., & Pecht, I. (1982). Resolution of two distinct electron transfer sites on Azurin. *Biochemistry*, *21*(l), 3556–3561.

Farver, O., & Pecht, I. (1991). Electron transfer in proteins: in search of preferential pathways. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *5*(11), 2554–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1868979

Fialho, A. M., Gupta, T. K. Das, & Chakrabarty, A. M. (2008). Promiscuous Drugs from Pathogenic Bacteria in the Post - Antibiotics Era. In *Promiscuous Drugs from Bacteria* (pp. 145–162).

Fialho, A. M., Salunkhe, P., Manna, S., Mahali, S., & Chakrabarty, A. M. (2012). Glioblastoma multiforme: novel therapeutic approaches. *ISRN neurology*, *2012*, 642345. doi:10.5402/2012/642345

Fiore, P., Polo, S., & Hofmann, K. (2003). receptors : a signalling connection. *Nature Reviews*, *4*, 491–497.

Fiucci, G., Ravid, D., Reich, R., & Liscovitch, M. (2002). Caveolin-1 inhibits anchorage-independent growth anoikis and invasiveness in MFC-7 human breast cancer cells. *Oncogene*, *21*, 2365–2375.

Gabellieri, E., Bucciantini, M., Stefani, M., & Cioni, P. (2011, December). Does azurin bind to the transactivation domain of p53? A Trp phosphorescence study. *Biophysical chemistry*. doi:10.1016/j.bpc.2011.07.008

Gilardi, G., Mei, G., Rosato, N., Canters, G. W., & Finazzi-Agrò, A. (1994). Unique environment of Trp48 in Pseudomonas aeruginosa azurin as probed by site-directed mutagenesis and dynamic fluorescence spectroscopy. *Biochemistry*, *33*(6), 1425–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8312262

Gottesman, M. M. (1993). How Cancer Cells Evade Chemotherapy : Sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Research*, *53*, 747–754.

Gu, D., Li, H., Wang, Z., Chen, Q., Jiang, J., & Zhu, H. (2007). Caveolin-1 inhibits the growth of human laryngeal squamous cell carcinoma and down regulates EGFR-MAPKs signaling pathway. *The Laryngoscope*, *117*(10), 1782–9. doi:10.1097/MLG.0b013e31811edd31

Gumbiner, B. M., & McCrea, P. D. (1993). Catenins as mediators of the cytoplasmic functions of cadherins. *Journal of cell science. Supplement*, *17*, 155–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8144692

Gupta, G. P., & Massagué, J. (2006, November 17). Cancer metastasis: building a framework. *Cell*. doi:10.1016/j.cell.2006.11.001

Haglund, K., & Dikic, I. (2012). The role of ubiquitylation in receptor endocytosis and endosomal sorting. *Journal of cell science*, *125*, 265–75. doi:10.1242/jcs.091280

Harris, C. (1996). Structure and Function of the p53 Tumor supressor gene.pdf. *Journal of the National Cancer Institute*, *88*(20), 1442–1455.

Higgins, C. F. (1995). The ABC of Channel Regulation. *Cell*, *82*(1992), 693–696.

Hirai, Y., Nose, A., Kobayashi, S., & Takeichi, M. (1989). Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. *Development*, *105*, 263–270.

Hitosugi, T., Sato, M., Sasaki, K., & Umezawa, Y. (2007). Lipid raft specific knockdown of SRC family kinase activity inhibits cell adhesion and cell cycle progression of breast cancer cells. *Cancer research*, *67*(17), 8139–48. doi:10.1158/0008-5472.CAN-06-4539

Ho, C., Huang, P., Huang, H., Chen, Y., Yang, P., & Hsu, S. (2002). Upregulated Caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. *American Journal of Pathology*, *161*(5), 1647–1656.

Ho, C.-C., Kuo, S.-H., Huang, P.-H., Huang, H.-Y., Yang, C.-H., & Yang, P.-C. (2008). Caveolin-1 expression is significantly associated with drug resistance and poor prognosis in advanced non-small cell lung cancer patients treated with gemcitabine-based chemotherapy. *Lung cancer*, *59*(1), 105–10. doi:10.1016/j.lungcan.2007.07.024

Hoitink, C. W., & Canters, G. W. (1992). The importance of Asn47 for structure and reactivity of azurin from Alcaligenes denitrificans as studied by site-directed mutagenesis and spectroscopy. *The Journal of biological chemistry*, *267*(20), 13836–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1321127

Hong, C., Yamada, T., Hashimoto, W., Fialho, A., Gupta, T., & Chakrabarty, A. (2006). Disrupting the entry barrier and attacking brain tumors.pdf. *Cell Cycle*, *5*(15), 1633–1641.

Kawula, T. H., Spinola, S. M., Klapper, D. G., & Cannon, J. G. (1987). Localization of a conserved epitope and an azurin-like domain in the H.8 protein of pathogenic Neisseria. *Molecular microbiology*, *1*(2), 179–85. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2452958

Kogo, H., Aiba, T., & Fujimoto, T. (2004). Cell type-specific occurrence of caveolin-1α and -1β in the lung caused by expression of distinct mRNAs. *The Journal of biological chemistry*, *279*(24), 25574–81. doi:10.1074/jbc.M310807200

Koleske, A. J., Baltimore, D., & Lisantit, M. P. (1995). Reduction of caveolin and caveolae in oncogenically transformed cells. *Proc Natl Acad Sci*, *92*(5), 1381–1385.

Langhorst, M. F., Jaeger, F., Mueller, S., Sven Hartmann, L., Luxenhofer, G., & Stuermer, C. (2008). Reggies/flotillins regulate cytoskeletal remodeling during neuronal differentiation via CAP/ponsin and Rho GTPases. *European journal of cell biology*, *87*(12), 921–31. doi:10.1016/j.ejcb.2008.07.001

Lavie, Y., Fiucci, G., & Liscovitch, M. (1998). Up-regulation of Caveolae and Caveolar Constituents in Multidrug-resistant Cancer Cells. *Journal of Biological Chemistry*, *273*(49), 32380–32383. doi:10.1074/jbc.273.49.32380

Lavie, Y., & Liscovitch, M. (2001). Changes in lipid and protein constituents of rafts and caveolae in multidrug resistant cancer cells and their functional consequences. *Glycoconjugate journal*, *17*, 253–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11201798

Leber, M. F., & Efferth, T. (2009, April). Molecular principles of cancer invasion and metastasis (review). *International journal of oncology*. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19287945

Li, W., Liu, P., Pilcher, B. K., & Anderson, R. G. W. (2001). Cell-specific targeting of caveolin-1 to caveolae, secretory vesicles, cytoplasm or mitochondria. *Journal of Cell Science*, *114*(7), 1397–1408.

Lisanti, M., Scherer, P., Tang, Z., & Sargiacomo, M. (1994). Caveolae, caveolin and caveolin-rich membrane domains: a signalling hypothesis. *Trends un Cell Biology*, *4*, 231–235.

Mehta, R.R., Yamada, T., Taylor, B. N., Christov, K., King, M. L., Majumdar, D., … Das Gupta, T. K. (2011). A cell penetrating peptide derived from azurin inhibits angiogenesis and tumor growth by inhibiting phosphorylation of VEGFR-2, FAK and Akt. *Angiogenesis*, *14*, 355–369.

Mehta, Rajeshwari R, Yamada, T., Taylor, B. N., Christov, K., King, M. L., Majumdar, D., … Das Gupta, T. K. (2011). A cell penetrating peptide derived from azurin inhibits angiogenesis and tumor growth by inhibiting phosphorylation of VEGFR-2, FAK and Akt. *Angiogenesis*, *14*(3), 355–69. doi:10.1007/s10456-011-9220-6

Murata, T., Lin, M. I., Huang, Y., Yu, J., Bauer, P. M., Giordano, F. J., & Sessa, W. C. (2007). Reexpression of caveolin-1 in endothelium rescues the vascular, cardiac, and pulmonary defects in global caveolin-1 knockout mice. *The Journal of experimental medicine*, *204*(10), 2373–82. doi:10.1084/jem.20062340

Nakada, M., Niska, J. A., Miyamori, H., Mcdonough, W. S., Wu, J., Sato, H., & Berens, M. E. (2004). The Phosphorylation of EphB2 Receptor Regulates Migration and Invasion of Human Glioma Cells. *Cancer Research*, *64*, 3179–3185.

Nelson, W. J. (2003). Epithelial cell polarity from the outside looking in. *News Physiol Sci.*, *18*, 143–146.

Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., … Lippincott-Schwartz, L. (2001). Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *The Journal of Cell Biology*, *153*(3), 529–541.

Noe, V., Fingleton, B., Jacobs, K., Crawford, H. C., Vermeulen, S., Steelant, W., … Mareel, M. (2000). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *Journal of Cell Science*, *114*(1), 111–118.

Okamoto, T., Schlegel, a., Scherer, P. E., & Lisanti, M. P. (1998). Caveolins, a Family of Scaffolding Proteins for Organizing "Preassembled Signaling Complexes" at the Plasma Membrane. *Journal of Biological Chemistry*, *273*(10), 5419–5422. doi:10.1074/jbc.273.10.5419

Okorokov, A. L., & Orlova, E. V. (2009). Structural biology of the p53 tumour suppressor. *Current opinion in structural biology*, *19*(2), 197–202. doi:10.1016/j.sbi.2009.02.003

Orlandi, P., & Fishman, P. (1998). Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *The Journal of cell biology*, *141*(4), 905–15. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2132770&tool=pmcentrez&rendertype=abstract

Otto, G. P., & Nichols, B. J. (2011). The roles of flotillin microdomains--endocytosis and beyond. *Journal of cell science*, *124*, 3933–40. doi:10.1242/jcs.092015

Paredes, J., Figueiredo, J., Albergaria, A., Oliveira, P., Carvalho, J., Ribeiro, A. S., … Carneiro, F. R. (2012). Epithelial E- and P-cadherins: role and clinical significance in cancer. *Biochimica et biophysica acta*, *1826*, 297–311.

Park, D. S., Woodman, S. E., Schubert, W., Cohen, A. W., Frank, P. G., Chandra, M., … Lisanti, M. P. (2002). Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype. *The American journal of pathology*, *160*(6), 2207–17. doi:10.1016/S0002-9440(10)61168-6

Parr, S. R., Barber, D., Greenwood, C., & Brunori, M. (1977). Electron-Transfer Reaction between Azurin and the Cytochrome c Oxidase from Pseudomonas aeruginosa. *Biochemistry Journal*, *167*, 447–455.

Patra, S. K. (2008). Dissecting lipid raft facilitated cell signaling pathways in cancer. *Biochimica et biophysica acta*, *1785*(2), 182–206. doi:10.1016/j.bbcan.2007.11.002

Pece, S., & Gutkind, S. (2000). Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of Epidermal Growth Factor Receptors upon cell-cell contact formation. *The Journal of Biological Chemistry*, *275*(52), 41227–41233.

Polo, S., Pece, S., & Fiore, P. (2004). Endocytosis and cancer.pdf. *Current Opinion in Cell Biology*, *16*, 156–161.

Pozdnyakova, I., Guidry, J., & Wittung-Stafshede, P. (2001). Copper stabilizes azurin by decreasing the unfolding rate. *Archives of biochemistry and biophysics*, *390*(1), 146–8. doi:10.1006/abbi.2000.2369

Punj, V., Bhattacharyya, S., Saint-Dic, D., Vasu, C., Cunningham, E. a, Graves, J., … Das Gupta, T. K. (2004). Bacterial cupredoxin azurin as an inducer of apoptosis and regression in human breast cancer. *Oncogene*, *23*(13), 2367–78. doi:10.1038/sj.onc.1207376

Quest, A. F. G., Leyton, L., & Párraga, M. (2004, February). Caveolins, caveolae, and lipid rafts in cellular transport, signaling, and disease. *Biochemistry and cell biology = Biochimie et biologie cellulaire*. doi:10.1139/o03-071

Quest, F. G., Lobos-González, L., Nuñez, S., Sanhueza, C., Fernández, J.-G., Aguirre, A., … Torres, V. (2013). The caveolin-1 connection to cell death and survival. *Current molecular medicine*, *13*(2), 266–81. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23228128

Rajendran, L., & Simons, K. (2004). Lipid rafts and membrane dynamics.pdf. *Journal of Cell Science*, *118*(6), 1099–1102.

Razani, B., & Lisanti, M. P. (2001). Caveolin-deficient mice : insights into caveolar function and human disease. *The Journal of Clinical Investigation*, *108*(11), 1553–1561. doi:10.1172/JCI200114611.In

Razani, B., Schlegel, A., & Lisanti, M. P. (2000). Caveolin proteins in signaling, oncogenic transformation and muscular dystrophy. *Journal of cell science*, *113*, 2103–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10825283

Razani, B., Woodman, S. E., & Lisanti, M. P. (2002). Caveolae: from cell biology to animal physiology. *Pharmacological reviews*, *54*(3), 431–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12223531

RCSB PDB. (n.d.). Retrieved November 17, 2013, from http://www.rcsb.org/pdb/explore/jmol.do?structureId=3UGE&bionumber=1

Ribeiro, A. S., Albergaria, A., Sousa, B., Correia, A. L., Bracke, M., Seruca, R., … Paredes, J. (2010). Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells. *Oncogene*, *29*, 392–402.

Shekhar, M., Pauley, R., & Heppner, G. (2003). Extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. *Breast cancer research*, *5*(3), 130–135.

Silvestrini, M. C., Tordi, M. G., Colosimo, a, Antonini, E., & Brunori, M. (1982). The kinetics of electron transfer between pseudomonas aeruginosa cytochrome c-551 and its oxidase. *The Biochemical journal*, *203*(2), 445–51. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1158249&tool=pmcentrez&rendertype=abstract

Simons, K., & Toomre, D. (2000). Lipid rafts and signal transduction. *Nature reviews. Molecular cell biology*, *1*(1), 31–9. doi:10.1038/35036052

Stan, R. V. (2005). Structure of caveolae. *Biochimica et biophysica acta*, *1746*(3), 334–48. doi:10.1016/j.bbamcr.2005.08.008

Staubach, S., & Hanisch, F. (2011, April). Lipid rafts: signaling and sorting platforms of cells and their roles in cancer. *Expert review of proteomics*. doi:10.1586/epr.11.2

Stuermer, C. (2009). The reggie/flotillin connection to growth. *Trends in Cell Biology*, *20*(1), 6–13.

Stuermer, C., Lang, D. M., Kirsch, F., Wiechers, M., Deininger, S. O., & Plattner, H. (2001). Glycosylphosphatidyl inositol-anchored proteins and fyn kinase assemble in noncaveolar plasma membrane microdomains defined by reggie-1 and -2. *Molecular biology of the cell*, *12*(10), 3031–45. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=60153&tool=pmcentrez&rendertype=abstract

Taylor, B., Rajeshwari, M., Yamada, T., & Al., E. (2009). Noncationic peptides obtained from az preferentially enters cancer cells.pdf. *Cancer Res*, *69*(2), 537–546.

Van Amsterdam, I. M. C., Ubbink, M., van den Bosh, M., Rotsaert, F., Sanders-Loehr, J., & Canters, G. W. (2002). A new type 2 copper cysteinate azurin: Involvement of an engineered exposed cysteine in copper binding through internal rearrangement. *J. Biol. Chem.*, *277*, 44121–44130.

Van Pouderoyen, G., Cigna, G., Rolli, G., Cutruzzola, F., Malatesta, F., Silvestrini, M. C., … Canters, G. W. (1997). Electron-transfer properties of Pseudomonas aeruginosa [Lys44 , Glu64] azurin. *Eur J. Biochem.*, *247*, 322–331.

Vogelstein, B., Lane, D., & Levine, a J. (2000). Surfing the p53 network. *Nature*, *408*(6810), 307–10. doi:10.1038/35042675

Volonté, D., Galbiati, F., Li, S., Nishiyama, K., Okamoto, T., & Lisanti, M. (1999). Flotillins/Cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with Caveolins in vivo. *The Journal of Biological Chemistry*, *274*(18), 12702–12709.

Weaver, A. M. (2006). Invadopodia: Specialized Cell Structures for Cancer Invasion. *Clin Exp Metastasis*, *23*, 97–105.

White, D. E., & Muller, W. J. (2007). Multifaceted roles of integrins in breast cancer metastasis. *Journal of mammary gland biology and neoplasia*, *12*(2-3), 135–42. doi:10.1007/s10911-007-9045-5

Williams, T. M., & Lisanti, M. P. (2004). The caveolin proteins. *Genome Biology*, *5*(3).

Wu, C., Calcagno, A., & Ambudkar, S. (2008). Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells : Evaluation of current strategies, *1*(2), 93–105.

Yamada, T., Fialho, A. M., Punj, V., Bratescu, L., Das Gupta, T. K., & Chakrabarty, A. M. (2005). Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity. *Cellular microbiology*, *7*(10), 1418–31. doi:10.1111/j.1462-5822.2005.00567.x

Yamada, T., Goto, M., Punj, V., Zaborina, O., Chen, M. L., Kimbara, K., … Chakrabarty, A. M. (2002). Bacterial redox protein azurin, tumor suppressor protein p53, and regression of cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(22), 14098–103. doi:10.1073/pnas.222539699

Yamada, T., Mehta, R. R., & Lekmine, F. (2009). A peptide fragment of azurin induces a p53-mediated cell cycle arrest in human breast cancer cells. *Mol Cancer Ther*, *8*(10), 2947–2958.

Yamaguchi, H., Takeo, Y., Yoshida, S., & Al., E. (2009). Lipid rafts and caveolin-1 are required for invadopodia formation and extracellular matrix degradation by human breast cancer cells. *Cancer Res*, *69*, 8594–8602.

Yang, C. P., Galbiati, F., Volonte, D., Horwitz, S. B., & Lisanti, M. P. (1998). Upregulation of caveolin-1 and caveolae organelles in Taxol-resistant A549 cells. *FEBS letters*, *439*(3), 368–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9845355

Zhuang, L., Lin, J., Lu, M., & Al., E. (2002). Cholesterolrich lipid rafts mediate Akt-regulated survival in prostate cancer cells. *Cancer Res*, *62*, 2227–2231.

8. Appendix A

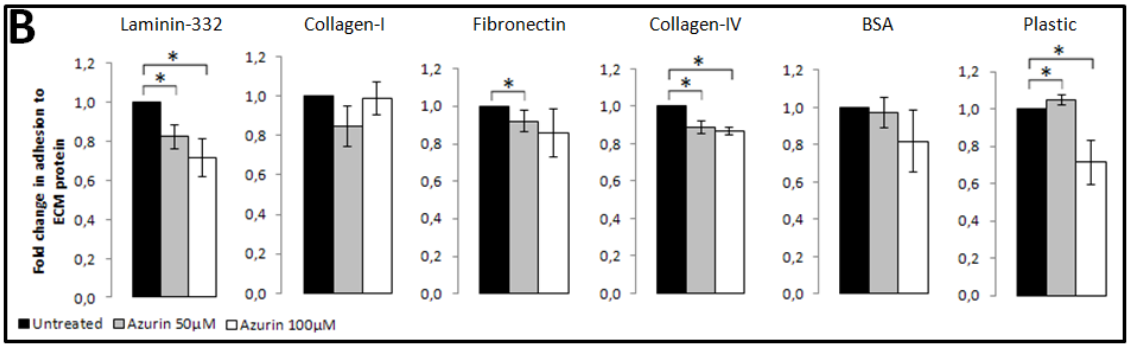

Figure 14. Adhesion assays in breast cancer cell line SUM149. SUM149 treated with azurin during 24h (B) and let to adhere during 30min in different ECM components (*: p<0.05). Adapted from (Bernardes *et al*., 2014).

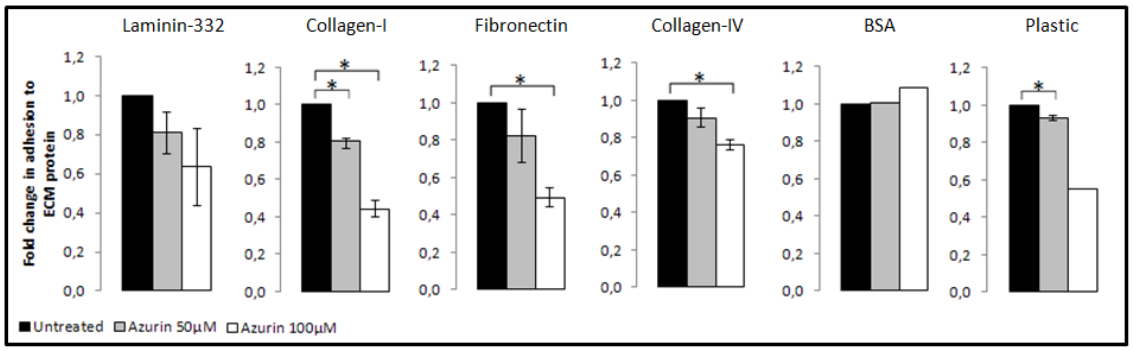

Figure 15. Azurin decreases adhesion in different ECM components (A549). A549 lung cancer cell line were treated with azurin during 48h and let to adhere during 30min in different ECM components (*: p<0.05)(Bernardes *et al*., in preparation).