The effects of azithromycin on cell signalling in human bronchial epithelium

Svanhvít Sigurjónsdóttir

Líf- og umhverfisvísindadeild
Háskóli Íslands
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14 eininga ritgerð sem er hluti af
Baccalaureus Scientiarum gráðu í Lífefna- og sameindalíffræði

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Líf- og umhverfisvísindadeild
Verkfræði- og náttúruvísindasvið
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Útdráttur

Fyrri rannsóknir hafa sýnt að meðferð með sýklalyfinu azithromycin (azm) getur bætt lungna-starfssemi sjúklinga með slímseigjusjúkdóm (cystic fibrosis) og hækkað viðnám (transepithelial electrical resistance, TER) frumna í frumurækt. Hvernig azm hefur þessi áhrif er þó ekki vitað. Í þessari rannsókn notaði ég VA10 frumur ræktaðar við aðstæður sem líkja eftir aðstæðum efri öndunarvegs (air-liquide interface, ALI) og Western blettun til að rannsaka innan frumu boðleiðir sem azm gæti haft áhrif á. Boðleiðir voru rannsakaðar sem höfðu áður verið tengdar við meðferð með makrólíðum eða við þéttitengi. Niðurstöður benda til þess að azm meðferð auki bæði tjáningu á PKCζ og fosfórýleringu þess, einnig benda niðurstöðurnar til þess að meðferðin dragi úr virkjun á bæði Akt og p38. Tjáning á EGFR virðist minnka með azm meðferð en virkjun þess virðist þó aukast lítillega. Þessar niðurstöður eru fyrsta skref í leit að boðleiðum sem azm hefur áhrif á og þær þarf að staðfesta með frekari prófunum.

Abstract

Treatment with azm has been shown to improve pulmonary function in cystic fibrosis patients, as well as increase TER in human bronchial cells in culture. It is however not clear how azm has this effect. This study utilised VA10 cells grown in ALI-culture, which simulates the conditions of the upper airway and Western blots to look for signalling pathways that could be affected by azm treatment. The results indicate some changes in certain signalling molecules that had previously been linked to macrolide treatment and tight junction proteins. Namely, these results indicated that there is an increase in PKCζ expression and its phosphorylation with azm treatment, and a reduction in phosphorylation was indicated in both Akt and p38, and finally that the expression of total EGFR is reduced although phosphorylation of EGFR(Y845) is increased with azm treatment. These results taken together give preliminary indications of the various signalling pathways involved in azm-mediated increased transepithelial electrical resistance.

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A special thanks to my family and to Arnar Ingi Bragason for their support, patience and encouragement.

Abbreviations

ALI	Air-liquid interface
Azm	Azithromycin
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
EGFR	Epidermal growth factor receptor
LPA	Lysophosphatic acid
MAPKs	Mitogen-activated protein kinase
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
RTK	Receptor tyrosine kinase
TER	Transepithelial electrical resistance
TJ	Tight junction
TLR	Toll-like receptor

1 Introduction

1.1 The human respiratory system

Every cell in the body requires oxygen for normal functions. The human respiratory system is responsible for the intake of oxygen and the expulsion of carbon dioxide which is achieved through respiration or breathing. Inhaled air travels through the pharynx and the larynx to the trachea, which divides into the left and right principle bronchi, which branches out in each lung finally producing approximately 6500 terminal bronchioles. The terminal bronchioles then branch into the respiratory bronchioles, from there air travels to the alveolar sacs where gas exchange occurs. Each lung contains around 150 million alveoli, giving the lungs a large amount of surface area for gas exchange. (ed. Susan Standring, PhD 2009; Martini et al. 2015)

The respiratory system has other functions besides gas exchange; it plays an important role in immune defence. The simplest form of this defence is the hair in the nasal cavity and the mucus in the pharynx that traps small particles so they do not reach the lungs. A more complex form of defence is the mucociliary airway epithelium; it maintains the structure and function of the lungs by trapping and removing pathogens without causing an inflammatory response. (Martini et al. 2015; Knowles & Boucher 2002; J. A. Whitsett 2002)

1.1.1 The human airway epithelium

The human airway epithelium is made up of multiple cell types that serve different purposes. The epithelium is pseudostratified, that is, all the cells are attached to the basement membrane but not all of the cells reach the surface of the layer (Karp et al. 2002). The three most common cell types in the airway epithelium are goblet cells that secrete mucus, ciliated cells that propel the mucus to clear the airway of particles and the basal cells that serve as stem cells (J. A. Whitsett 2002). The combination of the cell types varies in the airway, goblet cells and basal cells are more common in the upper trachea, while the ciliated cells are more common deeper in the lungs. Other cell types found in the airway epithelium include Clara, squamous, intermediate serous and neuroendocrine cells (Young et al. 2014; J. a. Whitsett 2002).

1.1.2 Lung disease

There are a number of lung diseases related to the innate immune defence of the airway epithelium; the most prevalent are cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD).

CF is an inherited disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. There are multiple mutations in the gene and different mutations cause different phenotypes of CF. The mutations that cause CF affect the mucociliary airway epithelium, the central hypothesis on CF is that the ion transport is interrupted causing dehydration of the airway epithelium (ed. Longo et al. 2012). This leads to lung infections and increases the inflammation response, because the lungs are unable to clear mucus from the airway (Davies et al. 2007). Because of this CF patients suffer from chronic infections by Staphylococcus aureus and Pseudomonas aeruginosa. Because of these infections CF patients are often prescribed antibiotics, one of these is azm. The effects of azm in clinical trials on both CF and COPD are discussed in chapter 1.3.

The main cause of COPD is tobacco smoking although there are other known factors such as pollution and genetics. The reduction of airflow is the main indicator of COPD and it is mostly caused by the inflammation of the lung tissue. The development of symptoms is complicated but it is believed to be an interaction between the recruitment of inflammatory cells, tissue destruction, apoptosis and other pathogenic mechanisms (Balkissoon et al. 2011).

1.2 The VA10 cell line

The complexity of the bronchial epithelium causes problems in establishing cell lines that accurately represent their *in vivo* counterparts. The VA10 cell line was made using primary bronchial epithelial cells transfected with retroviral constructs from human papilloma virus (HPV) 16 containing E6 and E7 oncogenes. The cell line expresses cytokeratins 5/6, 13, 14 and 17, indicating that the cell line includes cells with a basal phenotype. This was further supported by the fact that the cell line expresses α6β4 integrin and p63.

When the VA10 cell line is cultured in an air-liquid interface (ALI) it generates a pseudostratisfied epithelium. VA10 cells are also capable of generating high transepithelial electrical resistance (TER) in ALI culture denoting functional tight junctions (TJ) (Halldorsson & Asgrimsson 2007).

1.2.1 Air – liquid interface cell culture

When studying bronchial epithelium it is important to have a model that closely resembles the tissue *in vivo.* By culturing the VA10 cells in ALI culture the cells obtain a pseudostratified phenotype *in vitro*. These culture conditions (Figure 1) resemble the *in vivo* conditions; medium is supplied to the basal membrane and the apical surface is

exposed to air. Cells cultured in ALI conditions will secrete mucus, have cilia and measurable TER.

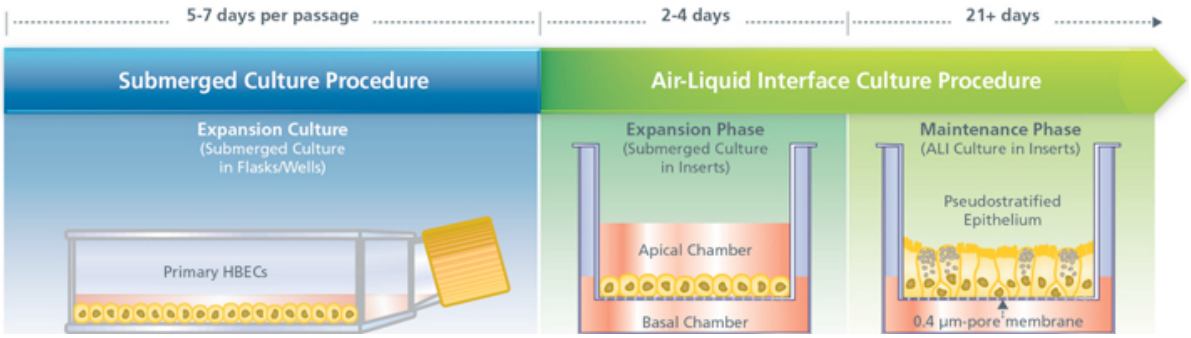

Figure 1: An example of ALI culture. Cells are originally cultured in a flask where they are completely submerged. From there the cells are transfered into wells with media in both the upper and lower chamber. Once the cells are confluent the medium is removed from the upper chamber and the apical side is exposed to air causing the cells to differentiate into a pseudostratified phenotype. (Stem Cell Technologies 2014)

1.3 Azithromycin

Macrolides are drugs with a macrocyclic lactone ring of 12 or more elements. Azithromycin (azm) is one of these drugs; it has a 15 membered lactone ring and has been shown to improve survival rates of patients with diffuse panbronchiolitis (DPB) as well as evidence that it improves lung function in patients with CF (Kanoh & Rubin 2010) and COPD (Herath & Poole 2014). Despite improvements in lung function among CF patients, there has been little change in the amount of *Pseudomonas aeruginosa* or *Staphylococcus aureus* in patients after treatment (Clement et al. 2006). This suggests that the improvement in CF patients after azm treatment is not caused by the eradication of these bacteria (Schultz 2004; Clement et al. 2006; Asgrimsson et al. 2006).

Infection by *P.aeruginosa* has been shown to decrease TER *in vitro* (Lee et al. 1999), and further research also indicates that epithelia with low TER is more receptive to *P.aeruginosa* binding than high TER epithelia (Fleiszig et al. 1997). Azm increases TER when added to the basolateral side of human airway epithelium *in vitro*, affecting TJ proteins but without effect on the viability of the cells or apoptosis (Asgrimsson et al. 2006). This increase in TER could therefore reduce the amount of *P.aeruginosa* that binds to the epithelium.

COPD patients have exacerbations of the disease; these are short periods of increased coughing and sputum. These exacerbations can range from mild to severe but always require some form of medical attention. In clinical trials, daily treatment with azm reduced the number of exacerbations patients experienced (Ishii & Kida 2014; Herath & Poole 2014). Patients that received azm but were also currently smokers did not have the same improvement as patients that had quit smoking. This could mean that the effects smoking has on mucociliary clearance and other factors of the innate immunity are working against the azm (Han et al. 2014).

It also appears that azm strengthens the epithelial integrity and maintains the polarity of the bronchial epithelium which helps protect against pathogens (Halldorsson et al. 2010; Cigana et al. 2006; Gillis & Iglewski 2004).

1.4 Cell signalling

Macrolides such as azm have various effects on cell signalling pathways and studies indicate that different macrolides can affect different pathways, or the same pathways in different degrees. Macrolides have been shown to primarily disturb pathways related to three types of membrane receptors: G-protein-coupled receptors (GPCR), receptor tyrosine kinases (RTKs) and Toll-like receptors (TLRs) (Kanoh & Rubin 2010). A number of signalling pathways have been implicated in macrolide treatment in previous studies, but this study focuses on a few signalling molecules, with special attention on those molecules that appeared to be involved in our model.

1.4.1 Protein kinases C

Protein kinases C (PKCs) are a family of serine/threonine specific enzymes. There are 12 known isozymes of PKCs that are divided into three subfamilies: conventional, novel and atypical. PKCζ is a member of the atypical family and it can be found associated to TJ proteins in the plasma membrane (González-Mariscal et al. 2008).

The different isoforms in the atypical PKC family are regulated by different factors; Ca^{2+} does not regulate PKCζ like the other isoforms. Studies have shown PKCζ to be regulated by ceramide (Lozano et al. 1994; Müller et al. 1995), and in intestinal epithelial cells it is regulated by phosphoinositide-dependent kinase 1 (PDK-1), a part of the phosphatidylinositol 3-kinase (PI3K) pathway (Mashukova et al. 2012). Furthermore, there have been indications that PKCζ plays a central role in TJ formation and the establishment of epithelial cell polarity and that PKCζ has a role in the activation of mitogen-activated protein kinases (MAPKs) (Eckert et al. 2004; Suzuki et al. 2001).

It has been shown that lysophosphatic acid (LPA), a naturally occurring peptide in body fluid that has a role in cell proliferation, increases TER in bronchial epithelial cell culture by enhancing the accumulation of E-cadherin (transmembrane protein, important in cell adhesion) at cell-cell junctions through a PKCζ mediated pathway (He et al. 2009). PKCζ has been shown to interact with the coiled-coil domain of occludin, an integral TJ protein (Nusrat et al. 2000).

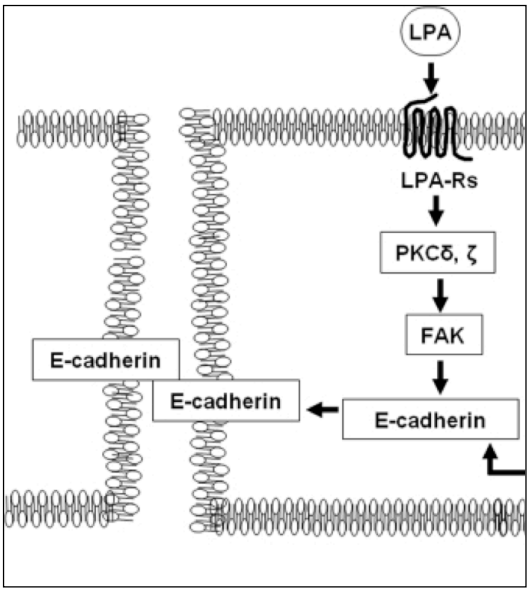

Figure 2: Proposed pathway of LPA regulation of E-cadherin. LPA ligation phos-phorylates PKCζ and another isozyme PKCδ. This leads to the accumilation of E-cadherin at cell-cell junctions (He et al. 2009).

1.4.2 Akt

Akt is a serine/threonine specific kinase that is also known as protein kinase B. It is important in various cell-signalling cascades such as cell proliferation, growth and apoptosis (Testa & Tsichlis 2005). Upstream activation of Akt by PI3K and mTOR is caused by activation of RTKs in both instances. PI3K activates Akt by phosphorylating the activation loop of Akt at T308 in a reaction that can be reversed by a phosphatase (PTEN phosphatase), but mTOR phosphorylates Akt at S473 which can be de-phosphorylated by a phosphatase (PHLPP phosphatase) (Manning & Cantley 2007; Engelman et al. 2006).

Akt has been associated Akt with TJ proteins, a study showed that occludin knockout reduced the phosphorylation of Akt in kidney epithelium (Du et al. 2010). Lin et al. showed in 2013 that TLR2, a toll like receptor, and TFF3 (Trefoil factor 3) peptide, protect against IL-1β-induced increase in TJ permeability in intestinal epithelium. This protection is dependent on the PI3K/Akt pathway (Lin et al. 2013).

1.4.3 Mitogen-activated protein kinases

MAPKs form a signalling network that responds to intracellular and extracellular stimuli. They play a vital role in cell proliferation, differentiation, inflammatory response and apoptosis. p38 kinases, of which there are four isoforms (α, β, γ, δ), are a subfamily of the MAPKs. The p38 kinases have a Thr-Gly-Tyr motif in their activation loop and phosphorylation of the threonine and tyrosine is essential for their activation. All isoforms can be phosphorylated by MKK6 (a MAPK kinase), while p38α, p38γ and p38δ can additionally be phosphorylated by MKK3 (Kanoh & Rubin 2010; Zhang & Liu 2002; Zhang & Dong 2005). The MKK responsible for activation of p38 is often cell-type and stimulus-specific (Cuenda & Rousseau 2007).

TLRs are key part of the innate immune system; they recognise specific microbial patterns and upon recognition and ligation trigger signal transduction events that lead to the activation of p38 and NFκB. This activation leads to the production of various inflammatory cytokines that are necessary for the eradication of the infection (Kanoh & Rubin 2010; Zhang & Dong 2005; Hashimoto et al. 2000). It has also been indicated that MAPKs can be regulated by E-cadherin through an EGFR mediated pathway (Pece & Gutkind 2000) and that the inhibition of PI3K negatively affects the activation of p38 (Laprise et al. 2002).

1.4.4 Epidermal growth factor receptor

RTKs are a family of cell surface receptors that have been shown to regulate important cellular processes. There are 58 known RTKs and they are divided into 20 subfamilies based on function. One of those families is the EGFR family, which the epidermal growth factor receptor (EGFR), also known as ErbB1, and 3 other receptors (Erb2, ErbB3 and Erb4) belong too (Hackel et al. 1999). When EGFR binds a ligand it causes domain rearrangements, which trigger transduction events. It has been shown that tyrosine phosphorylation of EGFR(Y845) contributes to epithelial repair (Allahverdian et al. 2010; Hyun 2012). Phosphorylation of EGFR(Y1045) leads to binding of c-Cbl, an adaptor protein that interacts with multiple proteins, that subsequently leads to ubiquitination and finally the degradation of the receptor (Levkowitz et al. 1999). When EGFR is stimulated by EGF it autophosphorylates at Y1173 which serves as a docking site for Src homology 2 a signalling molecule that activates ERK a member of the MAPK family (Hsu et al. 2011).

2 Aim of this project

A number of signalling molecules have been linked to the effects macrolides have on bronchial epithelium. In this project we wanted to study the expression and activation of some of these molecules in our model, a bronchial epithelium cultured in ALI-culture.

We focused on the aforementioned signalling molecules, p38, PKCζ, Akt and EGFR. The bronchial epithelial cell line VA10 was cultured in ALI-culture and treated with azm, which increases TER. Western blots were used to detect the expression of the signalling molecules in both their normal and activated states.

The purpose of the project was to further investigate the effects of azm on these specific signalling molecules in our model of human bronchial epithelium.

3 Methods and Material

3.1 Cell culture

VA10 cells were cultured at 37°C in 5% CO_2 in T25 flasks in BEGM media (Cell Applications, San Diego, USA) supplemented with retinoic acid (Cell Applications), and 50 IU/ml penicillin, 50 mg/ml streptomycin (Invitrogen, NY, USA). VA10 cells at passage 20 were used for ALI culture experiments. The media on the cells was changed every other day.

3.1.1 Air-liquid interface cell culture

For ALI cell culture, 12 mm Transwell inserts (Corning Inc., Corning, NY) with 0,4 μm pore size were used. The inserts were first treated with 0,5 mL of bovine collagen I (Advanced Biomatrix, San Diego, USA) diluted 1:44 in PBS. After 30 min at 37°C the liquid was aspirated and the inserts washed with PBS. Approximately 150.000 cells were seeded into the upper chamber of each insert and cultured with BEGM until confluent (approx. 2 days). After the cells were confluent the medium was changed to DMEM/F-12 (Gibco-Life Technologies, Carlsbad, USA) containing L-Glutamine and 15 mM HEPES and supplemented with 2% Ultroser G (Pall, Washington, USA) and penicillin/streptomycin. The media was changed every other day for one week. After this period air-liquid culture commenced.

ALI culture was started once the media in the upper chamber was removed, and for the duration of the experiment the media in the lower chamber was changed every second day. At the same time ALI commenced, treatment with azm was also started. Half of the total numbers of chambers were treated with 40 μg/mL of azm (Arzneimittel Gmbh, Ursensollen, Germany).

3.1.2 Transepithelial electrical resistance measurements

Once the filters were moved over to ALI culture, TER was measured every 2 days. In order to measure TER, pre-warmed DMEM/F-12 (1:1) (Gibco) + 5% P/S was added to the upper chamber and the samples incubated for a minimum of 20 minutes. Measurements were made using a Millicell-ERS voltohmmeter (Merck-Millipore, Darmstadt, Germany), whereby a probe was inserted into the upper and lower chamber of the Transwell (Figure 3). Resistance was measured between the shorter probe, on the apical side of the membrane, and the longer probe on the basolateral side. Finally the DMEM/F-12 was removed, the cells rinsed with PBS and returned to ALI culture.

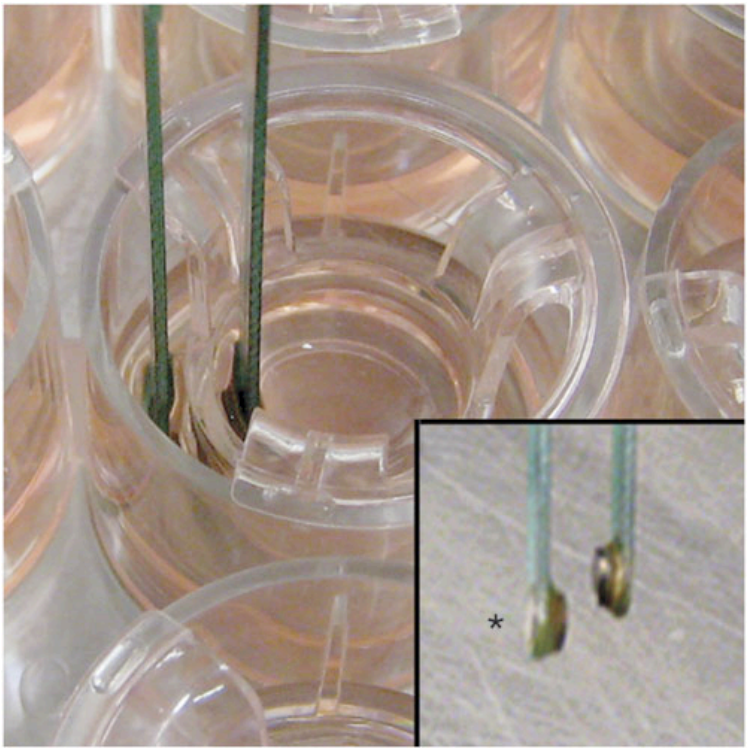

Figure 3: Measuring TER using a voltohmeter. A probe with two electrodes of different lengths is inserted into the cell culture. One electrode goes into the lower chamber (basolateral side of the membrane) while the other is in the upper chamber (apical side) then the voltohmeter measures the resistance between the two electrodes (Sonoda et al. 2009).

3.2 Protein extraction

At the conclusion of the experiment, the filters were removed from ALI culture and washed twice with cold PBS. RIPA buffer (20 mM Tris (pH 7.4), 1% Triton-X, 1% sodium deoxycholate, 10% glycerol, 150 mM NaCl, 2.5 mM EDTA 1% aprotinin, 1mM PMSF, 100mM Na_3VO_4,) containing additional proteinase and phosphatase inhibitors (1:100) (Thermo Scientific, Rockford IL) was prepared and 50 µL added to each filter. A cell scraper was used to disrupt the cells and the cells lysate was left to incubate for 10 minutes on ice. The cell lysate was then transferred to an eppendorf tube and frozen in liquid N_2 repeatedly (5 freeze/thaw cycles). After final thawing it was spun down and supernatant collected.

3.2.1 Bradford assay

A Bradford protein assay was used to determine the protein concentration in the cell lysates. Briefly, samples and blanks were measured in triplicate in a dilution series and plotted against a previously measured standard curve to estimate protein concentration.

3.3 Western blot

Equal amounts of protein (5 µg) were loaded onto NuPage 10% Bis-Tris gels (Novex, Carlsbad, USA) and run with 1x NuPage MES running buffer (Novex) at 175 V for 45 min. The protein was then transferred to a polyvinylidene difluoride (PVDF) membrane using semi-dry transfer at 20 V for 1 hour. The membrane was the blocked using a TBS blocking buffer (Odyssey, Lincoln, USA) for one hour at room temperature. The membrane was then incubated over night with the selected primary antibodies. On the second day the membrane was washed with TBS containing 0.1% Tween 20 and incubated for an hour with the secondary antibodies (Odyssey Li-Cor) diluted 1:20000 in the TBST wash buffer containing 0,02% SDS. The membranes were then imaged using an Odyssey LiCor imager.

3.3.1 Densitometry software

Image Studio software (Li-Cor) was used to quantify the bands of the Western blots. The intensity of the signal detected by the Li-Cor imager is proportional to the amount of protein; different bands can be quantified relative to a control (Li-Cor 2014).

3.3.2 Antibodies

Table 1: Antibodies used in Western blots

Antibody	Species	Isotype	Dilution	Company	Cat. #
Akt (pan)	Mouse	IgG1	1:2000	Cell Signaling	2920
pAkt (S473)	Rabbit	_	1:2000	Cell Signaling	4060
Erk1/2	Mouse	IgG1	1:1000	Cell Signaling	4696
Erk(pT202/Y204)	Rabbit	IgG	1:1000	Cell Signaling	4370
PKCζ	Rabbit	_	1:1000	Cell Signaling	9372
pPKCζ/λ	Rabbit	_	1:1000	Cell Signaling	9378
PKCδ	Rabbit	_	1:1000	Cell Signaling	2058
pPKCδ	Rabbit	_	1:1000	Cell Signaling	9374
P38α	Rabbit	_	1:1000	Santa Cruz	535
MAPKp38 (T180/Y182)	Rabbit	_	1:1000	Cell Signaling	4511
IL1B	Rabbit	_	1:1000	Santa Cruz	7884
EGFR	Rabbit	_	1:5000	Cell Signaling	4267
pEGFR(Y845)	Mouse	IgG1	1:1000	BD Biosciences	558381
pEGFR(Y1045)	Rabbit	_	1:1000	Cell Signaling	2237
pEGFR(Y1173)	Rabbit	_	1:1000	Cell Signaling	4407
JNK	Rabbit	_	1:1000	Santa Cruz	571
pJNK (T183/Y185)	Goat	_	1:1000	Santa Cruz	12882
NFκB	Rabbit	_	1:1000	Santa Cruz	372
IκB- α	Mouse	_	1:1000	Santa Cruz	1643
pIκB- α	Goat	_	1:1000	Santa Cruz	7977
IL6	Rabbit	_	1:1000	Santa Cruz	7920
Actin	Mouse	IgG1	1:5000	Abcam	3280
HSP90	Rabbit	_	1:1000	Santa Cruz	7947

3.4 Technical difficulties

There were a number of technical difficulties this study faced. The first problem was that the original cell culture was started with cells in passage 34 when the ALI culture was set up. After 2 weeks of azm treatment no change in TER was detected, the cause of this is most likely the high passage number. It is thought that the cells after several passages lose their ability to increase TER, this turned out to be the case. Additionally, in many Western blots neither the signalling molecules nor the loading control were detected - this turned out to be because of a contaminated TBS/0.1% Tween buffer. In a few cases the loading control was detected but not the sample protein with or without treatment, despite repeated Western blot attempts. These antibodies were against proteins that by all indications should be expressed in VA10 cells, a likely cause of this would be the quality of the antibodies used, eg. age, inappropriate storage. Due to time restraints and lack of materials it was not possible to order new antibodies to test this.

4 Results

To compare expression of signalling molecules in their total and activated form Western blots were performed on protein lysates from VA10 cells cultured in ALI culture with and without azm treatment. The cells treated with azm showed a two fold increase in TER over those that remained untreated. All lysates are from pooled Transwell inserts from one experiment, three inserts for the control and three for treatment with azm. The Western blots were performed only once due to time constraints and lack of materials, therefore no statistical analysis was performed on the data.

4.1 The expression and activation of PKCζ is increased with azm treatment

PKCζ is a signalling molecule that has a role in TJ formation and effects cell polarity, potentially having a role in the increase in TER associated with azm treated cells. There was a notable increase in phosphorylation of PKCζ after treatment with azm when compared to the control sample (Figure 4). **Figure 5** shows quantified intensity of the signals from Figure 4 relative to the actin loading control. There is an increase in the expression of total PKCζ, furthermore a considerable increase in the expression of phosphorylated PKCζ, indicating that azm treatment increases both the expression of total PKCζ and its activation in the VA10 cells.

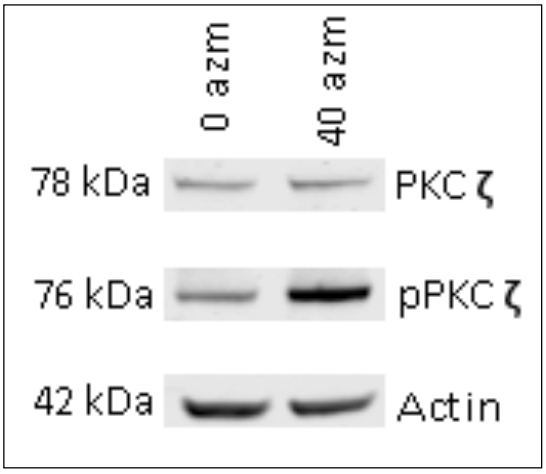

Figure 4: Protein expression of PKCζ and pPKCζ with and without azm treatment. Images of Western blots of PKCζ, pPKCζ and the actin loading control, showing the increase in intensity of pPKCζ after azm treatment.

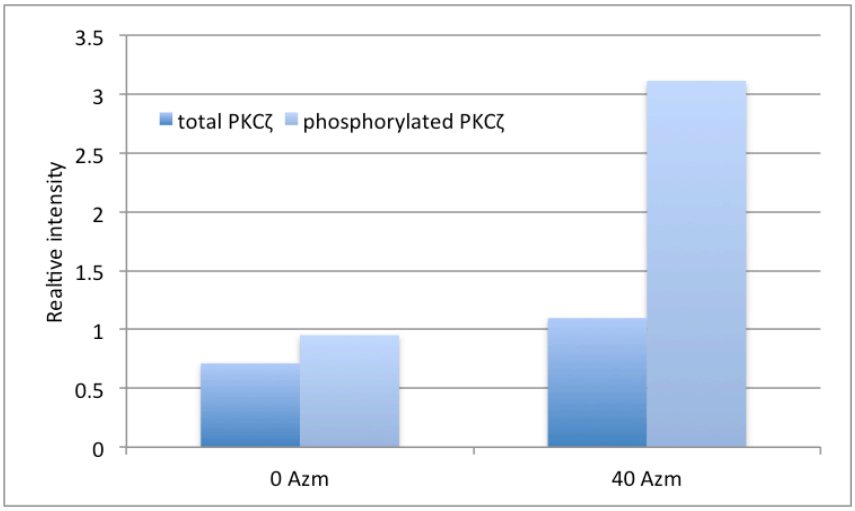

Figure 5: Intensity of PKC ζ and phosphorylated PKCζ relative to actin. The relative intensity of PKCζ and pPKCζ quantified from the Western blot in Figure 4.

4.2 Treatment with azm reduces the amount of phosphorylated Akt

Studies have shown that macrolides affect RTKs and that Akt is regulated by RTKs. Akt is also associated with TJ proteins, therefore Akt was one of the signalling molecules chosen for this study. There was a notable reduction in the phosphorylation of Akt in the azm treated samples compared to the control as can be seen in Figure 6. In Figure 7 the intensity of the phosphorylated Akt signals from Figure 6 were quantified relative to the intensity of the total Akt signals. The reduction of phosphorylated Akt is clear which indicates that azm treatment decreases the activation of Akt in the VA10 cells.

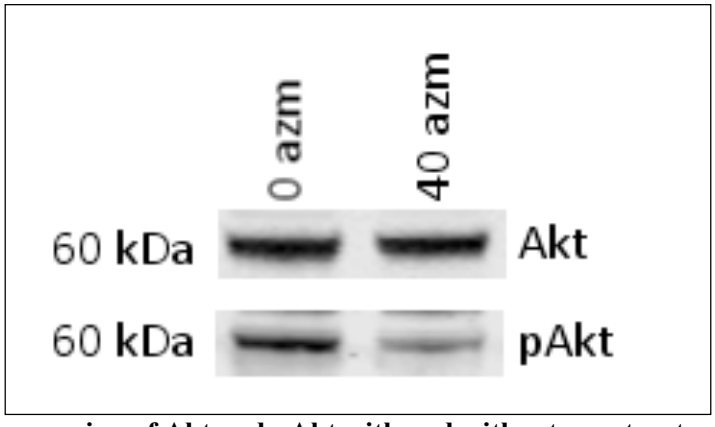

Figure 6: Protein expression of Akt and pAkt with and without azm treatment.

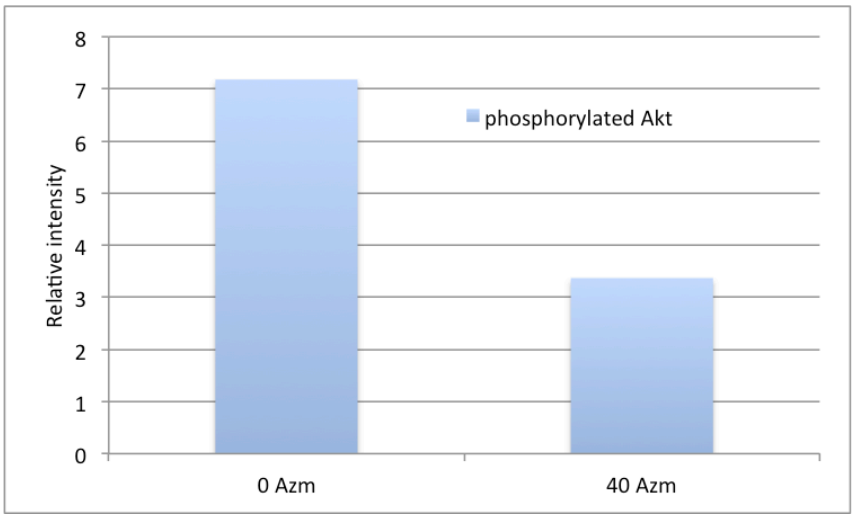

Figure 7: Intensity of phosphorylated Akt relative to total Akt. The relative intensity of pAkt quantified from the Western blot in **Figure 6**.

4.3 Treatment with azm decreases the phosphorylation of p38

p38 has been shown to be regulated by E-cadherin, a TJ protein, and by TLRs, membrane receptors affected by macrolides. This makes the p38 pathway interesting when studying the effects of azm treatment. In Figure 8 the Western blot results for p38 can be seen. The phosphorylated p38 signals from Figure 9 were quantified relative to the total p38 signals (Figure 8). There is a difference in the amount of protein loaded between the samples as can be seen with the HSP90 loading control (Figure 8). When the intensity of the signals is normalised and calculated relative to the total p38 signal the reduction in phosphorylation of p38 in the azm treated VA10 cells is considerable, indicating that azm treatment decreases or prevents the phosphorylation of p38.

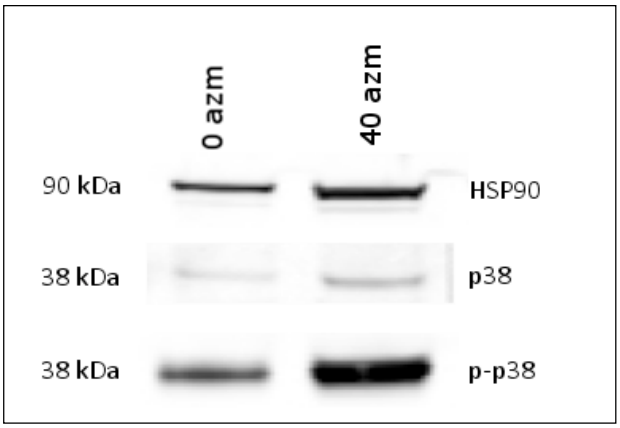

Figure 8: Protein expression of p38, p-p38 and the HSP90 loading control with and without azm treatment.

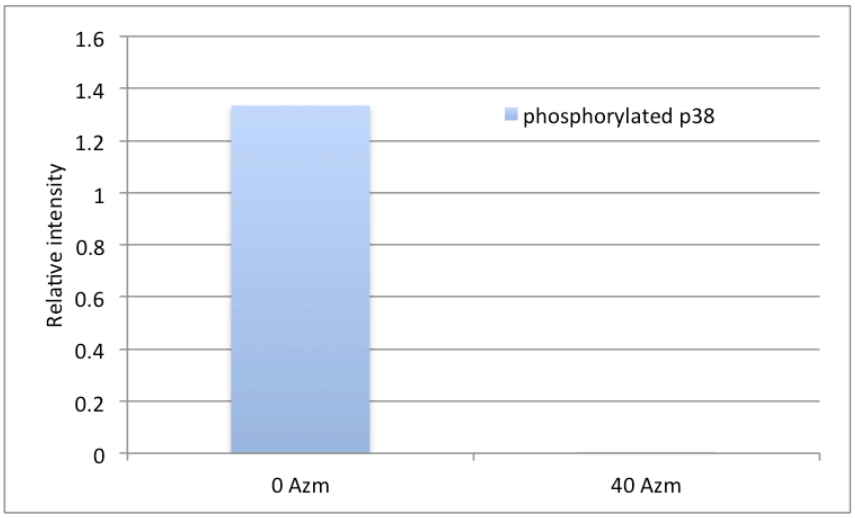

Figure 9: The intensity of phosphorylated p38 relative to total p38. The relative intensity of p38 quantified from the Western blot in **Figure 8**.

4.4 EGFR expression is decreased with azm treatment

It has been shown in previous studies that RTKs are affected by macrolides. In this study the affect of azm on both the expression of total EGFR and on the activation of EGFR was tested with Western blots. In Figure 10 the Western blot results of total EGFR are shown. The intensity of the signal of total EGFR was quantified relative to the HSP90 loading control and there was a notable reduction in the signal from the azm treated samples (Figure 11).

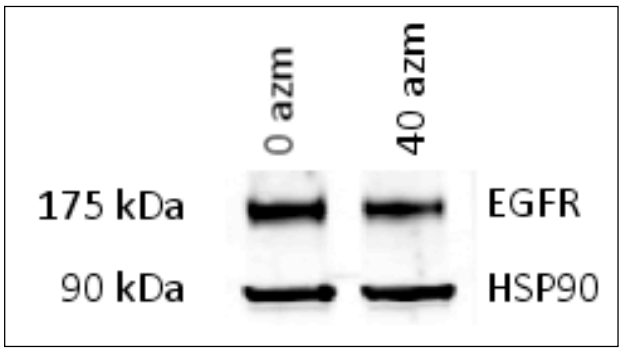

Figure 10: Protein expression of EGFR and the HSP90 loading control with and without azm treatment.

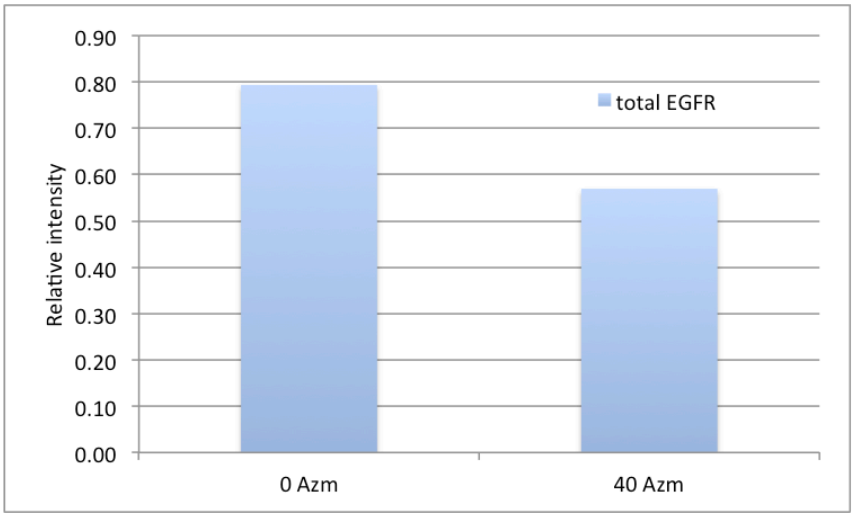

Figure 11: The intensity of total EGFR relative to HSP90. The intensity of the total EGFR signal relative to the HSP90 signal quantified from the Western blot in **Figure 10**.

4.5 Phosphorylation of EGFR(Y845) is increased with azm treatment

In Figure 12 the results of the Western blot of the phophorylated EGFR(Y845) can be seen. The intensity of the signal was quantified relative to the total EGFR signal (Figure 13) and there appeared to be an increase in the phosphorylation at Y845 site.

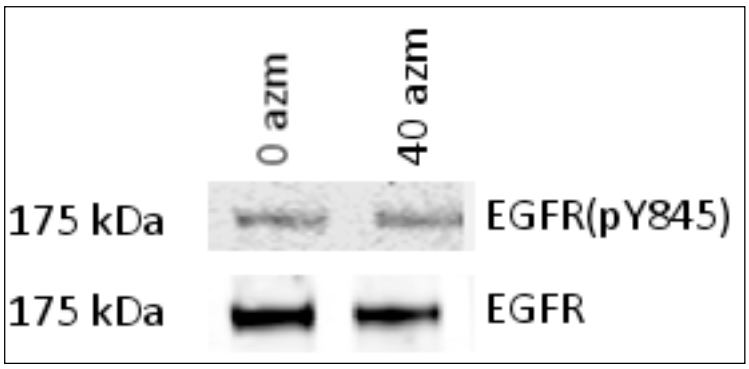

Figure 12: Protein expression of EGFR and EGFR(Y845), with and without azm treatment.

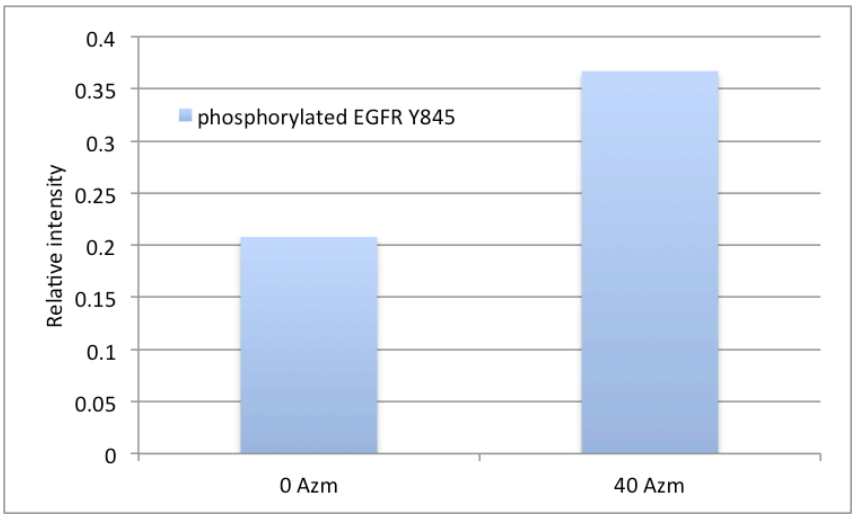

Figure 13: The intensity of phosphorylated EGFR(Y845) relative to total EGFR. The relative intensity of EGFR(Y845) quantified from the Western blot in **Figure 12**.

The phosphorylation of EGFR(Y1045) and EGFR(Y1173) were also tested with Western blots (Figure 14) but no signals were detected.

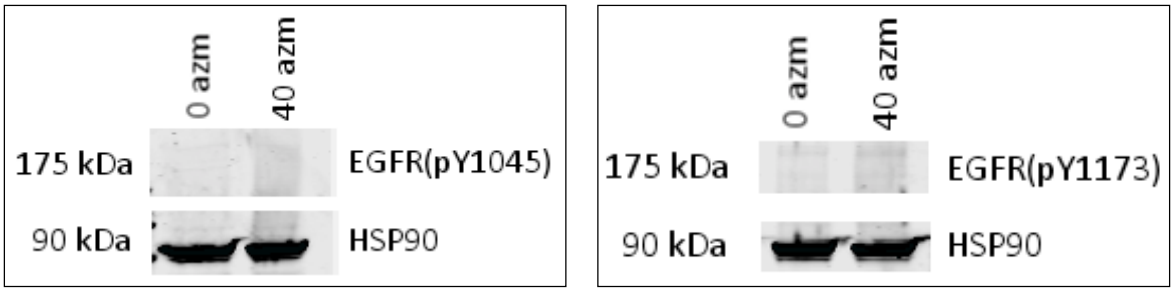

Figure 14: The protein expression of EGFR(Y1045) and EGFR(Y1173) and the HSP90 loading control. Neither protein was detected in the Western blots.

4.6 Other signalling molecules

Other signalling molecules were examined using Western blots, including NFκB, IκB, IL1β, JNK, ERK1/2, PKCδ and IL6. No signals were detected from these molecules although a loading control was always observed. Due to time restraints and lack of materials these blots could not be repeated in this study.

5 Discussion

It is clear that azm affects bronchial epithelial cells in the current model, whereby epithelial cells were grown on Transwell filters. This study supports the fact that azm treatment causes an increase in TER. It is known that in drug trials azm improves pulmonary function in patients with CF without killing the bacteria, and helps patients with COPD. It is not understood how azm exerts these effects. To search for signalling cascades that mediate these effects is a vast project and therefore, the aim of this research was primarily to be the first step in identifying affected signalling molecules in our model.

Signalling pathways are complicated: there is immense crosstalk between many pathways and multiple regulators in each cascade. To date, no signalling studies have been done on this model; thus, this is a primary study with the aim of locating affected cascades for further research. To choose signalling molecules for this study we looked at molecules that had been previously indicated to be affected by macrolides and, since azm has been shown to increase TER, were involved with TJs.

The results of this study indicate an increase in both PKCζ expression and activation. A previous study showed that LPA increased TER in bronchial epithelial cells, it also showed that the inhibition of PKCζ weakened these effects LPA had on TER. Future research could focus on observing if the inhibition of PKCζ has a similar effect on the azm-increased TER. PKCζ regulates both NFκB and IκB (Hirai 2003), therefore we thought it would be interesting to research the effects azm could have on their expression and activation. Unfortunately we were unable to demonstrate expression of neither NFkB nor IkB, so were unable to draw any further conclusions to the relationship or role of PKCζ in our model.

In a study done in 2002, Laprese et al. showed that the inhibition of PI3K reduced the activation of both Akt and p38α in human intestinal epithelial cells (Laprise et al. 2002). Studies done on human intestinal epithelium can be used as guidelines for human bronchial epithelium research; they share some common gene expression patterns and signalling events, and are made up of the same cell types although the composition differs. The similarities between the cell models could warrant further investigation into these effects in bronchial epithelium. The results of this study indicate that the activation of both Akt and p38 is reduced with azm treatment making the PI3K pathway an interesting option for further research.

Asgrimsson et al. (2006) researched the effects of azm on the TJ proteins claudin-1, caludin-4, E-cadherin, occludin and JAM-A. The study showed that azm affected the processing of all these proteins except E-cadherin. This suggests that the effects of azm are directly linked to the TJ proteins and the results of this study support these findings as stated previously, studies have shown occludin to affect regulation of Akt in kidney

epithelial cells (Asgrimsson et al. 2006; Du et al. 2010) and that occludin interacts with PKCζ in bronchial epithelial cells (Nusrat et al. 2000).

A study dating from 2010 linked the phosphorylation of EGFR(Y845) to epithelial repair. The study indicated that treatment with azm increases the phosphorylation of EGFR(Y845) (Allahverdian et al. 2010) potentially indicating the effects of azm on epithelial cells could also be related to epithelial repair processes. Allahverdian et al did not examine if EGFR was activated on additional phosphorylation sites, namely, EGFR(Y1045) and EGFR(Y1173). In the lysate used for this study, that is, harvested at the conclusion of ALI culture after 2 weeks, phosphorylation of these sites was not detected (Figure 14), with or without azm treatment. Although phosphorylation of EGFR(Y1045) was not detected here, it cannot be ruled out that this event has occurred. Phosphorylation of a particular site can be a rapid, transient event and therefore, the timing of harvesting lysate is crucial. Although speculative, it is possible that this event had already occurred, leading to the down-regulation of EGFR causing the lower signal of total EGFR in Figure 10. If this process is a rapid event it could be that the phosphorylation of EGFR(Y1045) would not be detected at the time point the lysates were harvested for this experiment.

Taken together, these results repeatedly indicate involvement of the PI3K pathway, which has roles in activation of PKCζ, Akt and p38. It would therefore, be a logical next step to research other components of this pathway. Other regulators that could have interesting results are the MKK kinases that phosphorylate p38.

This is an overview of several signalling cascades and all of these signalling molecules have various functions and each of them takes part in more than one pathway. However, it is worth noting for the direction of future studies that PKCζ, Akt and p38 all have a role in cell proliferation.

Due to time restraints and lack of material the results of this study have not been replicated. To verify these results Western blots need to be repeated, changes in expression verified with qPCR, and the effects of regulators, such as PKCζ, Akt and p38 could be verified by stimulating the cells in culture using inhibitors or activators. Studies into signalling cascades often include several time points to monitor the state of the molecules of interest. Since this was a preliminary study, this was not done, which raises additional questions about the various results observed. For example, it could give better answers about the EGFR(Y1045) phosphorylation if this experiment was repeated for various time points. These results also highlight similarities between the effects of the PI3K pathway on PKCζ, Akt and p38 in bronchial and intestinal epithelial cells.

After the conclusion of this study a gene expression study was performed comparing azm-treated and –untreated VA10 cells in ALI culture. It would be interesting to compare the results of the sequencing study with this one to verify the effects of azm shown here.

This study was the first step into researching the effects of azm treatment on signalling pathways. Trying to piece together signalling cascades that are affected by azm will be a long process but future research could start by investigating the effects azm has on molecules up- and downstream of PKCζ, Akt, p38 and EGFR.

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