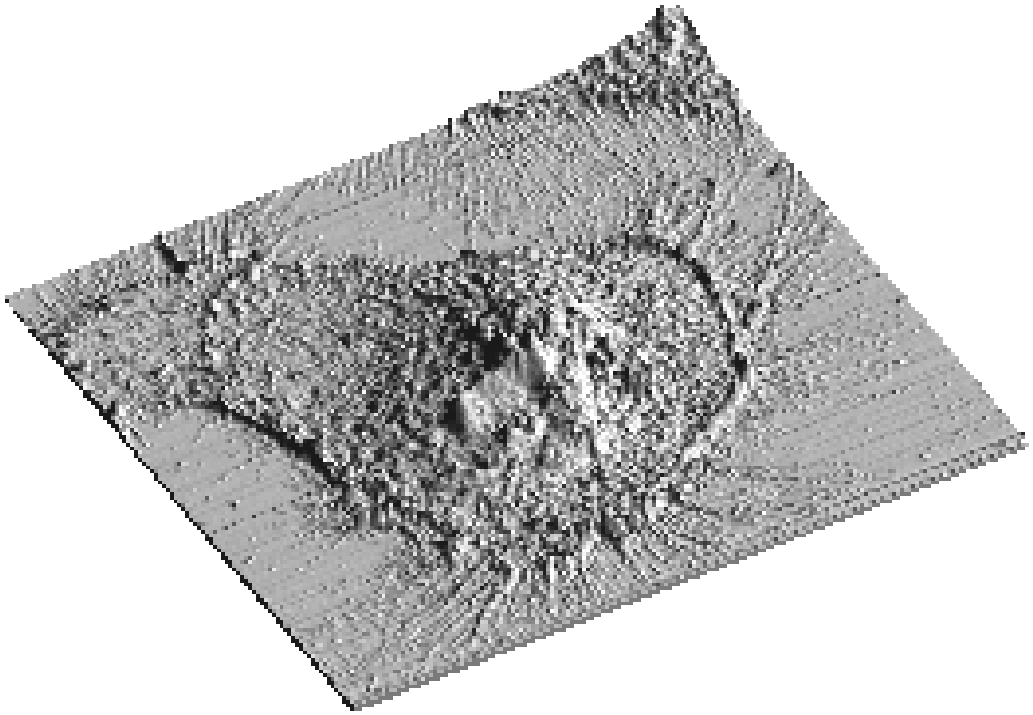
Resveratrol- and Rapamycin-induced Morphological Changes to Human Epithelial Colon Cancer DLD-1 Cells

Ping Fang & Jon Ambæk Durhuus



Supervisor: Associate Professor Ole Vang

Experimental Assistant: Ph.D. Stud Peter Kristian Kristoffersen

Molecular Biology; Department of Science, Systems and Models

2007/2008

ROSKILDE UNIVERSITY

Abstract

The prosperous polyphenol resveratrol have shown healthy benefits and anti-cancer properties, but the effect of resveratrol on cell morphology is less studied. The anti-suppressant rapamycin, targets mTOR (the mammalian target of rapamycin), which exist in two complexes in mammals (mTORC1 and -2), and have in recent studies controversially showed that both complexes are inhibited by rapamycin. Resveratrol seems to somehow to interact with the mTOR and PI3K-Akt and the treatment of rapamycin plus resveratrol is therefore interesting to study. The PI3K-Akt pathway is known as an important role of cell growth and migration by regulating F-actin (filamentous actin) assembly. The morphological changes to the human epithelial colon cancer cell line, DLD-1, treated with 60 μM resveratrol and 0.5 μM rapamycin was scanned with atomic force microscopy (AFM). The cells were incubated for 48 and 72 hours, fixated and scanned in air, and hereafter size and height were measured. The results show that resveratrol enhance the area about 8 times, when compared to untreated and rapamycin-treated cells. Resveratrol plus rapamycin increase the area but less than the sole resveratrol treatment. Incubation of resveratrol and/or rapamycin decreases the height the cells.

Keywords

Resveratrol, rapamycin, mTOR, AFM, Cell morphology, Akt.

Readers Guide

The report is build up with a theory section focusing on the biological processes on less on the physical aspects of the mechanism behind the AFM. The experiments used are briefly described so are the various techniques used in this project. Depending on the readers own experience these sections can be read according to his or hers level. Even Though we have tried to only describe the processes and substrates involved in our experiment, others have to mention because of the complexity of the pathways involved. Common used abbreviations are put in the bottom of this page.

Acknowledgement

Above all, we would like to thank our supervisor; Associate Professor Ole Vang, Roskilde University, for his inspiration and guidance.

Next we will like to thank Ph.D. Stud Peter Kristian Kristoffersen, for his many hours of technical support, his guidance and insight. Without his help this project would not have been possible.

We would also like to thank the lab-staff of Professor Ole Vang and Professor Birgitte Munch Petersen, for help when needed. We also like to thank the people of Cismi, for kindly providing us with the equipment of the Atomic Force Microscopy (AFM) and their software.

Abbreviations

4E-BP1, the eukaryotic initiation factor 4E (eIF4E) binding protein 1; **AFM**, atomic force microscopy; **Arp2/3 complex**, actin-related proteins 2/ 3; **BPAEC**, Bovine pulmonary artery endothelial cells; **FAT** domain, FRAP, ATM, TRAP; **F-actin**, filamentous-actin; **FKBP12**, FK506 Binding Protein; **FRB**, FKBP12 rapamycin-binding; **G-actin**, globular actin; **GβL**, G-protein β-subunit like protein; **Girdin/APE**, girders of actin filaments/Akt phosphorylation Enhancer; **mSIN1,** Mammalian Stress-activated protein kinase Interacting protein 1 **mTOR**, mammalian target of rapamycin; **mTORC1/2**, mammalian TOR complex 1/ 2; **NF-κB,** nuclear factor-kappa B; **PAK**, p21-activated kinase complex; **PDK**, protein dependent kinase; **PDK1/ 2**, phosphoinositide-dependent kinase 1/ 2; **PH** domain, pleckstrin homology domain; **PI3K**, Phosphoinositide 3-kinase; **PIP_2**, phosphatidylinositol (4,5)-biphosphate; **PIP_3** (phosphatidylinositol (3,4,5)-triphosphate; **PIX**, PAK-interacting protein; **PKB**, protein kinase B; **PKC**, protein kinase C; **PRAS40**, proline-rich Akt substrate of 40 kilodaltons; **PTEN**, Phosphatase and Tensin homolog; **Raptor**, regulatory associated protein of mTOR; **Rheb**, Ras homolog enriched in brain; **Rictor**, rapamycin-insensitive companion of mTOR; **S6K1**, p70-S6 Kinase 1; **TSC1/ 2**, tuberous sclerosis complex1/ 2;

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Problem formulation

How will the effect of resveratrol influence mTOR pathway of the human colon cancer cell line DLD-1, and what will we observe when combined with rapamycin and scanned in atomic force microscopy?

1. Introduction

Since the human genome has been available, novel therapeutic strategies has changed from a protein-centric view towards a more pathway-centric view. We will in this report try to add new knowledge to this global project. We have used a human colon cell line (DLD-1) and treated it with resveratrol and rapamycin. The morphological changes were observed by AFM (atomic force microscopy,) and the results are discussed in respects to new knowledge regarding the feasible pathways involved. Since the first microscope was used to study the scrapings of a tooth or some dirty rainwater, a new world of microorganisms and even smaller parts have emerged in front of us. We were given the opportunity to use a relative new microscope, in the biological regiment, to study changes on a cellular level.

Resveratrol is found in a range of consumer products including grapes, red wine and peanuts, and it is believed to give benefits to the health. One of the proven effects of resveratrol is an anti-proliferative effect on cancer cells [Aggarwal *et al.,* 2004; Aziz *et al*., 2003]. Resveratrol have shown to induce morphology changes on for example bovine pulmonary artery endothelial cells (BPAEC) [Bruder *et al*., 2001], human leukaemia cell line HL-60 and the human Hepatocellular carcinoma cell line HepG2 [Stervbo *et al.,* 2006b]. These changes in cell size have been observed on several occasions in our institute.

Rapamycin was initially used to suppress the immune system in patients with organ transplantations. Later rapamycin has been used as a cytostatic, showing to arrest cell growth in tumours and to block the mTOR pathway. Thus, we hypothesized that the morphological changes induced by resveratrol could be decreased or inhibited by adding rapamycin to the assay.

AFM is a relatively new tool for studying biological samples. We had to fixate the cells, which is necessary for scanning biological samples in air. The analysis of the cell area and heights, plus two and three dimensional pictures were obtained. These, to our knowledge, unique AFM pictures of resveratrol plus rapamycin treated cells, combined with new knowledge, shed light on some of the questions regarding the pathways and some of the many roles of resveratrol.

This project will not discuss the AFM technique in details and the role of resveratrol and rapamycin as an anti-carcinogenic drug. The scanning done by the AFM gave many options regarding analysis and literature search, we have chosen to focus on the pathway from the drugs; resveratrol and rapamycin, through mTOR and PI3K/Akt, to the F-actin assembly. In the analysis of the scanned cells we chose to focus on the actin structures in the cells. The peaks of the cells, observed in the resveratrol- and resveratrol plus rapamycin-treated cells, are an interesting field to, but will not be discussed in this report.

2. Theory

2.1 Resveratrol

Resveratrol, first isolated in 1940 from the roots of white hellebore (*Veratrum grandiflorum*) as an antifungal/antibacterial agent, showed later anti-inflammatory and anti-carcinogenic properties plus indicated a cardioprotective role, associated with the "French paradox" [Renaud and de Lorgeril, 1992].

The antioxidant resveratrol (3,5,4´-trihydroxystilbene) is a polyphenolic natural product synthesized by a range of plant species, including grapes, nuts, pines and berries. The concentration found in peanuts and berries are normally low. It is especially in the skin and seeds of red grapes that the concentration of resveratrol is high, but the bioavailability are negligible as no resveratrol can be found in plasma after ingestion [reviewed in Bertelli, 2007]. Red wine seems to be the major source in the western world. The concentration varies from grape species, product year, climate and other factors [Stervbo *et al.,* 2006a].The concentration of resveratrol seems to be higher in organic wines, compared to conventional produced wines, which might be due to the elevated risk of attack by fungicides and bacteria, making the grapes produce more antifungal/antibacterial agents [Dani *et al.,* 2007]. The Japanese knotweed has

a particular high concentration and is now used to produce commercial resveratrol supplements.

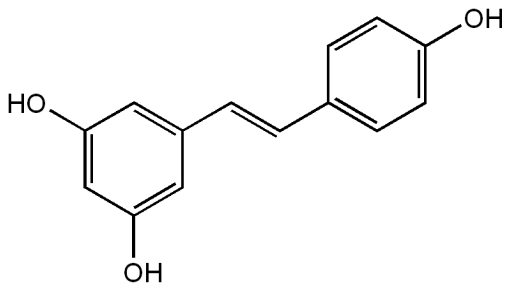


Figure 1 Resveratrol in its *trans*-form (3,5,4´-trihydroxystilbene) - a stilbene (two phenols, which are joined by a 3 carbon chain, containing a double bond in the middle) with 3 hydroxy groups. [www.wikipedia.org].

Resveratrol has shown to interfere with the pathways: PI3K/Akt, MAPK (Mitogen-activated protein MAP kinases) and NF-κB (nuclear factor-kappa B) and to induce necrosis and/or apoptosis.

In the PI3K/Akt pathway, resveratrol was found to inhibit cell transformation by blocking the EGF-induced (epidermal growth factors) activation PI3K/Akt in epidermal cells of mice and down regulate EGF in human endometrial adenocarcinoma cells (the Ishikawa cell line, derived from a 39 year-old Asian woman). In human breast cancer cells, resveratrol interfered with an estrogen receptor-associated PI3K pathway, thereby blocking PI3K activation. Resveratrol has also shown indications of interfering with the receptor tyrosine kinase pathway.

In the MAPK pathway, resveratrol inhibited the signalling in cervival carcinoma cells and has shown to require p53 activation and subsequent apoptosis. Resveratrol has shown to phosphorylate p53 in an epidermal mouse cancer cell line and in thyroid carcinoma cell lines. Different concentrations of resveratrol seem to influence the MAPK pathway differently in human neuroblastoma cells.

Resveratrol has furthermore shown to suppress angiotensin and LDL (low density lipids), inhibit proliferation and to induce apoptosis in smooth muscle cells. Resveratrol-induced angiotensin II-suppression in smooth muscle cells (in rats) has shown to activate PI3K/Akt, ERK1/2 and S6K1 [reviewed in Fulda and Debatin, 2006]. The cardio protective effects of resveratrol has recently been linked the activation of Akt, which generates a survival signal through Thioredoxin [Das *et al.,* 2008].

Resveratrol has also shown to inhibit glucose metabolism in epithelial ovarian cancer cells (human cell lines). Cancer cells, with elevated glycolysis, seem to be sensitive to glycolysis-inhibition, and many tumours often rely on glycolysis rather than oxidative phosphorylation for ATP production. This is called the Warburg effect [Warburg, 1956]. The autophagocytosis mimics that of caloric restriction. This might be due to Akt inhibition, which is often elevated in ovarian cancer. Studies have shown that resveratrol inhibits Akt and mTOR signalling in a time and dose-dependent manner in a human ovarian cancer cell line, A2780. However normal epithelial cells were not affected by resveratrol [Kueck *et al.,* 2007].

Studies of resveratrol and morphological changes in endothelial cells have shown that resveratrol changes the cell shape [Bruder *et al.,* 2001]. Stervbo *et al.* showed that resveratrol-treated HL-60 (human promyelocytic leukemia) and HepG2 cells (human hepatocellular carcinoma cell), increased in nuclear size and granularity. Increased cell granularity has been linked to apoptosis. The increased nuclear size is less studied, but the change in the nuclear membrane morphology, was proposed to be linked with the cell cycle arrest in the S-phase [Stervbo *et al.,* 2006b].

Other Studies of the effects of resveratrol on cell morphology in have indicated that resveratrol interfere with the signalling cascades involving in F-actin (filamentous-actin) assembly and that PKC (protein kinase C) signalling is a target of resveratrol [Slater *et al.,* 2003] (both described in the cell morphology section). It has been shown that resveratrol incorporates into the cell membrane [Garcia-Garcia *et al.,* 1999]. The results seem to support the contention that the beneficial effects of resveratrol may be mediated by an impact on membrane-associated PKC activity and the associated signalling networks. The effect of resveratrol on cell morphology, regarding its inhibitory effect on PKC isozymes, seems to explain some of resveratrol´s beneficial effects on cellular and cardiovascular functions. However resveratrol seems to bind to only some isozymes and especially when they are membrane bound [reviewed in Slater *et al.,* 2003]. For example binds resveratrol to PKCa with higher affinity than other PKC´s [Garcia-Garcia *et al.,* 1999] and to class II PI3K (described in the PI3K-Akt section) [Srivastava *et al.,* 2005].

Another study with the cell line HepG2 showed that resveratrol furthermore reduces the dNTP pool (deoxyribonucleoside triphosphates) [Horvath *et al.,* 2005].

Studies in prolonged lifespan due to caloric restriction, shows similarities with resveratrol treatment, which mimics the Sir2 dependent lifespan extension during

caloric restriction. SIRT1 is a mammalian homolog to Sir2, which is activated by resveratrol. SIRT1 is known to regulate longevity, apoptosis, DNA repair and to facilitate changes in nutritional levels by NAD^+ levels [Lagouge *et al.,* 2006].

2.2 Rapamycin

Rapamycin was found in the soil of the Easter Island Rapa Nui in 1965. Rapamycin is a macrocylic lactone product of the soil bacteria *Streptomyces hygroscopius* [Paghdal and Schwartz, 2007]. Macrocylic lactones are natural antibiotics belonging to a class called polyketides. Polyketides are secondary metabolites not directly involved in the normal growth, development and reproduction as primary metabolites, but involved in for example, defend and communication. Rapamycin was isolated in 1975 and first identified as an antifungal antibiotic [Vezina *et al. 1975*].

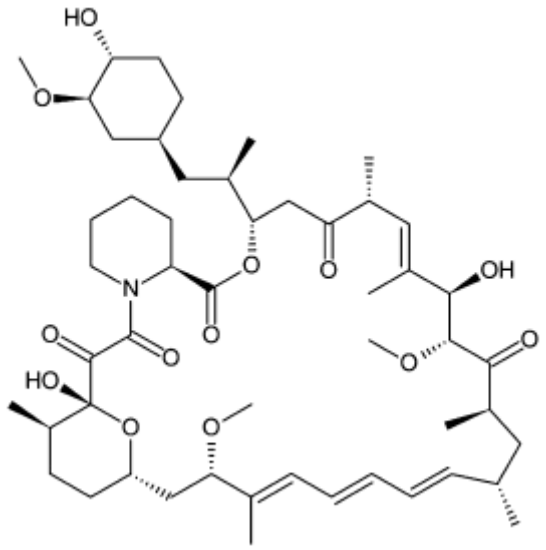


Figure 2 Structure of Rapamycin [Paghdal and Schwartz, 2007]

In 1999 rapamycin was approved by FDA (the Food and Drug Administration) as an immunosuppressive drug under the name Rapamune ® as a prophylaxis of organ rejection. In 2000 it was approved in Europe. Rapamycin is also known as sirolimus. Rapamycin was initially used to suppress the immune system in patients with organ transplantations. Later rapamycin has been used as a cytostatic, showing to arrest cell growth in tumours either by inducing apoptosis or arresting cells in the G1 phase, leading to a suppression of T-lymphocyte activation and proliferation. Rapamycin has also been used in studies and case reports for patients with tuberous sclerosis, psoriasis

Kaposi´s sarcoma, which is a AIDS defining illness caused by a human herpesvirus [Paghdal and Schwartz, 2007].

Rapamycin has shown to be a difficult clinical anti-cancer agent because of its poor water solubility and its poor stability in solution. Two analogues, CCL-779 and RAD001 (everolimus) with improved clinical use has been synthesized and have entered clinical trials.

Rapamycin binds to the intracellular receptor FKBP12 (FK506 Binding Protein) forming an inhibitory complex which interacts with the protein kinase mTOR (mammalian target of rapamycin) [Sarbassov *et al.* 2005]. FKBP12 is an immunophilin that binds to immunosuppresive drugs. FK506 or tacrolimus are other examples of an immunosuppresive drugs derived from soil bacteria, which binds to FKBP12 [Kino *et al.,* 1987]. Rapamycin binds to a region in the C terminus or TOR called FRB (FKBP12 rapamycin-binding) causing inhibition of TOR activity by binding to the mTORC1 (mTOR complex 1) [Hay and Sonenberg, 2004]. By its inhibition of mTOR, rapamycin has also shown to decrease VEGF concentration [Paghdal and Schwartz, 2007].

mTOR however, exists in two complexes, and "the classic theory" says that only TORC1 binds to the FKBP12-rapamycin complex [Sarbassov *et al.* 2005]. However recent studies have shown that mTORC2 is also affected by rapamycin [Sarbassov *et al.,* 2006] (explained in the mTOR section).

2.4 mTOR

mTOR (mammalian target of rapamycin) is essential for growth and proliferation. If the *TOR* gene is disrupted the result is lethal. In human beings, a dysfunctional mTOR pathway are seen many cancer forms and in diseases. mTOR was identified and cloned in 1994 shortly after the discovery of *TOR1* and *TOR2* in *s. Cerevisiae* (yeast) during a screen for resistance to rapamycin. Where *s. Cerevisiae* has two different TOR proteins, the human analogue is a single protein. The mammalian protein however, exists in two distinct complexes mTORC1 and mTORC2 [Sarbassov *et al.,* 2005a] both which are essential [Thedieck *et al.,* 2007]. *s. Cerevisiae* has often been used as a model for the mammalian analogue, so has dTOR from *Drosophila melanogaster* (fruitfly).

mTOR is made of 2549 amino acids and contains 20 tandem HEAT repeats in the N terminus. Each HEAT repeat contains 2 a helices with both hydrophobic and

hydrophilic residues. mTOR contains in a downstream order of; a FAT domain (FRAP, ATM, TRAP), FRB, a kinase domain, a negative regulatory domain (NRD) and a FATC domain in the C-terminal half. The TOR kinase is a highly conserved, central controller of cell growth and may be involved in more proteins than those described below [Hay and Sonnenberg, 2004]. FRAP is another name for mTOR.

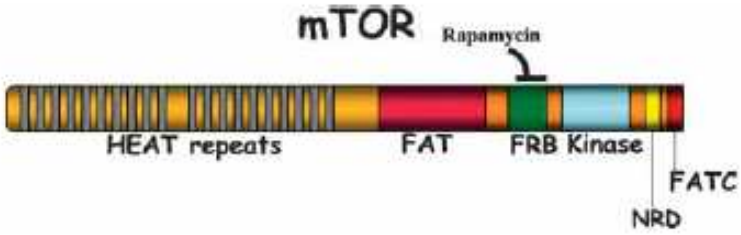


Figure 3 the primary structure of mTOR with the 20 HEAT repeats, the FAT domain (FRAP, ATM, TRAP), FRB (FKBP12 rapamycin-binding), kinase domain, NRD (negative regulatory domain) and the FATC domain (another FAT domain) [Hay and Sonnenberg, 2004].

Both mTOR complexes are composed of mTOR and mLST8/GβL. GβL (G-protein β-subunit like protein) is now most commonly referred to as mLST8, which is a mammalian ortholog to LST8 from *s. Cerevisiae*. However the two TOR complexes differs in some proteins and therefore they are regulated by different signals and drugs.

One of the differences is a protein called either Raptor (regulatory associated protein of mTOR) in mTORC1 or Rictor (rapamycin-insensitive companion of mTOR) in Rapamycin-insensitive complex [Sarbassov *et al.,* 2005a]. Whether Rictor is affected of rapamycin or not, will be presented in the mTORC2 section.

Upstream of mTOR

Two of the main components upstream of mTOR is the TSC1/2 complex and Rheb (Ras homolog enriched in brain). TSC (tuberous sclerosis complex) 1 and 2, is also known as hamartin and tuberin. The TSC heterodimer is a GTPase activating protein that inhibits Rheb. Rheb, is member of the Ras superfamily and a small GTPase. Rheb-GTP binds and activates mTORC1. Akt phosphorylates and inactivates TSC2, whereas AMPK (AMP activated protein kinase) phosphorylates and activates TSC2 [Thedieck *et al.,* 2007]

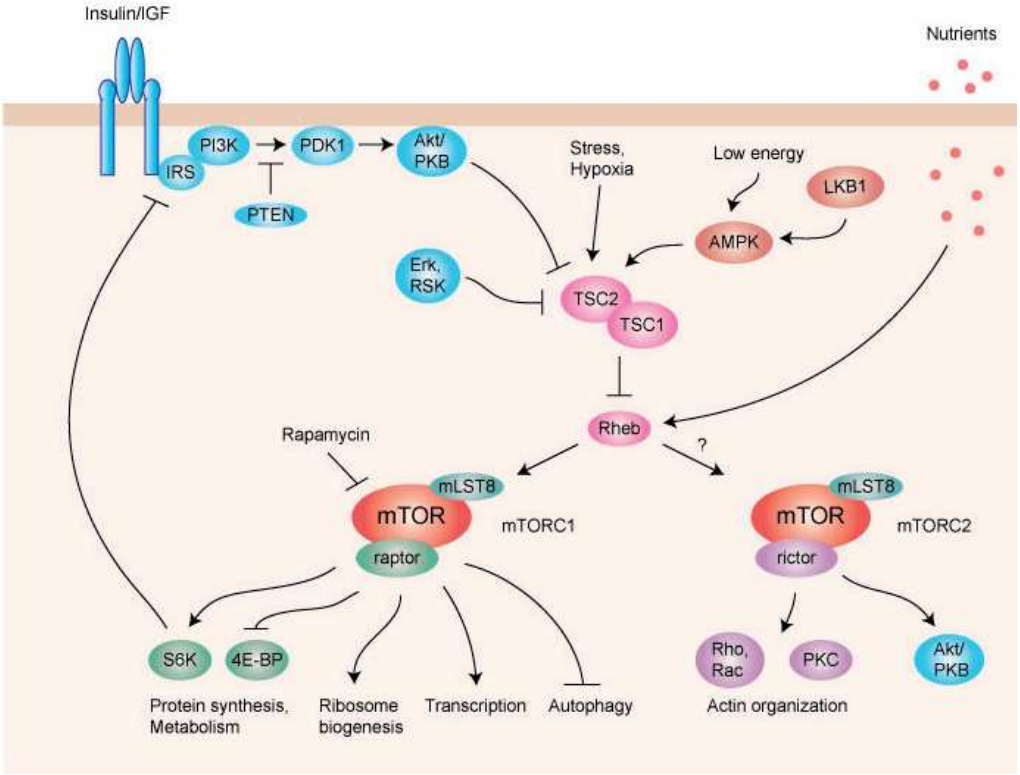


Figure 4 Pathway of mTOR and the substrates involved. PI3K activates Akt through PDK1. The TSC1/2 complex functions as a key negative regulator of mTOR and TSC1/2 is inhibited by Akt. Rheb is downstream of TSC1/2 and stimulates S6K1 and 4E-BP phosphorylation through mTORC1. S6K1 is involved in a feedback loop, which suppress the Akt pathway by inhibiting IRS (insulin receptor substrate). Rapamycin blocks mTORC1 directly [Hall *et al.*, 2005]. Rapamycin also blocks mTORC2 (not shown). Some novel substrates are not shown on the picture but explained in the theory section.

2.4.1 mTORC1

mTORC1 (The mammalian TOR complex 1) is composed of mTOR, Raptor, mLST8/GβL [Sarbassov *et al.,* 2004]. A novel component; PRAS40, has recently been associated with mTORC1 [Oshiro *et al.,* 2007, Thedieck *et al.,* 2007]. This complex functions as a nutrient/energy/redox sensor and a controller of protein synthesis. mTORC1 is activated by nutrients (amino acids), anabolic growth factors (such as insulin and insulin-like growth factor), hormones and cellular energy (ATP) [Jacinto *et al,* 2006, Hay and Sonnenberg, 2004] and inhibited by low nutrient levels, growth factor deprivation and reductive stress. Moreover mTORC1 is inhibited by a range of drugs including rapamycin [Jacinto *et al,* 2006] and curcumin (which is found in for example turmeric, where the yellow colour is a result of the polyphenol curcumin/ diferuloylmethane) [Beevers *at al.,* 2006]. mTORC1 controls transcription, protein synthesis, ribosome biogenesis, nutrient control and autophagy [Hay and

Sonnenberg, 2004]. Growth factor signals and energy status are transmitted to mTORC1 through TSC1/ 2. PI3K is also involved in mTORC1.

2.4.1.1 Raptor

Raptor was first found in *s. Cerevisiae* and was named KOG-1 (kontroller of growth-1) and has been identified as a positive regulator of TOR, because *s. Cerevisiae* with the phenotype KOG-1- resembles cells lacking TOR or rapamycin-treated cells [Loewith *et al,* 2002]. Raptor, consist of a conserved region in the N-terminal half, found in all Raptor othologs. Hereafter there are 3 HEAT repeats and 7 WD-40 repeats in the C-terminal half. WD-repeat proteins are a large family found eukaryotes and are implicated in a variety of functions including: Signalling, transcription, cell cycle and apoptosis. WD-40 repeats are short amino acid motifs (about 40 amino acids long), often terminating in a Trp-Asp (W-D) dipeptide.

The 20 HEAT repeats in the N-terminal of mTOR binds with Raptor, but the C-terminal of mTOR can also bind to Raptor, although this bonding is weaker. During nutrition deprivation mTOR-Raptor inhibits further mTOR activity [Kim *et al,* 2002]. The Raptor-mTOR complex has shown to positively regulate cell size and a number of processes including mRNA translation [Richter *et al.,* 2005], ribosome biogenesis [Hannan *et al.,* 2003], nutrient metabolism [Peng *et al.,* 2002] and autophagy [Meijer and Codogno 2004]. Inhibition of the complex has shown to cause decrease in size. When mTORC1 is activated, S6K1 (p70-S6 Kinase 1) and 4E-BP1 (the eukaryotic initiation factor 4E (eIF4E) binding protein 1), becomes phosphorylated. S6K1 and 4E-BP1 are downstream regulators of mTOR, which increases protein synthesis [Jacinto *et al,* 2006]. Both Raptor and mLST8/GβL are positive regulators of mTORC1 activities.

2.4.1.2 PRAS40

PRAS40 (proline-rich Akt substrate of 40 kilodaltons), is an upstream negative regulator of mTORC1. It binds to mTORC1 via Raptor, but requires mTOR kinase activity. The PRAS40 phosphorylation may contribute to the mTORC1-interaction. PRAS40 inhibits mTORC1 autophosphorylation and mTORC1 kinase activity towards S6K1 and 4E-BP1 [Thedieck *et al.,* 2007, Oshiro *et al.,* 2007].

mLST8/GβL

mLST8/GβL interacts with the kinase domain of mTOR (see Figure 3), independently of Raptor and plays a positive role in mTOR activation by nutrients. It has been shown that mLST8/GβL stabilizes mTOR-Raptor and it is therefore likely that mLST8/GβL, Raptor

and mTOR comprise a nutrient-sensitive complex and that mLST8/GβL regulates mTOR-Raptor stabilization under different nutrient conditions [Kim *et al,* 2003]. However studies have shown that rapamycin induces mTOR-Raptor dissociation but amino acid deprivation does not alter mTOR-Raptor association [Oshiro *et al.,* 2004]. mLST8 is also a substrate of mTORC2.

2.4.2 Downstream of mTORC1

S6K1 was the first component of the pathway to be identified and despite it has been well studied, exactly how S6K1 regulates cell size remains still unclear [Sarbassov *et al.,* 2005a].

S6K1phosphorylation state is a convenient measure of the activity of the Raptor branch of the pathway [Sarbassov *et al.,* 2005a]. mTORC1 phosphorylates S6K1 in the hydrophobic motif, which stimulates the subsequent phosphorylation of S6K1 by PDK1. Active S6K1 can stimulate the initiation of protein synthesis. Mice lacking S6K1 have small cells [Pende *et al.,* 2004]. Most mammalian cells becomes more reduced in size by rapamycin than by S6K1 inhibition, indicating that more growth regulators must exist downstream of raptor. [Kim *et al,* 2002]. S6K1 can also participate in a positive feedback loop with mTORC1.

Opposite S6K1, the role of 4E-BP1 is better understood. When 4E-BP1 is in its non-phosphorylated it represses the cap-dependent mRNA translation by binding to the translation initiation factor eIF4E. eIF4E is a cap-binding protein which interacts with to the eIF-4G protein. Upon phosphorylation by mTORC1, 4E-BP1 releases eIF4E, allowing it to restore cap-dependent translation and will no longer inhibit the binding of mRNA to 4E-BP1 [Sarbassov *et al.,* 2005a].

Both S6K1 and 4E-BP1 are downstream effectors of mTOR and recruited by Raptor. The interaction is mediated by the TOS motif (TOR signalling) which is found in the N termini of both S6K1 and 4E-BP1 [Kim *et al,* 2002] and is a conserved shorts sequence. Raptor recognizes the TOS motif, which is required for efficient phosphorylation [Sarbassov *et al.,* 2005a]. Rapamycin inhibits the mTOR-Raptor interaction and results in non-phosphorylated S6K1 and 4E-BP1 [Kim *et al.,* 2002].

The lack of conservation between S6K1 and 4E-BP1 phosphorylation sites and the high frequency of TOS-motif-like sequences in many proteins makes identification of mTOR substrates difficult. S6K1 and 4E-BP1 seems to be direct substrates of raptor-mTOR [Sarbassov *et al.,* 2005a].

2.4.3 mTORC2

mTORC2 (The mammalian TOR complex 2) is composed of mTOR, Rictor/mAVO3 and mLST8/GβL [Jacinto 2004/Sarbassov 2004] and newly discovered proteins named mSIN1/MIP [Jacinto *et al.,* 2006] and the mTORC2 associated protein, PRR5L/Protor [Woo *et al.,* 2007]. TORC2 in *s. Cerevisiae* contains TOR2, LST8, AVO1, AVO2, AVO3 and BIT61 [Loewith *et al.,* 2002]. AVO3 is the ortholog of Rictor. AVO1 might be related to *hsin1* (human stress activated protein kinase interacting protein 1), because of theirs functional similarities [Loewith *et al.,* 2002].

mTORC2 seems to respond to growth factors, possibly via TSC1/2 [Yang *et al.,* 2006]. Biochemical studies has shown that mTORC2 is able to phosphorylate Akt at ser473 in the hydrophobic motif and thereby activates Akt toward substrates such as FOXO and BAD [Hresko and Mueckler, 2005, Sarbassov *et al.,* 2005b, Jaquinto *et al.,* 2006].

mTORC2 is futhermore involved in actin cytoskeleton reorganization and via Rho-family small GTPases and PKCa (protein kinase C) (described in the morphology section), which thereby determines cell shape and maybe the mobility of the cell [Jaquinto *et al.,* 2004]. The actin cytoskeleton-regulation by Rictor-mTOR may still involve unknown mechanisms [Sarbassov *et al.,* 2004, Jacinto *et al.,* 2004]. It has been shown that mTORC2 directly phosphorylates Akt at hydrophobic motif site Ser-473 [Sarbassov *et al.,* 2005b].

2.4.3.1 Rictor

Rictor is less studied than Raptor, mainly because it can not bind FKBP12-rapamycin and the discovery happened later. It is unclear why FKBP12-rapamycin does not bind the Rictor-cointaning mTOR complex. It might be Rictor or another component of mTORC2, which blocks the binding site [Sarbassov *et al.,* 2005a].

The findings leading to the discovery that it is Rictor-mTOR, which is the hydrophobic motif kinase of a rapamycin-resistant mutant of S6K1 (and not Raptor –mTOR) led to the discovering of a substrate of Rictor-mTOR [Ali and Sabatini, 2005]. This led to the realization that it is Rictor-mTOR, who is the hydrophobic motif kinase for Akt and plays an important role in Akt activation [Sarbassov *et al.,* 2005b].

The Rictor-mTOR has previously been regarded as rapamycine-insensitive; however recent studies have shown that this is not entirely true. The mTORC2 complex does not

interact with FKBP12-rapamycin and is in this matter not affected by rapamycin. Sabatini *et al.* have showed that prolonged rapamycin treatment (more then 24h) inhibits mTORC2 assembly and thereby phosphorylation and activation of Akt. Although there results varied from cell type to cell type, they showed that Rictor decreased after 24 hours. They explain it with the newly synthesized free mTOR and the mTOR complexes turnover, which binds to rapamycin-FKBP12, preventing the subsequent binding of Rictor. They tested a variety of cell lines and found highly sensitive, partially sensitive and insentive cell lines. The sensitivity could be due to their in kinase activity toward Akt [Sarbassov *et al.,* 2006].

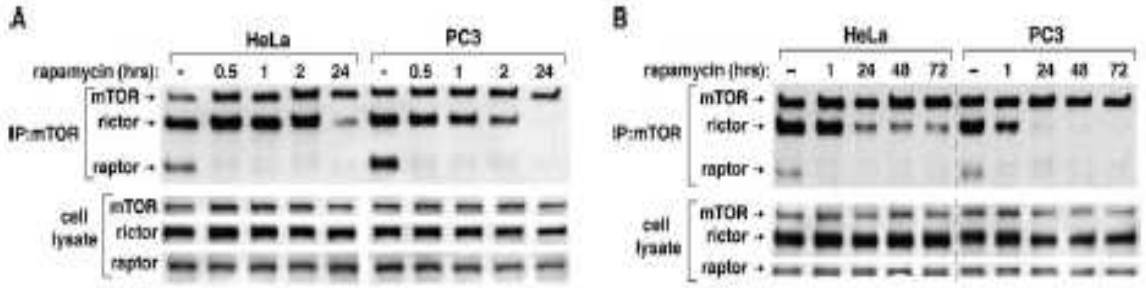


Figure 5 (A) shows the effect of prolonged treatment with 100 nM rapamycin in two different cell lines; HeLa and PC3. HeLa is a cervical cancer line and PC3 is a prostate cancer cell line. Samples were taken before treatment and at 0.5, 1, 2 and 24 hours after rapamycin-treatment. IP is short for immunoprecipitate. Raptor decreases immediately and can not be seen after half an hour. Rictor is absent after 24 hours. (B) Here samples are also taken after 48 and 72 hours, Rictor-inhibiton is most profound in PC3 cells [Sarbassov *et al.,* 2006]

2.4.3.2 SIN1/MIP1

SIN1/MIP1 is protein recently associated with mTORC2. It has been shown that SIN1 regulates the Akt pathway, by phosphorylating Akt-Ser473 and thereby activating the Akt [Jacinto *et al.,* 2006].Studies have shown that the SIN1-Rictor-mTOR complex is likely the principal if not the sole phosphoionsitide-dependent protein kinase 2 (PDK2) for Akt. In the absence of SIN1, phosphorylation of Ser473, but not Thr308 is abolished demonstrating that the PDK1-mediated Akt Thr308 phosphorylation is not dependent of a prior phosphorylation of Ser473 by PDK2 [Jacinto *et al.,* 2006]. However another study has shown that both Ser473 and Thr308 phosphorylation of Akt was diminished in Rictor and mTOR knockdown cells [Sarbassov *et al.,* 2005b].Futhermore, $SIN1^{-/-}$ cells have shown increased susceptibility to stress-induced apoptosis. SIN1 is not required for mTORC1, which have been indicated by the observation that TSC2 is phosphorylated normally in $SIN1^{-/-}$ cells and that SIN1 knockout and knockdown cells have an enhanced S6K1 phosphorylation at Thr389 [Jacinto *et al.,* 2006].

Akt-Ser473 phosphorylation is not required for TORC1 activation, which can be due to the phosphorylation of Thr308 Akt fulfil this function or because Akt play a minor role in TSC2 phosphorylation. TSC2 is also phosphorylated and inactivated by other kinases such as AMPK, ERK1/2 and RSK at other sites than the Akt target.

It is not clear if the SIN1-mediated TORC2 function involved the MEKK/MAPK cascades, but evidence suggests that the mTOR-Akt pathway is regulated by MAPKs. However Akt Thr308 and Ser473 phosphorylation is not affected in either MEKK2-deficient or –activated cells, and MEKK2 do not interact with mTOR nor Rictor. However enhanced JNK and p38 activation are found in SIN1$^{-/-}$ cells, suggesting a parallel function of SIN1 in the MAPK pathway [Jacinto *et al.,* 2006].

An interesting discovery is that mSIN1 exist in different isoforms and 3 of them assembles 3 different mTORC2s, indicating that the distinct mTORC2 isoforms phosphorylates Akt in response different substrates. The mSIN1 isoforms which assembled into mTORC2 were named mSIN1.1, mSIN1.2 and mSIN1.5. mSIN1.1 and mSIN1.2 showed to phosphorylate Akt in response to insulin [Frias *et al.,* 2006]. The upstream regulator of mSIN1.5 has, to our knowledge, not yet been found. The prolonged effect on Rictor by rapamycin [Sarbassov *et al.,* 2006], which is shown on Figure 5, also affects mSIN1.1 and mSIN1.2 after 24 hours when treated with 100 nm rapamycin. The two studies combined suggest that the novel discovery of mTORC2 inhibition by rapamycin is a result of the short lived mSIN1 proteins affected by rapamycin [Frias *et al.,* 2006].

2.4.3.3 PPR5L/ Protor

PRR5L (Proline-rich protein 5-like) is another protein recently associated with mTORC2. PRR5L interacts with rictor , independent of mTOR. PRR5L, unlike mSIN1, is not important for the mTOR-Rictor complex assembly or for the mTOR activity towards Akt phosphorylation. Despite no significant effect of PRR5L on mTORC2-mediated Akt phosphorylation, studies have shown that PRR5 silencing inhibits Akt and S6K1 phosphorylation and reduces cell proliferation. The inhibition of Akt and S6K1 phosphorylation by PRR5 knock down correlates with reduction in the expression level of platelet-derived growth factor receptor β (PDGFRβ). PRR5L silencing impairs PDGF-stimulated phosphorylation of S6K1 and Akt but moderately reduces epidermal growth factor- and insulin-stimulated phosphorylation [Woo *et al.,* 2007].

In another study, where HeLa and HEK293 cells were used, PRR5L binds to mTORC2 by SIN1 and/or Rictor. PRR5L was phosphorylated by both mTORC1 and mTORC2. The results suggested that PRR5L is not considered an mTORC2 upstream regulator or an integral component of mTORC2, but a downstream effector of mTORC2. PRR5L has also shown to promote apoptosis in a matter similar to TSC1/2 deficiency [Thedieck *et al.,* 2007].

2.3 PI3K/Akt pathway

Akt (also known as PKB (protein kinase B)) is a serine/threonine kinase, and is recognized as a primary mediator of the downstream effects of PI3K, making it a controller of responses to extrinsic stimuli. Akt is involved in a range of signalling pathways involved in cell proliferation, apoptosis, glucose metabolism or angionesis [Fulda and Debatin, 2006]. Akt is activated by growth factors, cytokines, hormones, neurotransmitters [Yang *et al.,* 2004] and DNA damage. Following the p53 pathway, the PI3K-PTEN-Akt signalling pathway is one of the most mutated pathways associated with cancer [Parcellier *et al.,* 2008]. Akt appears to be a substrate of mTORC2 [Hresko and Mueckler, 2005; Sarbassov *et al.,* 2005b]. The role of Akt in rearrangement of cytoskeleton is still studied and novel components emerge frequently.

Humans and mice have 3 isoforms of Akt, named Akt1, Akt2 and Akt3 (also named PKBa, PKBβ and PKBγ), whereas yeast, flies and worms only have one Akt protein. They are located on different genes but share 80 % amino acid identity and with only minor differences between man and mice [Yang *et al.,* 2004]. By studies with knock-out mice, the functions of the various Akt isoforms have been determined. Akt1 has been linked with placental development, growth and adipogenesis. Adipogenesis is the development of fat cells from pre-adipocytes. Akt2 is involved in glucose metabolism, adipogenesis and maintenance of growth. Akt3 is located in the brain and has been linked with postnatal brain growth.

This was illustrated by *Akt1*$^{-}$ mutants, who were smaller than wt mice, but without apparent defect in glucose homeostasis and often with apoptosis occurred spontaneously in thymus and testis. *Akt2* null mice had impaired glucose metabolism shown as severe diabetes but without severe growth defects. *Akt3* null mice have small brains, but neither growth defects nor impaired glucose metabolism. Double

knockout of Akt isoforms give different phenotypes; Akt1/3 double knockout mice are embryonic lethal, while Akt1/2 double knockout mice die soon after birth. [Yang *et al.,* 2004, Kato *et al.,* 2007].

Akt requires three ordered processes to be activated:

1. Translocation to the plasma membrane through PH domain binding to PIP_3.
2. Phosphorylation of Ser473, which increases the kinase activity 10-fold and is necessary for Thr308 phosphorylation.
3. PDK1 is recruited and activated by PIP_3, thereby phosphorylates Thr308, resulting in a 100-fold kinase activity, which is a 1000-fold increase in total [Sasaki and Firtel, 2006].

When Akt is stimulated, it activates PI3K (Phosphoinositide 3-kinase) through receptor tyrosine kinases, which recruits Akt to the membrane where Akt gets phosphorylated [Yang *et al.,* 2004]. Akt possesses a kinase domain and a PH domain (pleckstrin homology domain), which binds to phoshoinositides, such as PIP2 (phosphatidylinositol (4,5)-biphosphate) or PIP3 (phosphatidylinositol (3,4,5)-triphosphate), with high affinity. When a receptor tyrosine kinase binds with growth factors, the receptor is phosphorylated which recruits PI3K to the plasma membrane. PI3K are classified into three classes based on its structure; class I PI3K is primarily responsible for PIP_3 production due to extracellular stimulation. Class II is not well understood and phosphorylates PIP_2 although only poorly. It is class II PI3K, which have been connected with resveratrol [Srivastava *et al.,* 2005]. Class III PI3K regulates intracellular vesicle trafficking and have shown to be involved in mTOR activation in response to amino acids [Sasaki and Firtel 2006]. PI3K mediates PIP2 into PIP3, which leads to the recruitment downstream targets to the cell membrane and their subsequent activation [Parcellier *et al.,* 2008], by PIP_3 recruiting Akt to the membrane, where Akt becomes phosphorylated at thr308 and ser473, by the upstream kinases PDK1 (phosphoinositide-dependent kinase 1) [Yang *et al.,* 2004] and mTORC2. The identity of the kinase, responsible for Ser473 phosphorylation, have remained unidentified until lately [Hresko and Mueckler, 2005; Sarbassov *et al.,* 2005b]. Knockdown of Rictor completely blocks Ser473 phosphorylation of Akt, however knockdown of Raptor enhances Ser473 phosphorylation, which could be explained by depletion of mTORC1 increases mTORC2.

PTEN (Phosphatase and Tensin homolog) is another important protein involved in the Akt pathway. *PTEN* is a tumor suppressor, which blocks Akt and thereby uncontrolled growth, by dephosphorylating PIP_3.PTEN is normally localized at the plasma membrane but moves from the plasma membrane to the cytosol after chemoattractant stimulation. During chemotaxis, PTEN is excluded from the leading edge and becomes localized to the lateral sides and back of the cell

Although the molecular mechanisms by which Akt and Girdin/APE regulate chemotaxis remain to be uncovered, the findings are particularly interesting because Girdin/APE maybe is the missing link between Akt and F-actin polymerization, and it may participate in the suggested feedback activation loop between Ras/PI3K and F-actin

Akt has been shown too been to be required for chemotaxis in mammalian leukocytes and *dictyostelium* (slime mould) [Chung *et al*., 2001]. A growing body of evidence indicates that Akt promotes cell motility in mammalian fibroblasts and tumour cells [Higuchi *et al.,* 2001]. P13K and PTEN have been shown to be essential for cell migration [Merlot and Firtel, 2003]. Furthermore over expression of constitutively active Akt enhances cell migration. mTORC2 phosphorylates Akt, which leads to the phosphorylation of Girdin/APE, leading to the formation of F-actin [Sasaki and Firtel 2006].

Girdin/APE

Girdin/APE (girders of actin filaments/Akt phosphorylation Enhancer) binds to actin filament at the plasma membrane, whereas during cell migration, Akt-mediated phosphorylation allows Girdin/APE to localize at the leading edge and contribute the formation of short-branched filaments in the lamellipodia[Enomoto *et al.,* 2005]. Girdin/APE contains an Akt phosphorylation site at the C-terminus and this is responsible for the localizing to the plasma membrane. Knockdown of Girdin/APE completely abolish cell motility. Girdin/APE represents the molecule missing between Akt and F-actin polymerization and may participate in a feedback loop between PI3K and F-actin [Sasaki and Firtel 2006].

2.5 Cell morphology

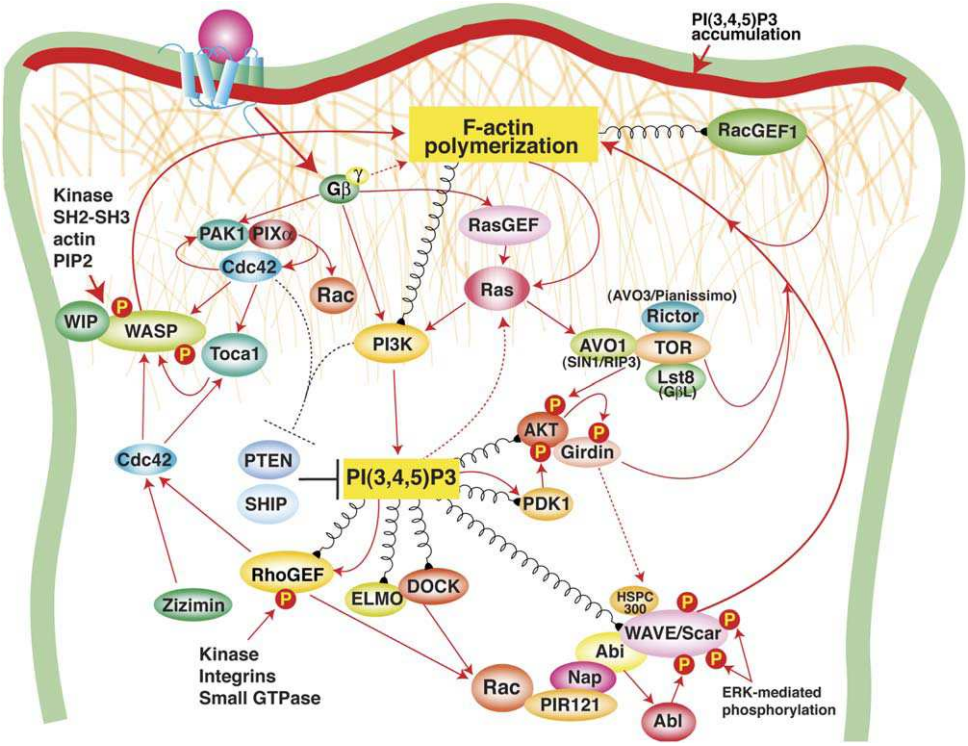


Figure 6 F-actin polymerization. This model shows how F-actin assembles at the leading edge in a chemotaxing cell. The feedback loop from PIP_3 involves TORC2, Akt-Girdin/APE, the WAVE/Scar complex and RhoGEF, Cdc42 and WASP. The external stimuli are mediated through the chemoattractant which activates the G- protein βγ subunit (Gβγ). Gβγ induces F-actin polymerization, Cdc42 activation and PI3K activation through Ras [Sasaki and Firtel, 2006]. The proteins are described in the text below.

2.5.1 Actin filaments

The rearrangement of the cytoskeleton is a fundamental function seen in all eukaryotic cells from amoeba to advanced neural cells. Chemotaxis, or directed cell movement up a chemical play an essential role in a range of functions including: development, homeostasis, wound healing, cancer cell metastasis and moving towards food or away from toxicants in protozoa. Where an amoeba relies on F-actin assembly an adhesion receptor mediated migration has been found in higher developed cells including fibroblasts and myoblasts. This migration depends on adhesion receptor and/or cell-substrate interaction and creates a remodelling of the extracellular matrix [Sasaki and Firtel, 2006].

Rearrangement of the cytoskeleton is central to the morphological changes. The amoeboid crawling system, which emerged about a billion years ago has been conserved through evolution from Dictyostelium (slime moulds) to leukocytes (white blood cells) and depends on cytoskeleton-mediated cell movement in which the assembly of a branched F-actin network provides movement.

Actin filament is one of the three major components of the cytoskeleton. G-actin is small globular proteins forming helix filaments (F-actin). Actin filament has two dynamic structures; network and bundle structure. Lamellipodia is arranged in a network structure, whereas filopodia and stress fiber have bundle structures. Filopodia moves in one dimension, and lamellipodia moves parallel with substratum in two dimension. The net filaments of Lamellipodia can assembly at one end and move forward. The filaments can be seen as protrusions out of the cell, providing movement or as skeletal structure inside the cell, giving strength and stability to the cell [Alberts *et al.,* The Cell].

When a cell is treated with a substrate, which binds to the receptors on the plasma membrane, the receptors convert it into an intracellular signal, making the cell undergo dynamic changes. This can cause asymmetric localization of the cellular components. Treatment with an F-actin polymerization inhibitor, such as latrunculin A, induces a rounded shape and cell immobilization [Sasaki and Firtel, 2006].

2.5.2 Rho family of small GTPases

The Rho family of small GTPases are key regulators of the actin cytoskeleton during chemotaxis. The most well known members are Cdc42 (Cell division cycle 42), Rac and Rho, which are present in all mammalian cells. Cdc42, Rac and Rho are responsible for the rearrangement of actin filaments. They act as molecular switches, which cycle between GTP-bound (guanosine-5'-triphosphate) and GDP-bound (guanosine diphosphate) states. When a Rho GTPase is in its GTP-bound state it is active, and inactive when GDP-bound [Alberts *et al.,* The Cell]. This is regulated by GAP (GTPase-activating proteins), GEF (guanine nucleotide exchange factors) and nucleotide dissociation inhibitors [Sasaki and Firtel, 2006].

Activated Rac and Cdc42 induce morphologically distinct actin protrusions at the membrane. Where the activation of Cdc42 induces filipodia-extensions, the activation of Rac makes the lamellipodia extend [Carryway *et al.,* 1998] Mammalians have tree Rac isoforms; Rac1, Rac2 and Rac3. Rac1 is expressed throughout the

body, Rac2 is only expressed in hematopoietic cells and Rac3 is expressed in the developing nervous system and adult brain.

Although the inhibition of Cdc42 does not block cell movement and pseudopodia formation, it suppresses persistent leading edge formation and induces non-directional F-actin projection. Cdc42 can induce Rac activation by recruiting and activating of PAK (p21-activated kinase complex) and PIX (PAK-interacting protein). PIX have GEF activity toward Rac and Cdc42, see Figure 6.

An important mediator for F-actin protrusions is the Arp2/3 complex (Actin-related proteins). Rac and Cdc42 induce protrusions through Arp2/3, which creates rapidly growing branched actin filaments, providing a mechanical force to move the membrane.
Rac-Wave/Scar and Cdc42-WASP (Wiskott-Aldrich syndrome protein) both activates the Arp2/3 complex. WASP also relies on PIP_2 to be activated but can also bind to PIP_3 and $–SH_3$ containing molecules. WASP binds G-actin and Arp2/3, which makes Arp2/3 to assemble the G-actin into F-actin. Recent studies have shown that most WASPs bind to member of the WIP proteins (WASP-interacting protein). Another protein, Toca-1 (trans-inducer of Cdc42-dependent actin assembly) has been identified and shown to bind specifically to GTP bound Cdc42 and has furthermore shown to be essential for the neural Cdc42/WASP mediated F-actin polymerization.
The *WAVE /Scar* genes are found in three places in mammalians and named *WAVE1, WAVE2* and *WAVE3*. The expression of WAVE1 and -3 are mainly expressed in the brain, whereas WAVE2 are found everywhere. WAVE/Scar knockdown cells have significantly reduced lamellipodial formation. Rac binds to PIR121, causing WAVE-mediated Arp2/3 activation. Cdc42 and Rac are only activated at the leading edge. The activation of Rac is achieved by a chemoattractant-mediated activation of RhoGEF. The human genome contains more than 60 RhoGEF and many of those are activated due to PIP_3 production [reviewed in Sasaki and Firtel, 2006].

2.5.2 Regulation of cytoskeleton

The role of mTORC1 in actin rearrangement is not yet clear [Jacinto *et al.,* 2004], but the upstream negative regulator, the TSC1/2 complex can induce actin rearrangement by Rho GTPases and induces Rac activation. TSC1 activates RhoA and induces stress fibers [Sasaki and Firtel, 2006]. Knockdown of the Raptor (in

fibroblasts) does not cause a detectable defect of the actin cytoskeleton [Jacinto *et al.,* 2004].

One of the major roles of mTORC2 is rearrangement of the actin cytoskeleton. Formation of stress fiber and lamellipodia caused by activation of Rho and Rac has been observed in NIH 3T3 cells (a fibroblast cell line), which suggests that mTORC2 may signal to the actin cytoskeleton through Rho and Rac. Rho and Rac are downstream substrates of mTORC2 and when mTORC2 is blocked, they are activated. (Figure 4) [Jacinto *et al.*, 2004].

Another study of HeLa cells suggests that mTORC2 signals actin cytoskeleton through PKCa (Protein Kinase C a), which is also downstream of mTORC2. This is consistent with the studies in *S. cerevisiae* which demonstrate that TORC2 controls cell polarity through a PKC-Rho GTPase pathway [Sasaki and Firtel, 2006]. The activation of PKCa is reduced in Rictor and mTORC2 knock-down cells [Sarbassov *et al.,* 2004].

Only few studies have been published regarding resveratrol and cell morphological changes in the cytoskeleton. Scientists have observed that resveratrol significantly affects the shape of endothelial cells *in vitro*, changing the cells from a cobblestone-like appearance (Figure 7 A) to an elongated shape with long protrusions (B) [Bruder *et al.,* 2001].

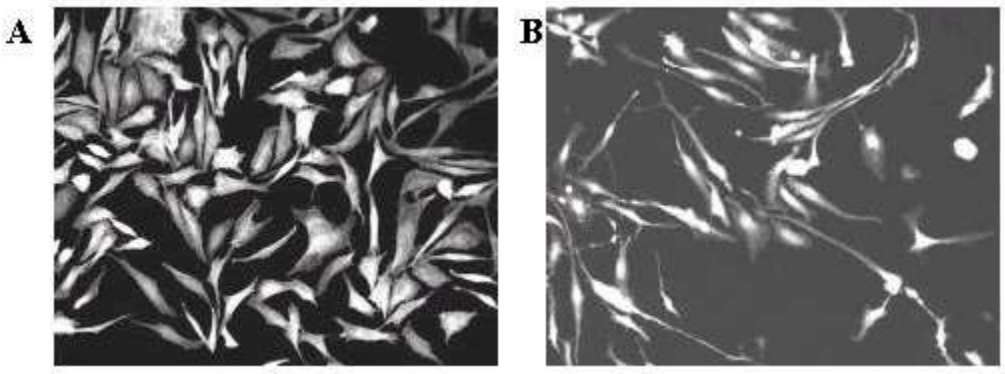


Figure 7 Resveratrol treatment produces elongation of BPAEC (Bovine pulmonary artery endothelial cells). (A) Example of stellar, cobblestone-like morphology characteristic of normal BPAECs grown *in vitro*. (B) Example of elongated, spindle-shaped morphology characteristic of resveratrol treated cells. The cells were treated with 100 µM resveratrol and were viewed with 20X objective [Bruder *et al.,* 2001].

2.6 AFM

Atomic force Microscope (AFM) was invented by Binning and colleagues in 1986 [Binning G *et al.,* 1986]. The AFM was invented to scan solid materials and have later showed to be useful for biological samples. The soft structure of for example a cell or bacteria can thereby be problematic, when the tip (probe) of the cantilever interacts with the sample, altering the structure. The tip of the AFM interacts with the sample either in a very close distance or in contact with the surface of the sample. The interaction is named probe-sample interaction. The local probe is a sharp tip, with a radius around 10 nm, and with a stretched pyramid-like structure. During optimal conditions the interaction is only between the outermost atom of the tip and a single atom on the sample [Morris *et al.,* 1999; Güntherodt and Wiesendanger, 1994]. Therefore very small structures and objects can be seen in AFM.

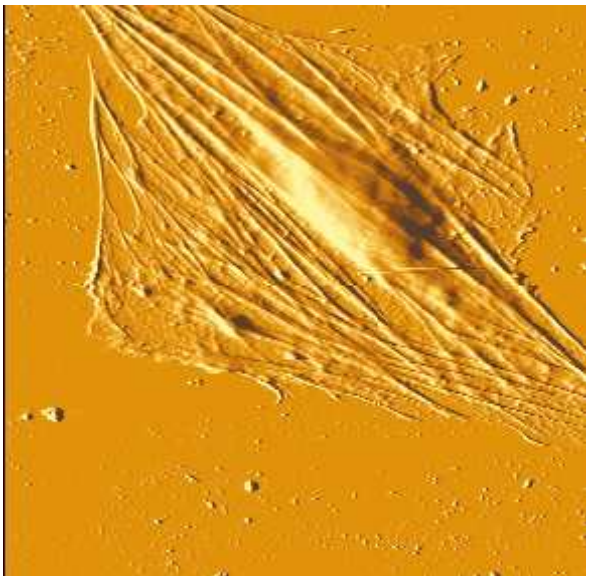


Figure 8 Example of a scanned cell with AFM in DC-mode (contact) in liquid showing cytoskeleton fibers of a live osteoblast [Lehenkari *et al.,* 2000].

Optical detection method in AFM

There are several modes for detecting tip sample information in AFM. The most common method is the optical detection method. In the optical detection method, a laser beam hits the back of the cantilever and is reflected to a photodetector, which is an array of photodiodes. The photodetector records the surface information. The tip-sample interaction causes a deflection of the cantilever, which is monitored by the photodetector, recording information of the x,y and z-scale of the sample, thereby giving a true three-dimensional image [Morris *et al.*, 1999].

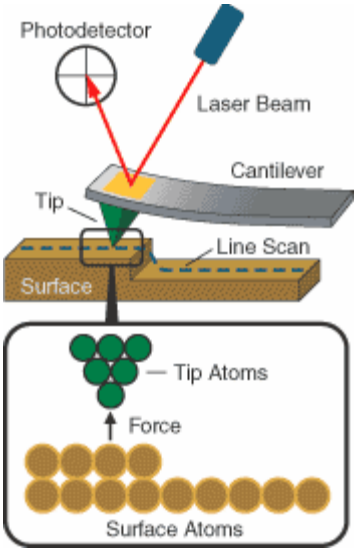


Figure 9 Principle of optical detection method in atomic force microscopy (AFM) and the interaction between the tip and the sample. The laser beam is reflected to the photodetector, recording changes in the surface which is mediated through the tip and cantilever. The enlarged picture of the atoms of the tip and surface, illustrates the repulsive forces between the two objects [sdu.dk/visualisering].

DC and AC mode are two different modes used with AFM. We have only used AC mode but both modes are described to illustrate the differences and to give some background information.

DC Mode

In DC-Mode, the tip is in contact with the sample during scanning. The cantilever tries to maintain a constant deflection during the scan. The constant deflection is equal to a constant interaction force. However, as the height of the surface changes, the forces of the two surfaces changes too, making the interaction change leading to the change of the deflection. The photodetector records these changes [Morris *et al*., 1999].

When scanning in air a thin water layer might be present on the sample surface. The capillary forces will make the water adhere to the tip adding to the shear force already present by scanning the tip in contact with the surface [Braga and Ricci, 2004]. Biological samples are often sensitive to shear force and the alternate, AC-mode where shear force is negligible, is therefore often used as an alternative.

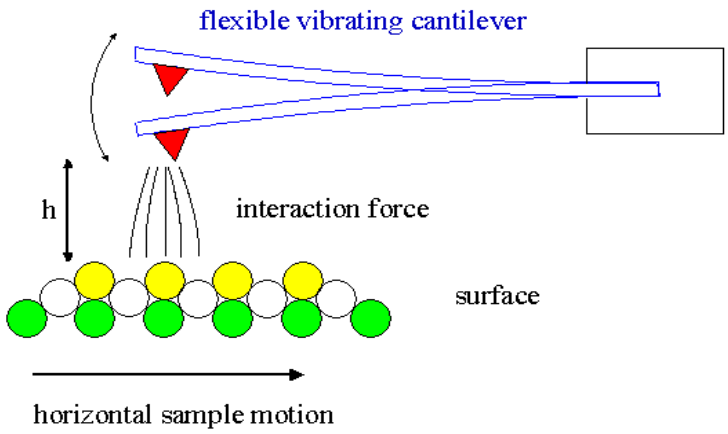


Figure 10 Interaction between the tip and sample in AC-Mode. The picture illustrates compulsive forces between the point of the tip and outer nuclear layer of a sample [Chem.ox.uk].

AC-Mode

The AC-mode has no direct contact with the sample or only intermediate contact with the sample, therefore, AC mode effect biological sample less, when compared with DC-mode. In AC-mode a shaker mechanism oscillates the cantilever at its resonance frequency. When the tip, at the end of the cantilever, comes in intermediate contact with the sample the amplitude of the generated oscillation is dampened. The degree of dampening is equivalent to the force interaction between tip and sample. Scanning the sample surface the amplitude of the oscillating cantilever changes in accordance with sample height. The scanner compensates the change in amplitude, moving towards or away from the sample, maintaining constant amplitude [Morris *et al*., 1999; Braga and Ricci 2004].

The technique of scanning in liquid has been developed both in DC-mode and AC-mode. However the procedure of scanning biological samples in liquid is still under development [Morris *et al.,* 1999]. Thus, it is so far, more reliable to scan biological samples in air combined with some fixation.

There are many different fixation techniques, we chose the fixation with glutaryldehyde and osmium tetroxide, because it seems to affect the samples less and have been used before at our institute (as well as others) making comparison to prior experiments easier.

3. Materials and Methods

3.1 Culture Medium, chemicals etc.

McCoy's 5A media w. GlutaMAX™ (10 % FBS), D-PBS, Gemtamicin and Trypsin (from Gibco). Resveratrol, Rapamycin and poly-L-lysine were from Sigma-Aldrich. NaOH (highest grade available).

The chemicals used to the fixation; Glutaraldehyde 25%, Osmium tetroxide(s) and sodium cacodylate were obtained from Electron Microscopy Science, all EM grade. Nunc™ culture flasks. Cover slips (Knittel Gläser, Germany). 10 cm Nunc™ Petri dishes. 10 cm Petri dishes (glass). Parafilm (Pechiney Plastic Packaging Company).

Resveratrol Stock solution: 600 mM resveratrol, dissolved in DMSO, stored at -18°C, and diluted to 60 µM for the experiments. Rapamycin was diluted to 0.5 µM. Poly-L-lysine 500 µg/ml dissolved in MilliQ-water, stored at -18 °C.

3.2 Instruments and Software

DME Dual scope™ DC 95-50, Dual Scope™ C-21. Dual Scope/Rasterscope™ version 1.4.1.12. DME-SPM software version 5.03.2600.2180 was used for analyze the cells and to measure the height. Scan conditions: AC mode was used throughout the project, force 0.1–0.15 nN, scan speed 30-80 µm/s, resolution from 128×128 to 512×51.2. Range from 50×50 to 200×200 µm.

ImageJ version 1,38x was used to measure cell size. Cantilevers (Nano World Pointprobe) glued on Dual Scope™ Plug and Play mounting rings. The cantilevers where pyramidal in shape with the measurements of; 120-130 µm in length, 25-35 µm in width and 3.5-4.5 µm thick..

Phase contrast Microscope Leica DM IRA witLeica DC 300F cam. **Coulter counter**: Coulter® Partikle count & size analyser Z_2 (Beckmann Coulter Inc.) Software: Multisizer program AccuComp. **Osciliscope** used for measuring the resonance when working in AC-mode**.**

3.3 Cell line and cultivation

The Cells used in this work were derived from Human colon epithelia colorectal adenocarcinomas, DLD-1 (American Type Culture Collection , and were cultured in Nunc™ culture flask (75 cm^2) in 5a McCoy media with 10% FBS serum and Geneticin and held at 37°C, 95 % humidity and 5 % CO_2. The cells were subcultured twice a week, and cells used for the experiments were 50% to 80% confluent. Cells used for experiments were counted in Coulter Counter and kept on ice.

3.4 AFM Sample Preparation

Before attachment of the cells, cover slips were marked with a glass cutter so specific single cells later on can be identified. The cover slips were then incubated with 2M NaOH for 2 hours so the poly-L-lysine can stick to the cover slips better. The cover slips were coated with 500µg/ml poly-L-lysine for 10 to 15 min so that the cell can attach more easily to the glass surface. After incubation with poly-L-lysine, the cover slips were washed twice with MilliQ, sterilized in 70% ethanol and washed in PBS once. They were then transferred to sterile petri dishes for further usage. The poly-L- lysine coated cover slips was placed in 10 cm sterile petri dishes and a cell suspension containing around 6400 cells/cm^2 in 12 ml medium The cell suspension was obtained by trypsination of the growing cell culture and kept on ice during counting to avoid cell aggregates, 10 ml of the cell culture are transferred to a 50 ml Nunc™ tube with 20 ml media in on ice. The cells, which normally attach to the surface of Nunc™ tubes, were not able to attach when they are stored on ice. Samples from the Nunc™ tube are were transferred to the coulter counter to determine the cell concentration. Each petri dish ends up with a density of 6400 cells/cm^2, which in our case is 500 000 cells in 12 ml. The cells attached to the cover slips overnight.

3.5 Resveratrol and rapamycin treatment

The cells attached o the cover slips with cells were washed twice with PBS and transferred to new sterile petri dishes. By transferring the cover slips to new petri dishes, only the cells attached to the cover slips would be further treated. Two or three cover slips were prepared for each treatment at both 48 and 72 hours. New media containing resveratrol, rapamycin, a combination or a control, were added into petri dishes.

Three experiments have been performed. In experiment 1, cells were treated with 0.1% DMSO (control) or 60 µM resveratrol for 48 and 72 hours treatment. In the

following two experiments, the cells were treated with: 0.1% DMSO (control), 60 µM resveratrol, 0.5 µM rapamycin or 60 µM resveratrol and 0,5 µM rapamycin for 48 and 72 hours treatment.

After 48 or 72 hours of incubation, phase contrast pictures were prepared using Leica microscope with a 200x magnification. Then, the cells were fixed and dehydrated for AFM scanning (method described below). If not scanned immediately, cells were stored in petri dishes, wrapped in Parafilm at 4°C. The best pictures were obtained by taking the pictures before the old medium was removed. Ethanol (after fixation) changed the focal point and to long exposure in PBS stressed the cells.

3.6 Fixation with glutaraldehyde and osmium tetroxide

When cells were treated with glutaraldehyde, the cells are quickly killed as glutaraldehyde cross-links the cellular proteins. Glutaraldehyde has two aldehyde groups, separated by a flexible chain of 3 methylene bridges and its chemical formula is $HCO\text{-}(CH_2)_3\text{-}CHO$. It can form polymers (Figure 11). There is a free aldehyde group sticking out of the side of each unit of the polymer molecule, as well as one at each end. All these -CHO groups will combine with nitrogen in for example proteins (Figure 12). In this way, the proteins in the membrane and the inside of the cell are fixed [Kiernan, 2000].

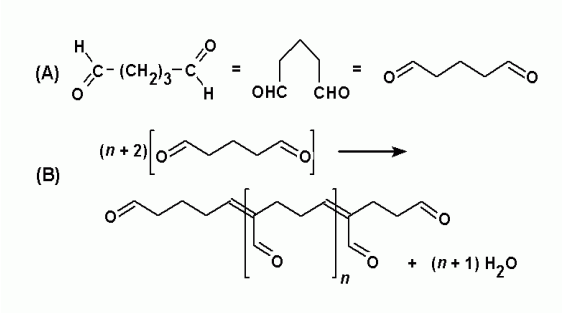


Figure 11 (A) Three representations of a molecular structure of glutaraldehyde: Two aldehyde groups –CHO connected by 3 ethylene group. (B) Glutaraldehyde form polymers, an aldehyde side-chain is on each unit of the polymer [Kiernan, 2000].

Secondly, osmium tetroxide was used to react with the lipids, as well as stabilize cell membrane and membranes of organelle, and to insolubilise proteins without coagulation. Osmium tetroxide reacts with the membrane-bound proteins, and is kept in the cell during this fixation [Glauert, 1975].

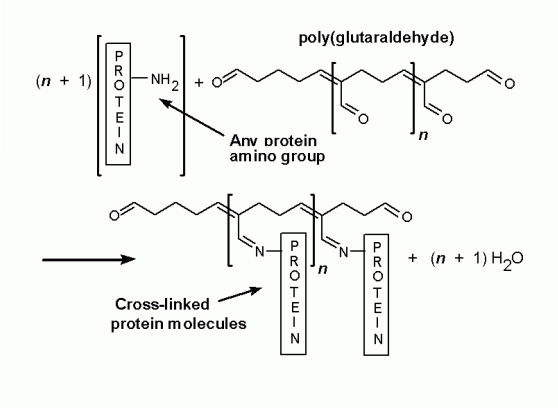


Figure 12 The mechanism of poly-glutaraldehyde reacting with protein. Poly-glutaraldehyde cross linked with nitrogen in amino groups of proteins [Kiernan, 2000].

The fixation is followed by dehydration in ethanol and acetone. Ethanol and acetone were added with increase concentration slowly without collapsing the whole cell. In this way, it was able to measure the height of the cell in order to investigate the effect of resveratrol and rapamycin on cells [Glausert, 1975].

The Preparation and the procedure of the fixation and dehydration

Sodium cacodylate buffer: 0.2 Sodium cacodylate dissolved in MilliQ-water to 0.1 M.

Glutaraldehyde solution: 1.58ml 25 % glutaraldehyde + 8.42ml MilliQ-water + 0,2M 10ml sodium cacodylate buffer. The sodium cacodylate solution is 0.1 M and the glutaraldehyde is 2% during the fixation.

Osmium tetroxide solution: 0.25g osmium tetroxide was dissolved in 25ml 0.1M sodium cacodylate buffer at least 30 min before usage and was vigorously shaken with the whillmix to dissolve it.

After washing with PBS, the cover slips were placed in plastic petri dishes in primary fixative (2% glutaraldehyde in 0.1M sodium cacodylate buffer), for 120min at 4°C after which they were washed twice in 0.1M sodium cacodylate buffer for 30min at 4°C.

The secondary fixation was done using 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 90min at 4°C (glass dishes) as the osmium tetroxide can react with plastic, and this step was followed by three time wash in MilliQ for 5min at room temperature (RT).

Dehydration of the samples were done by exchange the water with ethanol and then acetone (the cover slips should be covered by the solutions): 25% ethanol for 15 min, (RT); 50% ethanol for 15 min, (RT); 75% ethanol for 15 min, (RT); 96% ethanol for 15 min, (RT); 96% ethanol for 30 min (RT); 1/3 acetone in 2/3ethanol for 15 min (RT); 1/2 acetone in 1/2 ethanol for 15 min (RT); 2/3 acetone in 1/3 ethanol for 15min (RT); 100% acetone for 15 min (RT). Lastly, acetone was removed, the samples were left in fume hood to dry, and were transferred to new petri dishes. If not scanned immediately the samples were kept dry and refrigerated. (The fixation technique was kindly provided by Nadja Møberg from Copenhagen University).

3.7 AFM Scanning

The samples where transferred to the scanner stage, with the cell side up and fixed to the stage with a single drop of ethanol under the cover slip to avoid any movement or sliding of the cover slip. The crosses in the cover slips are found compared with the pictures from the phase microscopy. The function AC (tapping) mode was used through the entire project with retrace signal turned on. Oscillation frequency and amplitude were observed during scan. The cells were scanned at a minimum of two or three times, and the scans are saved. Scan speed or force was reduced when a shrieking sound occurred. Abrupt shifts in height seemed to induce shrieking. Shriek is a phenomenon, which occur when the resonance is not calibrated, which can be measured on the oscilloscope and the parameters changed in the DME software.

Plane correction was used to optimize the later analysis. An effect called "eye balling" occurs when the fixed arm of the cantilever scans the cells. The artificial elevated corners are flattened, which is necessary for area and height measurements.

DME-SME

DME-SPM was used to analyze the scanned cells and measure the heights. The program was used to obtain clear images of the cell outline. By adjusting the light bar, one can get a more detailed picture of either the top or bottom of the cell. When the light bar was lowered, the nucleus is pictured as a bright but blurred structure. However, the edge of the lamellipodia and the filopodia, become clear and more detailed. If the color bar is at maximum, the details of the actin filaments become blurred and hard to distinguish from the surface of the poly-L lysine treated cover slip. Instead the nucleus gets richer in details. The colors (Californian sunset) seen in the AFM scans, are used instead of a gray scale because the height difference becomes clearer.

ImageJ

ImageJ was used to calculate the area of the cells, by framing the cell along the lamellipodia and the filopodia. This is done by ImageJ by counting pixels. The program need the information of length and height of the picture, which are written

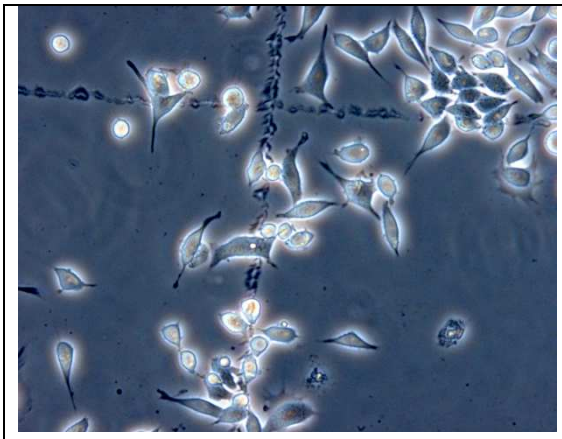
in the lower right and in the upper left respectively, of the pictures below. Figure 1 show examples of framed cells and the calculated cell size. The examples show the drawn lines and the calculated size. All examples are from the experiment after 48h of exposure. All the cells scanned by AFM from experiment 2 and experiment 3 were measured. 3 to 6 measurements were made for each treatment. High resolution pictures were used because the low-resolution AFM images made the analysis more difficult and less precise.

4 Results

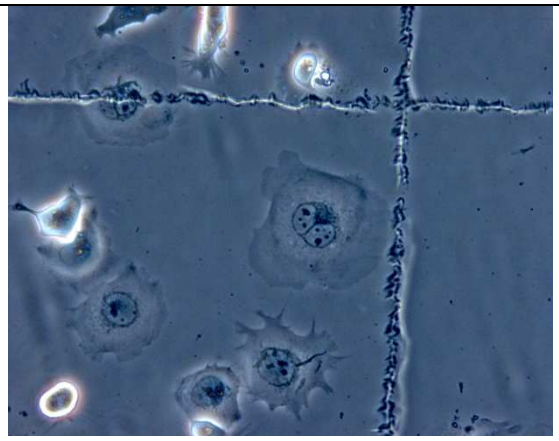
4.1 phase contrast images

The pictures obtained by the phase contrast microscope before the fixation and dehydration procedure are interesting because they show the differences of the two microscopes and before/after fixation and dehydration. The phase contrast pictures also give an impression of the general cell structure in the various treatments.

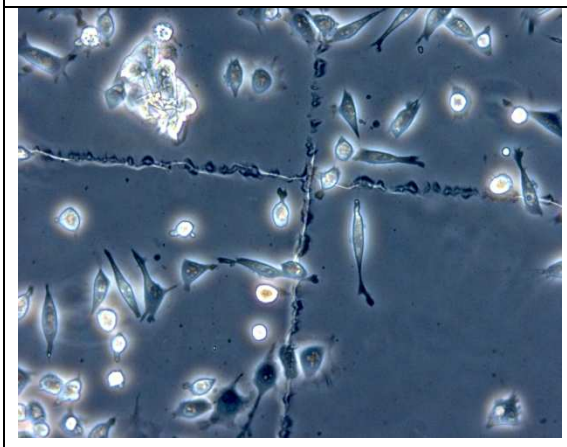
The control cells have less pronounced protrusions compared to the resveratrol-treated cells. The typical resveratrol cell has the appearance of a fried egg; a rounded shape and tend to extend in size compared with the control. However we have also observed astrocyte-like cells; less cytosol and long protrusion. The peaks of the nucleoli appear as black dots in the phase contrast (can be seen in b) resveratrol and d) resveratrol plus rapamycin, in Figure 13). These upright protrusions with microvillus-like appearance have been described in previous studies [Kristoffersen, 2006]. The shape of the rapamycin-treated cells looks similar in shape to the control cells. The cells treated with the combination, both have the rounded shape cells, seen in the resveratrol-treated cells and other cell shapes as well.



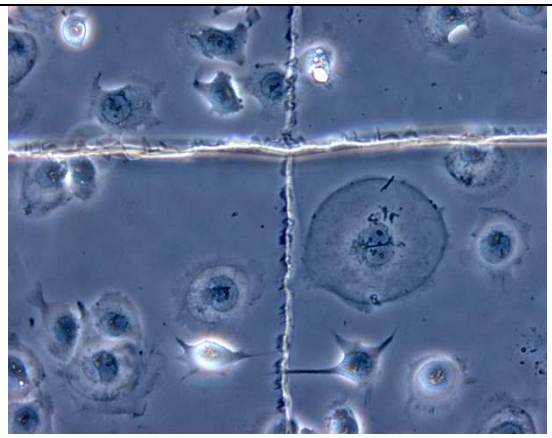
a) Control



b) Resveratrol



c) Rapamycin



d) Resveratrol + rapamycin

Figure 13 Pictures taken with the phase contrast microscope with 20 times magnification, in experiment 3 after 48 hours of incubation. The crosses are the man-made identification marks used for to find the same single cells in the AFM.

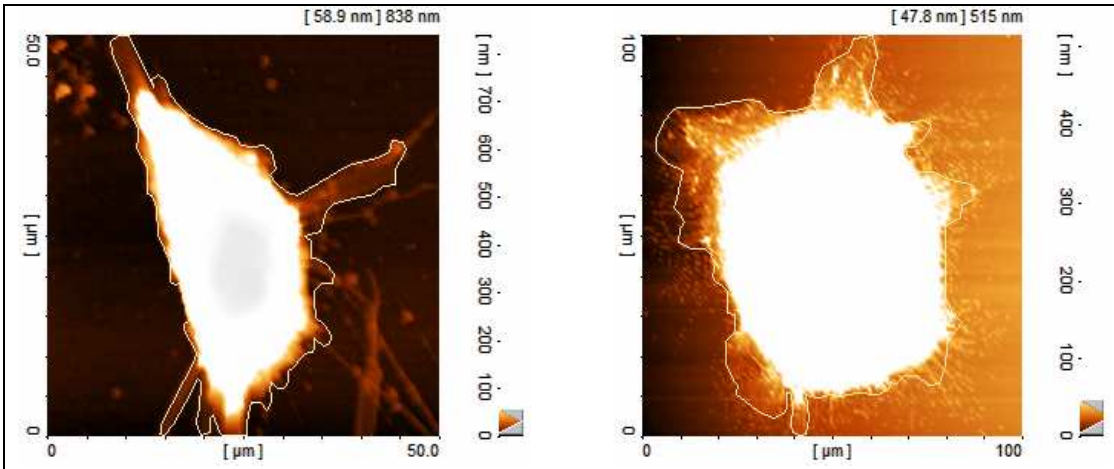
Picture after the fixation were also taken by the phase contrast (not shown), to see the effect of the fixation to the cells. They look more crystal-like but not with any visible different morphology regarding the protrusions compared to the AFM scans.

4.2 Area

The scanned DLD-1 cells from the AFM were analyzed with the help of DME-SPM and ImageJ to obtain cell size and height. Morphology changes were also observed using visual inspection.

Examples of analyzed cells are given in Figure 14 and Figure 17, and data are obtained for analyzing 3-6 measurement for each treatment, time and experiment.

As showed in Figure 16, resveratrol treated cell has the biggest area, whereas the treated combination cell also trends to increase in size. However, the rapamycin-treated cells do not appear to increase in size.



A) Control

B) Resveratrol

C) Rapamycin

D) Resveratrol + rapamycin

Figure 14 Examples of determination of cell area of DLD-1 cells by ImageJ. Cells was exposed, for 48 h, to DMSO (Control) (A), 60 µM Resveratrol (B), 100 nM Rapamycin and 60 µM Resveratrol (C), or 100 nM Rapamycin (D). Area of the cells: 705 (A), 4781 (B), 3970 (C) and 778 (D) µm².

The nucleus of the resveratrol-treated cells tends to increase in size too, as illustrated in the pictures below. This has previously been observed (Stervbo *et al.* 2006B].

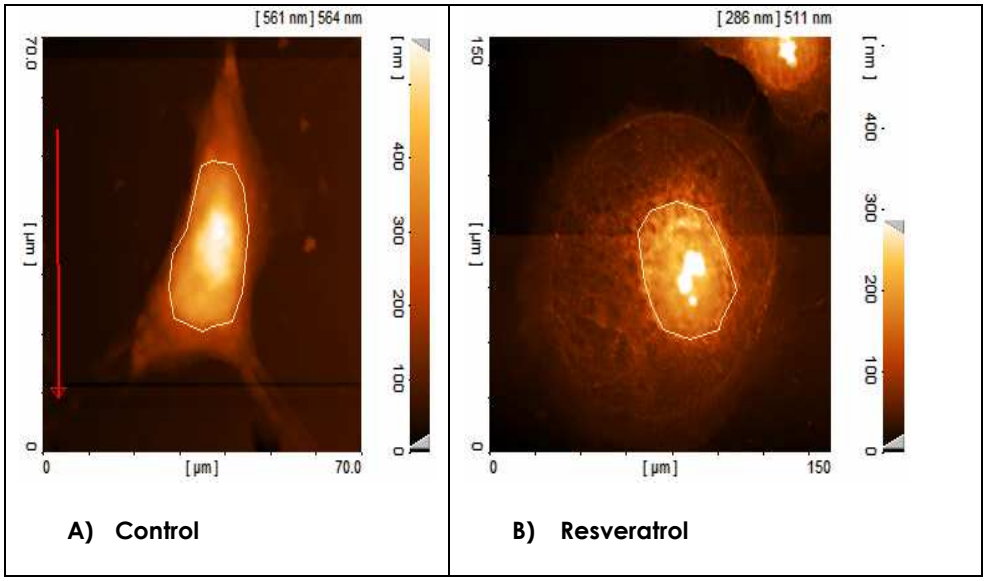


Figure 15 Nucleus size of DLD-1 cells is increased by resveratrol. Cells was exposed, for 48 hours, to DMSO (Control) (A) or 60 µM Resveratrol (B) with a nucleus size of 343 µm² and 1537 µm² respectively.

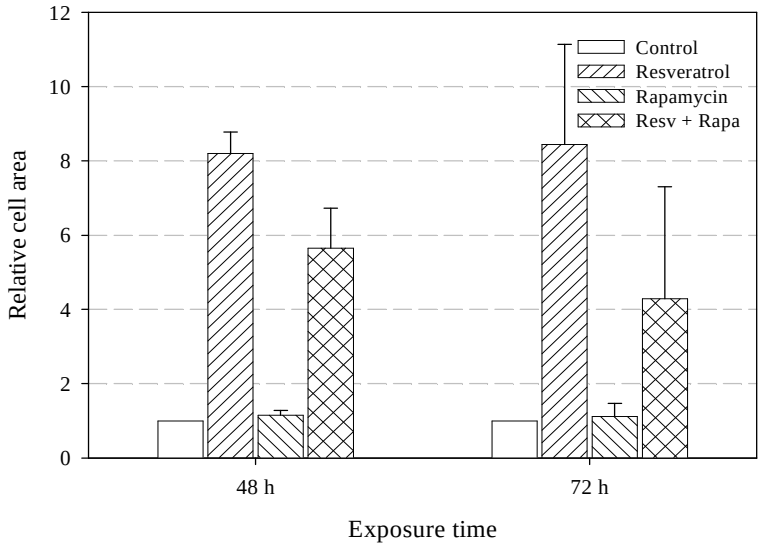
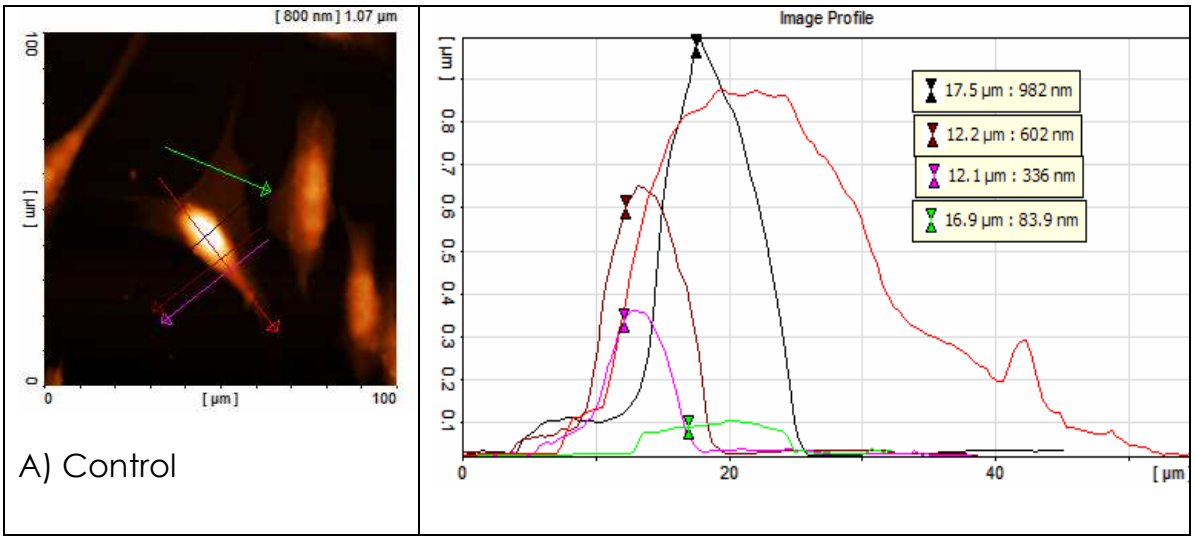
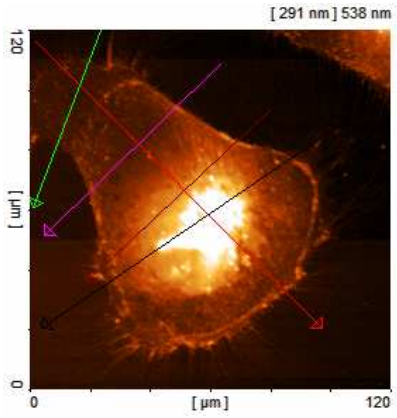


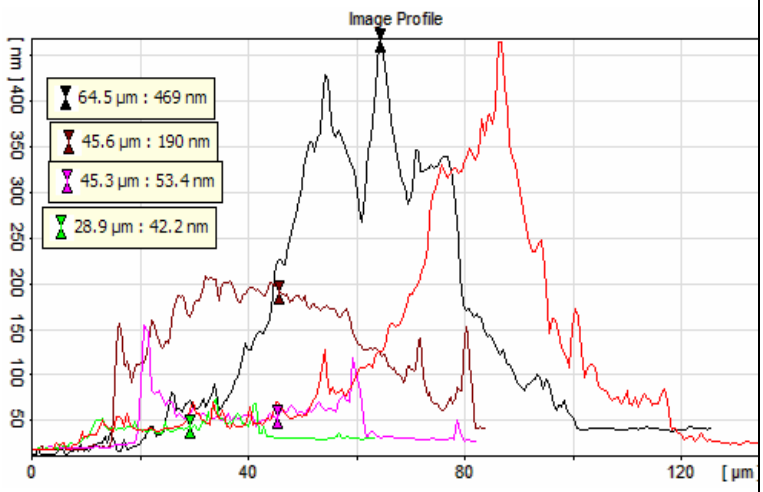
Figure 16 Effect of resveratrol and rapamycin on the cell area of DLD-1 cells. The numbers are an average of two experiments ±STD following exposure to 60 µM resveratrol and 0.5 µM rapamycin. Treatment of cells, fixation and AFM scanning was done as described in the material and method section. The mean area of control cells at 48 h and 72 h were 805±151 µm² and 907±179 µm², respectively.

4.3 Height

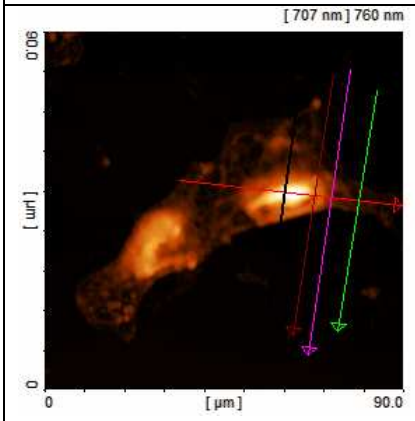


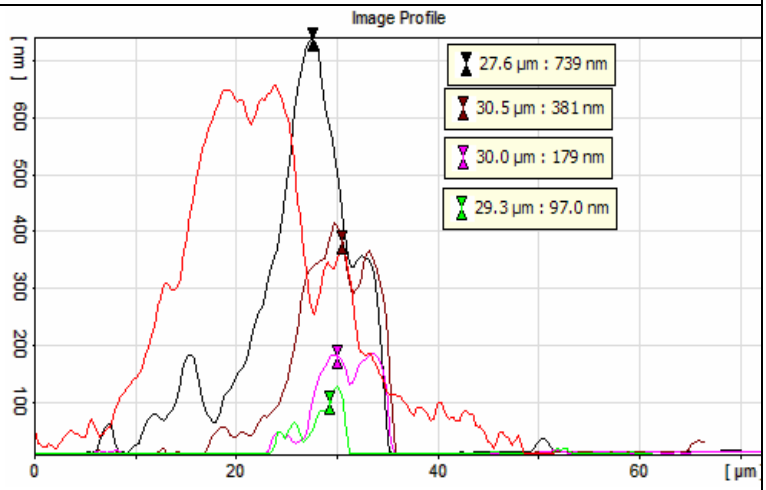
A) Control



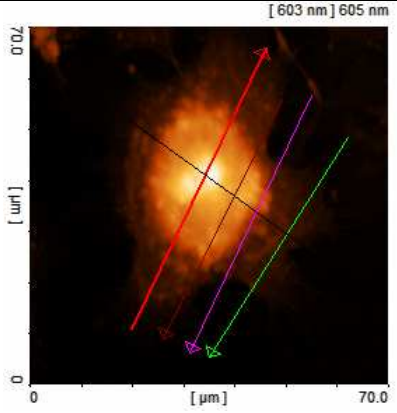


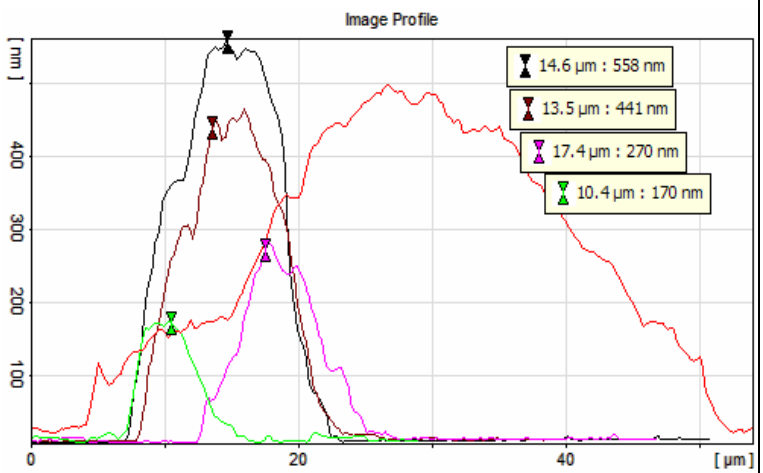
B) Resveratrol





C) Rapamycin





D) Resveratrol + rapamycin

Figure 17 Examples of height determinations in 4 single cells exposed to DMSO (control), resveratrol, rapamycin and resveratrol plus rapamycin. All the examples are after 72 hours of treatment in experiment 3. The height of nucleoli, nucleus, and cytosol and lamellipodia were draw by black, brown, fuchshsia (purple) and green respectively. The red line is the overview of the cell height. All the cells scanned by AFM from experiment 2 and 3 were drawn using this technique (3-6 measurement for each treatment, time and experiment). The edges between the different cell parts can be difficult to distinguish, but adjusting the light bar and flattening the edges helps this.

The untreated cells have the highest structures with a more soft hill-like structure, whereas the resveratrol-treated cells seem to collapse in especially the nucleus and have a more rigid cortex compared to the untreated. The Rapamycin-treated cells have also lower heights than untreated cells but not the rigid cortex appearance of the cells incubated with resveratrol. The cells treated with the combination are higher than the resveratrol-treated cells.

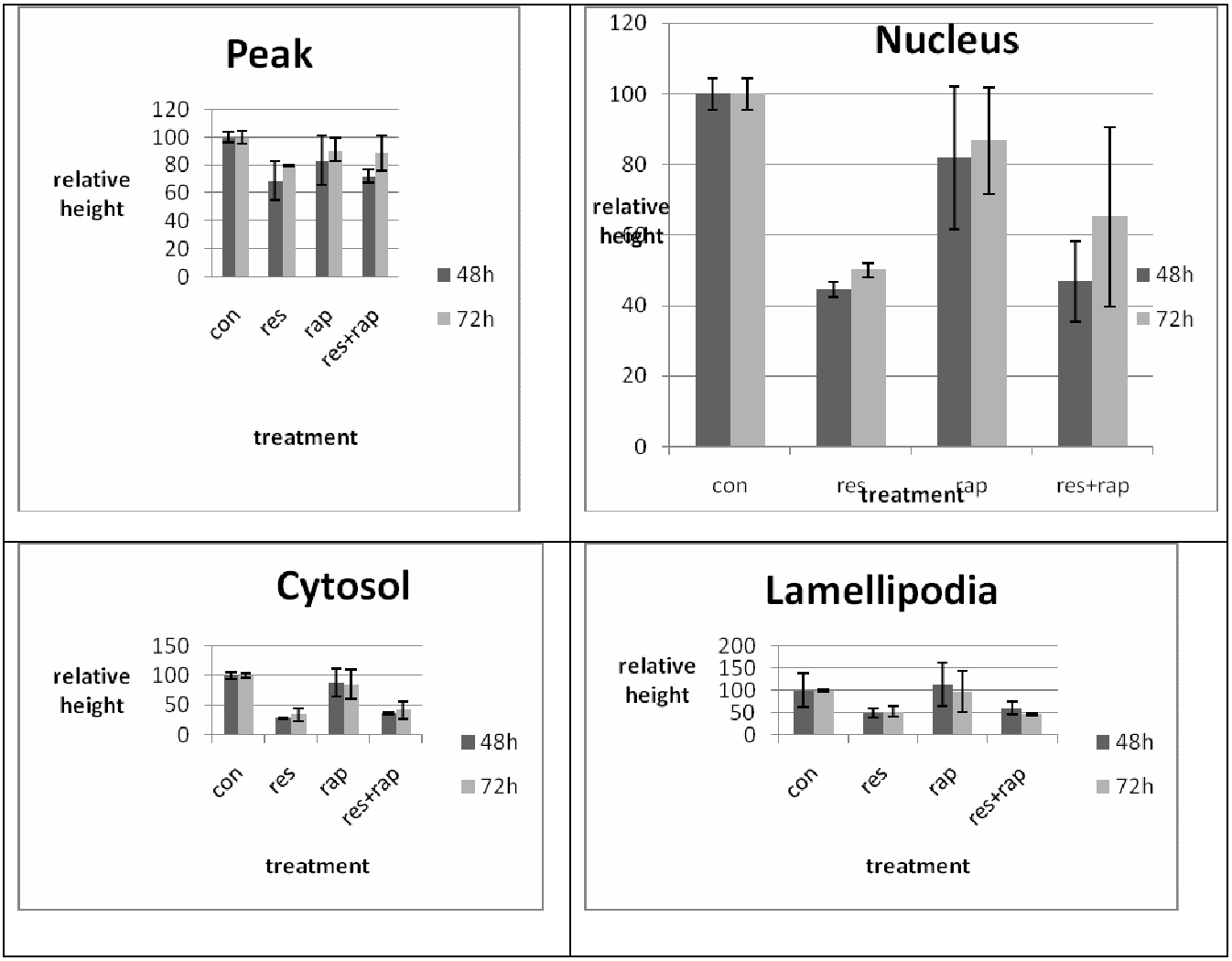


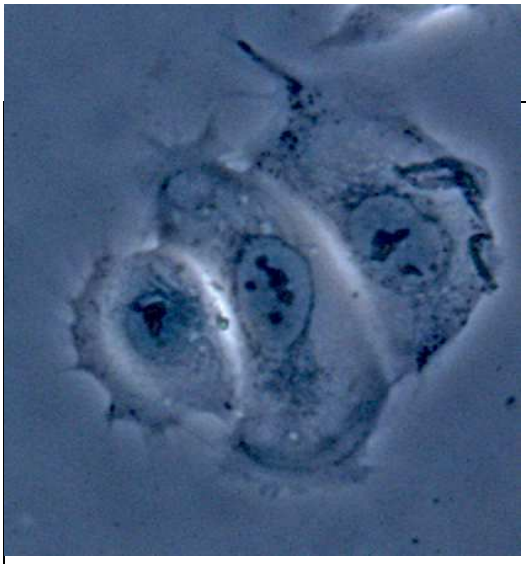
Figure 18 height of nucleoli, nucleus, and cytosol and lamellipodia in nm. The four columns in each figure represents the measured height after 48 and 72 hours treatment of DMSO (control), 60 µM resveratrol, 0.5 µM rapamycin or 60 µM resveratrol plus 0,5 µM rapamycin. The diagrams show the mean values of experiment 2 and 3 combined, with the dark gray scale showing 48 hours after treatment and the light gray as 72 hours. The ranges of the observations are showed with error bars. No statistic analyses were made due to the small number of observations. The untreated cells are had mean values of 630, 414, 241 and 100 for the peak, nucleus, cytosol and lamellipodia, respectively.

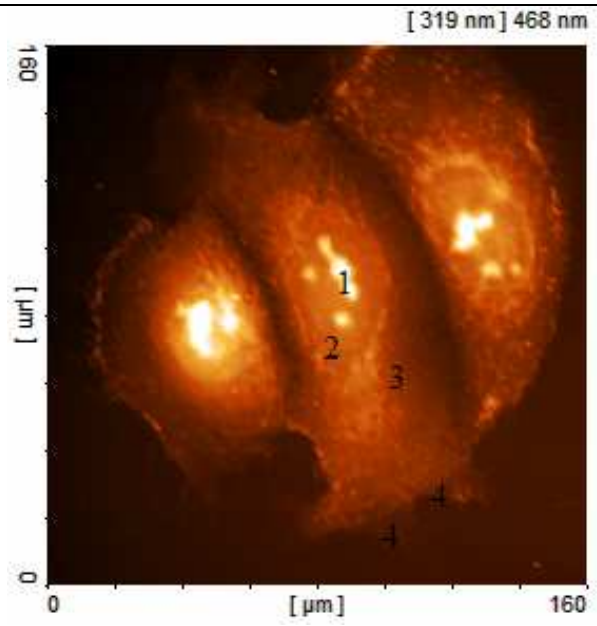
4.3 AFM images

In Figure 19 the two pictures are of the same resveratrol-treated cell (48 hours, experiment 3), comparing the phase contrast image and the AFM image.

The three dimensional image shows the surface information as a two dimensional image. The bright sports in the two dimensional images (**Figure 19**B) appear as peaks in the three dimension images

Figure 20). The control cell has less signs of nuclear collapse or no collapse at all. Resveratrol treated cell tend to collapse a lot and with the typical appearance of a fried egg. The information matches the data analysis.





A) phase contrast picture	B) AFM

Figure 19 A) is obtained with a phase contrast microscope with a 20 times magnification before fixation. The peaks appear black in the phase contrast microscope and are thereby not a result of the fixation itself. B) The same cell was later identified and scanned in the AFM (atomic force microscope.) with numbers on; the peak of the nucleoli (1), nucleus (2), cytosol (3) and lamellipodia (4). Here the peaks of the nucleoli are illustrated as bright spots due to the light bar (not shown), which have been set so all 4 elements appear.

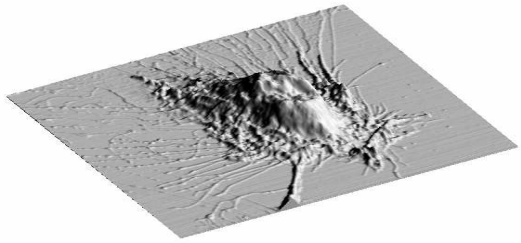
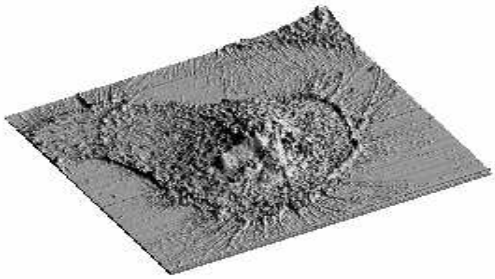
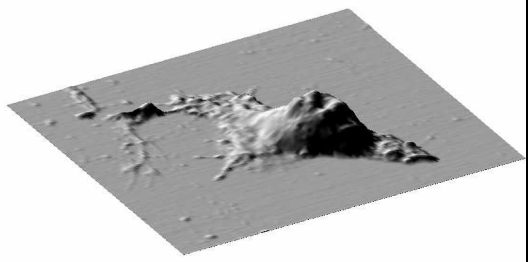
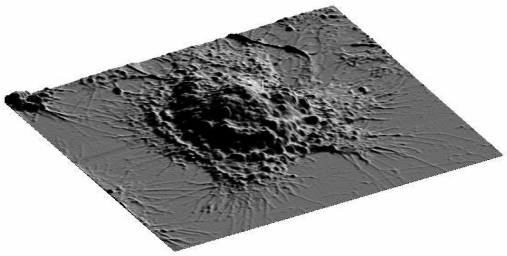
	
A) control	B) resveratrol
	
C)rapamycin	D)Resveratrol +rapamycin

Figure 20 three dimensional, grey scale pictures of cells obtained in AFM (atomic force microscopy). The height has been elevated for an illustrative effect, otherwise the cells would appear so flat that the details would be hard to distinguish, due to the height-area ratio (The cells are very flat and long).

4. Discussion

As the samples obtained at 48 and 72 hours do not differ significantly, the discussion will combine these time points.

The samples scanned in the AFM should not be used for quantity analysis, which can show statistic results but instead for high detailed pictures, with informative details. However some quantitative effects were used during the project to elevate the cell size difference in the different samples. This was also done by the help of phase contrast microscope, which is better suited for imaging many cells. By the help of the phase contrast we could observe the most abundant cell shape and then scan a cell with this morphology in the AFM. In for example the resveratrol treated samples, the cells have many different shapes but the most frequently cell shape was the fried egg-like cell, with a large area and very flat compared the untreated cells. Resveratrol also induced a different morphology, which looked astrocyte-like, with visible and long protrusions and a smaller area compared to the fried egg-like cells.

The resveratrol treated cells have a bigger area, compared with the other groups. This observation is not new or differs from previously observations. We observed that the area of the resveratrol treated cells is about 8 times bigger than untreated cells. The rapamycin treated cells, have sizes corresponding to those of the controls, is interesting because as the rapamycin block both mTORC1 and mTORC2, we would aspect cell proliferation, cell size and actin organisation to be affected too. Therefore we state that resveratrol affects more then one substrate in the pathway to actin organisation and that the different isoforms of for example Akt are affected differently by rapamycin (mTORC1/2 inhibition) and resveratrol. Another explanation could be due to an elevated Akt activation in our cell culture, or that rapamycin enhance Akt activation when mTORC1 is blocked (and maybe also when mTORC2 is later blocked too). However other instruments/cells maybe give other results regarding rapamycin and cell size. Resveratrol seems to be involved in mTORC2 and the PI3K-Akt pathway, due to the effect on PKCa [Garcia-Garcia *et al.,* 1999; Slater *et al.,* 2003] and PI3K class II [Srivastava *et al.,* 2005].

We only analyzed on cells after 48 and 72 hours, and according to Sarbassov *et al.* rapamycin inhibits mTORC2 after 24 hours [Sarbassov *et al.,* 2006].

The cells treated with rapamycin plus resveratrol have an area size larger than the rapamycin-treated, but smaller than the resveratrol treated. It seems that both drugs decreases the effects of the other, but not inhibits or adds an additive effect of the drugs combined.

Maybe the area increase is a result of the cell structure collapsing; the large-area resveratrol-treated cells, showed to be very flat compared to the control. The rapamycin-treated cells seem to be flat too. The structures of the nucleus and cytosol are affected most regarding height decrease- This could be a result of the cell collapse due to the fixation and hydration procedure or due decreased protein concentration. The AFM stresses the cell when the tip taps the sample but we would anticipate the same sheer stress to all the cells and that the stress is minimal due to mode we scan in. However the rigid structures of the membrane could maybe increase this friction. We have based our assumptions on the thesis that the fixation does not affect the cells differently. The target of rapamycin is intracellular, but resveratrol targets membrane bound substrates such as PI3K and PKCa (and probably others). We don not how the effects of the fixation technique influence this. This also applies for the active membrane bound Akt.

The cells with visible upright protrusions (also described as peaks of nucleoli) seem only to appear in the cells treated with resveratrol (also in a combination with rapamycin), but as resveratrol tend to flatten the cell, it is not yet possible to say if the peaks is visible because the rest of the cell is collapsed or if resveratrol makes the cell produce them. The peak of the different treated cells have almost the same heights and the could therefore also be present in the untreated and rapamycin-treated cells, although not visible in the phase contrast images. We can observe that resveratrol changes the morphology of the horizontal protrusions, which are made of actin. It could be tempting to view the upright protrusions as actin-containing microvillus-like structures due to similarities with epithelial colon cells *in vivo* and the effect resveratrol on colon cancer. However the protrusions have previously been described as peak of nucleoli, our colon cancer cells have been cultured *in vitro* and we will therefore not state otherwise.

6. Conclusion and Perspective

In the beginning of this project, we suspected resveratrol to be involved in mTOR and by using a well known mTOR inhibitor, we hoped to prove this. We did not know, at that time, that mTORC2 was sensitive to rapamycin, but based on the different cell sizes and height, we can conclude that resveratrol is indeed involved in the mTOR pathway. Resveratrol still changes the cell morphology (although to a lesser degree), when mTORC2 is inhibited by rapamycin, and some of the effects of resveratrol are thereby not mTORC2-dependent. This statement is based on that we did actually block mTORC2 completely.

Because we treated our cells with rapamycin for more than 24 hours, Rictor was inhibited as well. The mTORC2 complex act as PDK2 and phosphorylates Akt, which is involved in cell morphology. We thereby diminished the effect of resveratrol by treating with the combination. The isoforms of mTORC2 and mSIN1 might not all be affected and this could be the reason for an effect of the combination, somehow between the two sole treatments. This could also be true for Akt isoforms and proteins such as Cdc42 and PKCa, which are involved in F-actin assembly.

We suggest that if a similar experiment was done if could benefit if samples of, say 12 and 24 hours also were included. Hereby the effects of rapamycin, before it inhibited mTORC2 assembly, could be monitored. If the samples also were marked with fluorescence, the actin-filaments could be identified. We also suggest an immunoblotting of the proteins involved in the two mTOR complexes, and the isoforms of mTORC2, Akt, mSIN1 plus the classes and isoforms of PI3K. It could also be interesting to change the amount of nutrients to the cells, to see if the morphology of cells exposed to caloric restriction would mimic the effects of resveratrol when scanned in the AFM. When using cancer cells, the energy pathways could also be investigated as rapid growing cancer cells tend to rely on glycolysis.

We chose to focus on the cytoskeleton, but other parts of the cell could also be interesting to investigate. Other cell lines could be interesting to study too, due to the knowledge of cancer cells with blocked, mutated and up- or down-regulated expressions of different proteins and pathway. The same could be done to more primitive organism such as yeast, roundworms or slime moulds with well-described and less complex pathways.

The AFM provides new possibilities for the areas of biological, medical and chemical research. Its major advantage is the detailed three dimensional pictures. It is time consuming and a certain technical knowledge is necessary to work with the AFM. We had to use a lot of Ph.D. Stud P.K. Kristoffersens time as we were not sufficient skilled in this device ourselves. A bigger study group or more time is therefore required if the AFM combined with other techniques are used. Fewer scanned cells could maybe help to overcome this problem. Scanning living cells in liquid would remove the uncertainties regarding the fixation and dehydration before scanning cells in air. We look forward to see the progress of this evolve at our institute.

Because of the now proteins involved in the pathways described in this project, a more detailed image should be presented. We hope to be able to present this for the evaluation. As mTOR and Akt is still evolving, there would maybe be a new conclusion when new knowledge as added.

7. Sources

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