HSP90 inhibition as a way to treat cancer

Master thesis in Medicine

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1. Abstract

Myxoid liposarcoma (MLS) is a rare soft-tissue tumor. An important oncogenic factor driving the tumor is abnormal proliferative signals through tyrosine kinases. Tyrosine kinase inhibitors have been tested as a treatment, but with little effect. An explanation to this might be that different tyrosine kinases can form heterodimers and autophosphorylate each other in the cytoplasm of MLS cells, sustaining a proliferative signal. A protein that can promote this autoactivation process is Heat shock protein 90 (HSP90). HSP90 is a chaperone, a protein involved in modifying misfolded proteins and helping protein complex formation. In tumors, HSP90 has been linked with phosphorylation of tyrosine kinases and promotion of cell proliferation. HSP90 inhibition has been developed as a method to treat cancer. Experiments on mice with MLS and on MLS cell lines have shown that HSP90 inhibition has great potential. The aim of this study was to elucidate the mechanism of HSP90 inhibitions in MLS and the connection to tyrosine kinase signaling, using an HSP90 inhibitor called 17-DMAG.

Cultured tumor cells have been analyzed regarding to cell proliferation, expression of target proteins and signaling pathways. The results showed that 17-DMAG diluted in cell medium with 5 % FBS reduced cell proliferation in MLS cell lines. Fibroblasts, used as control cells, were not affected by the drug. Untreated fibroblasts had in general a significantly lower expression of 17-DMAG target proteins than untreated tumor cells. In MLS cell lines, protein quantification showed that activation of tyrosine kinase receptors RET and ErbB3 were reduced after addition of the drug. This indicates that 17-DMAG specifically affects cells dependent on tyrosine kinase signaling, decreasing proliferation and causing cell death. Tyrosine kinase signaling is an important oncogenic factor in myxoid liposarcoma, suggesting that HSP90 inhibition is a possible method for treatment of tumor cells without affecting normal tissue.

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2. Background

2.1 Cancer

Cancer is one of the biggest health problems in the world, having increased both in incidence and in mortality since the middle of the 20th century (1, 2). Estimated life-time probability of cancer differs between countries, but is in Sweden approximately 33% and in the US approximately 45% (1, 2). Cancer is the second most common cause of death after cardiovascular diseases, accounting for every fourth death in both Sweden and the US.

Cancer is a collective term for malignant tumors. Malignant tumors can either originate from epithelial cells or mesenchymal tissue. Malignant tumors originating from epithelial cells are referred to as carcinomas, whereas malignant tumors originating from mesenchymal tissue are referred to as sarcomas. Cancer is a group of diseases caused by an abnormal proliferation of cells. The most common cancers are prostatic cancer, breast cancer, skin cancer, lung cancer and colon cancer (fig.1). All these malignant tumors are carcinomas. Sarcomas, on the other hand, are very rare tumors, accounting for less than 1 % of all tumors.

2.2 Sarcomas

Approximately 350 new cases of Sarcomas are diagnosed in Sweden every year, according to Cancerfonden and Socialstyrelsen (2). One quarter of these tumors originate from the bones, whereas three quarters of the tumors originate from soft-tissue, such as fibrous tissue, muscles, nerves, blood vessels and lymphatic vessels (2). Liposarcomas account for 20 % of all sarcomas (3), making them the most common form of all mesenchymal tumors. The five year disease-specific survival rate of liposarcoma is approximately 77 % (4) , but depends on the histological subgroup (3).

The three main groups of liposarcomas are well-differentiated liposarcoma, pleomorphic liposarcomas and Round-cell/Myxoid liposarcoma (Fig.1). These can be further divided into

histological subtypes. The different groups of liposarcomas have some histological and genetic differences, as well as diversity in incidence, localization and survival rate (3). Myxoid liposarcoma (MLS) is the tumor that we will focus on in this paper.

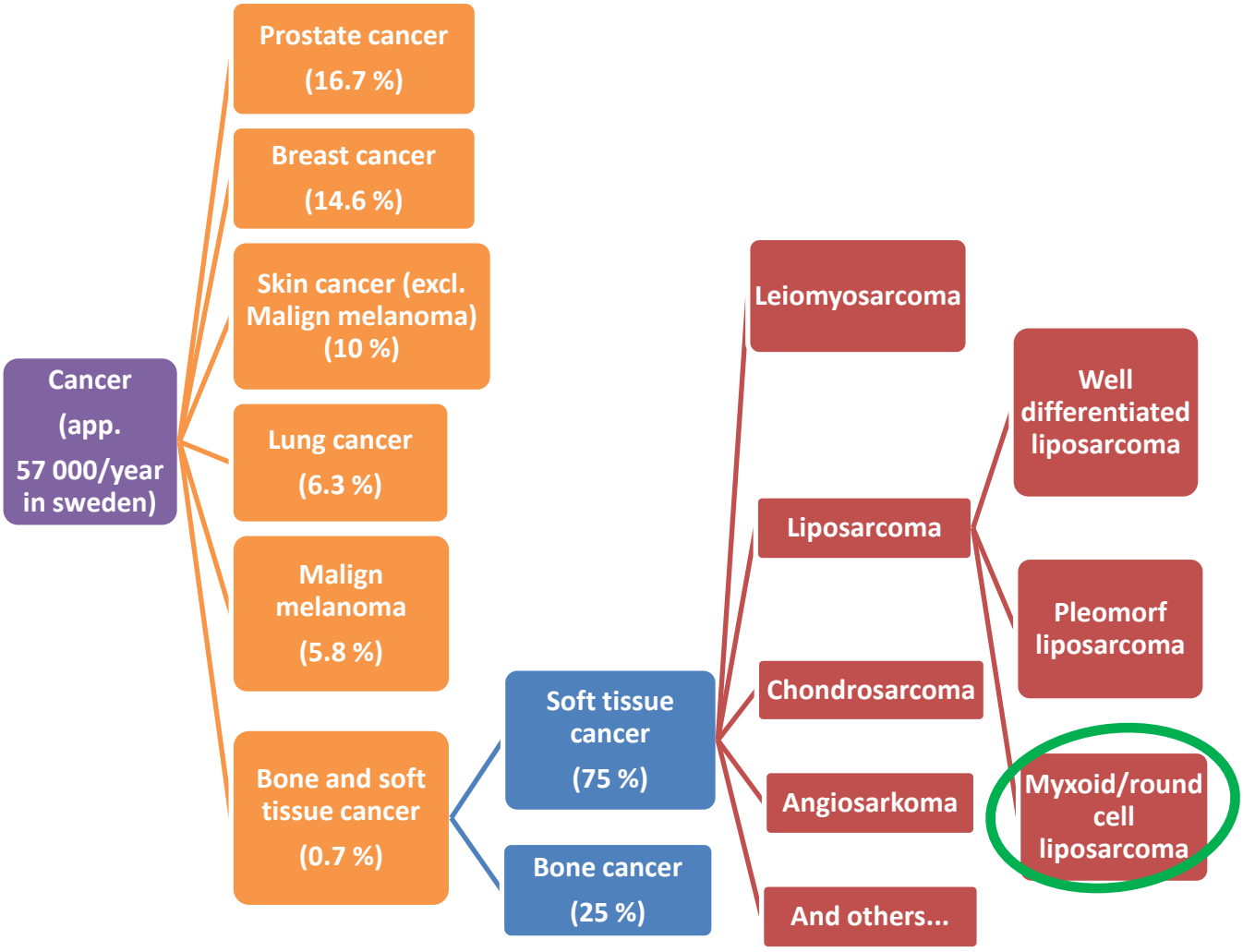


Fig.1 Diagram showing the most common forms of malignant tumors. Myxoid liposarcoma (MLS) is a very rare tumor, belonging to the sarcoma group of tumors (2)

2.3 Myxoid liposarcoma

Myxoid liposarcoma (MLS) is the second most common liposarcoma, representing approximately 30-35 % of all the cases. It occurs most frequently in patients between 35-55

years old. The tumors grow deep down in muscles, under the muscle fascia, and the majority of tumors occur in the thighs (3). Histologically, MLS contain few, spindle-shaped cells, on a myxoid background. Adipocyte precursor cells, lipoblast are often present; they are monovacuolated and located in perivascular areas. The tumors are often hypocellular with hypercellular areas, where the cells are more round-shaped (Fig.2). Some tumors are dominated by these hypercellular areas and they are referred to as round-cell liposarcoma (RCLS). In the old tumor-classification from WHO, RCLS and MLS are seen as two different subgroups of liposarcoma. Nowadays, it is believed that RCLS is a more aggressive form of MLS, since a transition from hypocellular to hypercellular tumors is common in MLS. Cytogenetic profiling also confirms this model, since MLS and RCLS share the same cytogenetic profile (3).

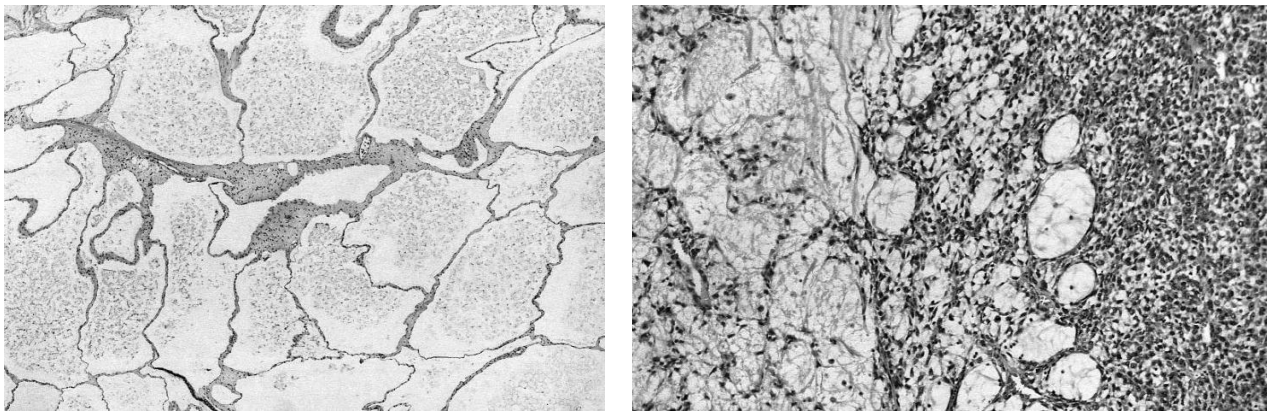


Fig.2 Histological sections of MLS. Left picture show the spindle-shaped cells on a myxoid background. Right picture shows a transition from a hypocellular to a hypercellular tumor (3).

The five year survival rate (5-YSR) of MLS is 70 %. If only considering the localized pure myxoid MLS, 5-YSR is 93 %, whereas the more aggressive subgroup RCLS has a 20 % 5-YSR (3, 5). Metastatic progression of pure myxoid MLS is rare but occurs in 10-20 % of the cases (6). Metastases are more common in RCLS. Some common locations for metastases are the retroperitoneum, peritoneum, peritoneal cavity, axilla, skeleton and the lungs (5-7).

2.3.1 Treatment for myxoid liposarcoma

The most common treatment for liposarcomas is surgery, often in combination with radiation to reduce the risk of local recurrence. Chemotherapy is used as a treatment for tumors larger than 10 cm and when metastasis has occurred (5). The most commonly used drug in MLS is doxorubicin, belonging to the antracycline group (8-10). It acts by inhibiting topoisomerase 2, thus blocking replication. Doxorubicin is being used as a mono-therapy drug. Combination with contumumab, a monoclonal antibody against death receptor 5 has been tried without any increased efficiency (11).

The response rate of doxorubicin is around 20-30 % and shows an objective diminution in tumor size (12, 13). Treatment with doxorubicin is associated with a progression free survival of 3-4 months. After this period, the tumors get resistant to the drug and the patient face a quick progression of the disease. No effect on overall survival rate has been seen, and toxicity is high with side effects such as neutropenia, systolic dysfunction, mucositis and alopecia (8).

Another drug that is currently used as second line therapy is trabectedin. Trabectedin is a DNA minor groove alkylator that causes cell cycle arrest in S and G2 phase. It shows an overall survival rate of 21 months, in comparison to 12-14 months in untreated controls (14). The most serious side effects of this drug are leukocytopenia and thrombocytopenia. The most common side effects are nausea, fatigue, diarrhea, constipation and anemia (14).

A third drug that has been used against MLS, mostly in combination with doxorubicin, is ifosfamide. Ifosfamide is an alkylating drug with a mono-therapy response rate around 10-20 % (12, 15). Combining ifosfamide and doxorubicin significantly increases progression free survival rate (12). There is no other cytostatic with good effect against metastatic MLS at the market today.

2.3.2 Cytogenetic

Human sarcomas are generally known for their genomic instability, meaning that the tumors have a complex and unbalanced karyotype. The heterogeneity between tumors of the same type is vast and usually there is not a single mutation that drives the oncogenesis (16).

MLS on the other hand are genetically stable and characterized by a single chromosomal translocation between chromosome 12 and 16, t(12;16)(q13;p11) (17). This translocation is prevalent in more than 90 % of all MLS and creates a fusion between the protein FUS (also called TLS and GADD153) and DDIT3 (also known as CHOP), resulting in a fusion protein called FUSDDIT3 (TLSCHOP). FUS is an RNA-binding protein, which is involved in transcriptional activation. It belongs to the FET-group of proteins, including FUS, EWSR1 and TAF15, which are all important genes in tumor specific translocations. DDIT3 is a stress-induced protein that is involved in cell-stress and apoptosis. The fusion protein FUSDDIT3 is an abnormal transcription factor believed to be important for the tumor development, affecting well-known downstream targets such as NFKB, IL6, C/EBP ß, IL-8, p53 and MDM2 (3, 18, 19).

2.4 Tyrosine kinase receptors in MLS

It has been shown that increased tyrosine kinase receptor activity is an important oncogenic factor in MLS (20). Tyrosine kinase receptors are receptors that exist in several cell types in our body. Their normal function is to bind their ligand and dimerize with another tyrosine kinase receptor. Upon dimerization, a kinase-activity is started, which causes phosphorylation and formation of large protein complexes around the receptors. This starts a signal cascade that ultimately generates a response in the cells. Typically, they are involved in cell proliferation, differentiation, migration and cell cycle control (21).

Overactivity of tyrosine kinase receptors is common in tumors, and can occur because of activating point mutations, autocrine stimulation, receptor overexpression or chromosomal

rearrangement (21). These mutations cause the tyrosine kinase receptor to remain in a phosphorylated state and sustain abnormal cell proliferation stimulating signals.

In MLS, it has been reported that multiple tyrosine kinase receptors have increased activity, among them RET, EGFR, ErbB3 and Flt1. It has also been shown that different tyrosine kinase receptors can cross-talk and activate each other by heterodimerization (20). Unpublished material from Åman group, confirmed that MLS cell lines have a higher expression of RET and EGFR than a fibroblast control cell line. Experiments comparing MLS cell lines and the fibroblast cell line, regarding expression of ErbB3 and FLT1 have not been done.

RET encodes a tyrosine kinase receptor that stimulates cell proliferation. Overexpression of RET is linked with different forms of human tumors, most commonly the multiple endocrine neoplasia type 2 syndrome (MEN2-syndrome) causing medullary thyroid carcinoma, pheochromocytoma and primary hyperparathyroidism. The four natural ligands for RET are called PSPN, GDNF, NRTN and ARTN, and among them, PSPN is expressed by MLS cells (22).

EGFR is also a cell surface tyrosine kinase receptor that stimulates cell proliferation. It belongs to the ErbB family of receptor tyrosine kinases. Overexpression of this protein is linked with cancer (lung cancer, anal cancer, glioblastoma multiforme). EGFR is activated by a number of ligands, among them EGF and TGF-α. Upon activation, EGFR creates homodimers with other EGFR, or heterodimers with other tyrosine kinase receptors from the ErbB family (23) .

ErbB3 is a cellular receptor also belonging to the ErbB family of receptor tyrosine kinases. This receptor does not have any kinase domain on its own, and upon activation, is required to form heterodimers with other members in the ErbB family to convey its signal (24). The

ligands of ErbB3 are called neuregulin 1 and 2. ErbB3 is often seen in tumors that express high levels of ErbB2, but has not been proved to cause tumors on its own. ErbB3 helps activating the phosphatidylinositol 3-kinase pathway (PI3K), a famous pathway involved in cell growth, cell proliferation, cell migration and tumor development (25, 26).

2.5 Tyrosine kinase receptor inhibition in MLS

Unpublished experiments made by Åman group, showed that RET is phosphorylated, hence activated in MLS cell lines and human tumors. DNA sequencing showed that the protein is not mutated. When treating MLS cell lines with RET tyrosine kinase receptor specific antibody vandetanib, no effect on phosphorylation or cell viability could be seen. This suggests that RET can be activated by a ligand independent mechanism.

It is known that activated EGFR can phosphorylate and activate RET by heterodimerization in the cytoplasm in thyroid cancer cells (27). ErbB3 and EGFR are very similar in their function and in MLS, ErbB3 has been shown to dimerize with RET. Nevertheless, inhibiting antibodies against tyrosine kinase receptors ErbB3 and EGFR also fail to reduce phosphorylation of RET, shows unpublished material from Åman group.

Heterodimerization of different tyrosine kinase receptors might be an explanation for this tyrosine kinase receptor inhibitor resistance. Blocking one tyrosine kinase receptor pathway may not be enough to block this autophosphorylation process and therefore does not reduce the level of phosphorylated RET.

2.6 Heat Shock Protein 90

A protein that have RET, ErbB3 and EGFR as target proteins and that might be of interest in the heterodimerization process described in the previous section is Heat shock protein 90 (HSP90). HSP90 is a chaperone, a protein being involved in protein folding, modification and complex formation. It helps newly synthesized proteins to obtain their biologically active

structure and creates protein complexes, while helping misfolded proteins to be repaired or degraded (28). HSP90 corresponds to 1-3 % of the cellular total protein load, has more than 20 co-chaperones modifying its own activity and has an effect on more than 400 client proteins (29). In cancer, HSP90 is believed to maintain cell proliferation pathways by preserving important proteins for cell signaling. HSP90 has also been linked to some other important hallmarks of cancer such as overcoming environmental stress, reduce nutrient and oxygen demands, prevention of destruction by the immune system, angiogenesis and promotion of metastasis (28). Inhibiting HSP90 could therefore have an effect on tumor cells in multiple aspects.

2.7 HSP90 inhibition

HSP 90 inhibition has become a very interesting mechanism for antitumor drug development and at least 17 HSP90 inhibitors are available on the market today (28, 29). It has mostly been tested against advanced tumors of high grade such as breast cancer, prostate cancer, melanoma, multiple melanoma and ovarian cancer (28). The earliest versions of HSP90 inhibitors have not proven to be very efficient, and had a very common side effect in liver toxicity. Recently, a number of new HSP90 inhibitors have emerged on the market with greater potential and less side effects (30).

2.7.1 17-AAG

Unpublished experiments on MLS cell lines with an HSP90 inhibitor called tanespimycin (17-AAG, 17-allylamino-17-demethoxygeldanamycin), have been done by Åman group. 17-AAG was one of the first HSP90 inhibitors on the market. The results were promising and showed that HSP90 inhibition diminished MLS cell lines while having little effect on control cell lines such as fibroblasts. The problem with this drug is that it is not water soluble, making it hard to infuse in animals. It also has a major side effect in liver toxicity, making it hard to increase

the dose to optimal treatment dose (28). Since the clinical potential was low, no further experiments with this drug on MLS cell lines have been done.

2.7.2 17-DMAG

Alvespimycin, (17-DMAG, 17-Dimethylaminoethylamino-17-Demethoxygeldanamycin) belongs to a newer generation of HSP90 inhibitors. This drug is more water soluble and potent than its precursor 17-AAG. Because of its water solubility and improved pharmacodynamics, it has fewer problems with liver toxicity. It has been documented to have contributed to a complete response in a patient with castration resistant prostate cancer, verified with CT and PSA (31). In other studies, it has shown a partial response against breast cancer.(32)

Phase 1 trials have shown that the drug is tolerable up to a maximum dose of 21 mg/m^2 infused twice a week with fatigue, nausea, musculoskeletal pain as major side effects (33).

Unpublished experiments on MLS cell lines, treated with 17-DMAG, have been done by Åman group. In cell culture, a drug titration curve of 17-DMAG was performed on MLS cell lines, showing that 30 nM of the drug is effective for inhibiting cell proliferation (Fig.3). In 2013, experiments with injections of 17-DMAG in immunodeficient xenograft mice incorporated with human MLS were started. Preliminary results indicated that treatment with 17-DMAG reduced tumor surface area up to 60 %, without any obvious side effects. Histological examinations of tumors showed reduction in cell number, apoptosis, necrosis and vessel bleedings (fig.4).

These results were of immense interest and have given rise to the initiative of a phase 2 trial with a HSP90 inhibitor against MLS. The exact mechanism of action, optimal treatment conditions and treatment interval for this drug against MLS are still not fully investigated, and more research is required to fully understand the potential of HSP90 inhibition.

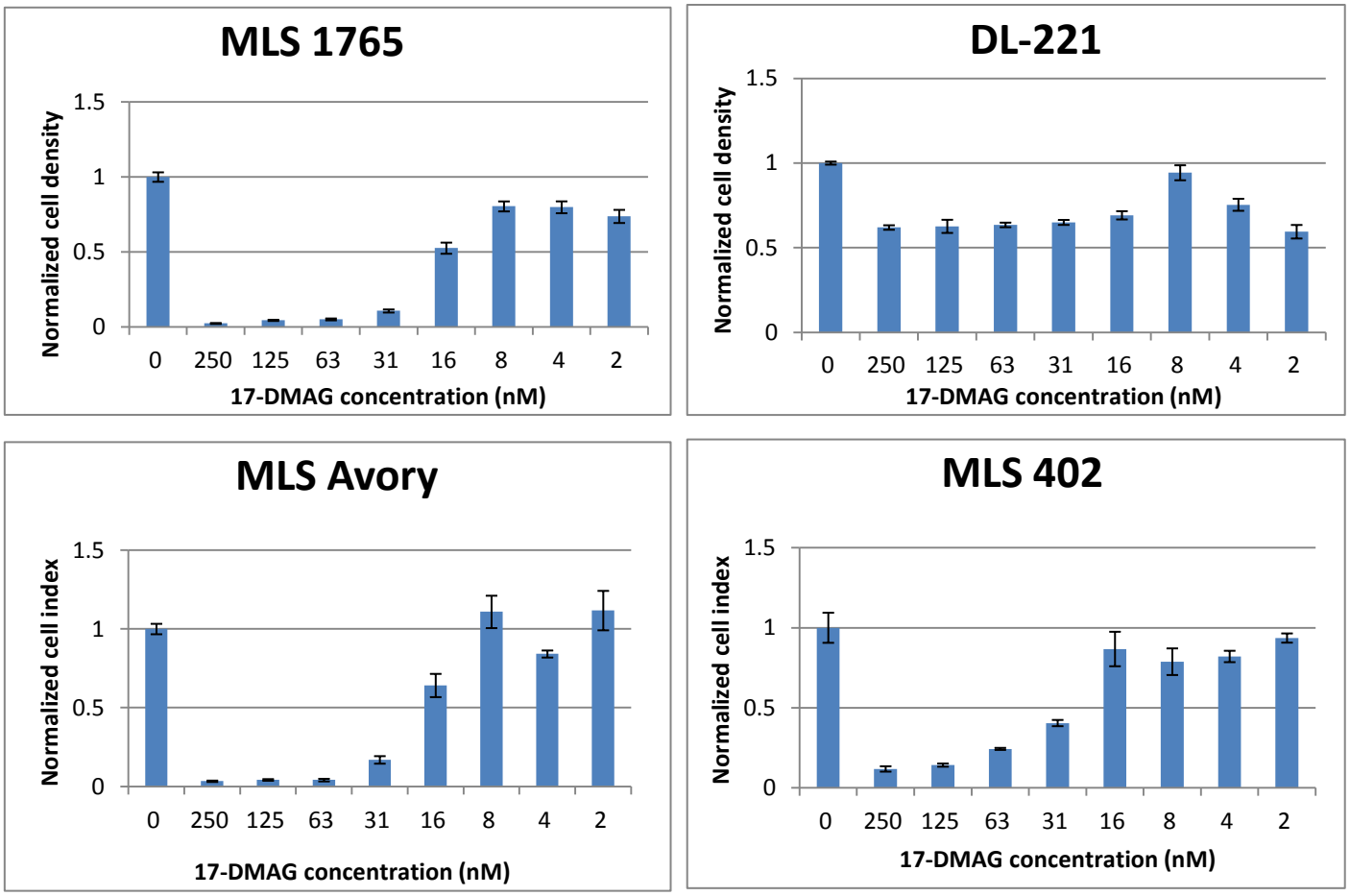


Fig.3. Three of four MLS cell lines have a sufficient response of around 30 nM 17-DMAG after 72 hours. DL221 does not seem to be sensitive to 17-DMAG (Unpublished material from Åman group)

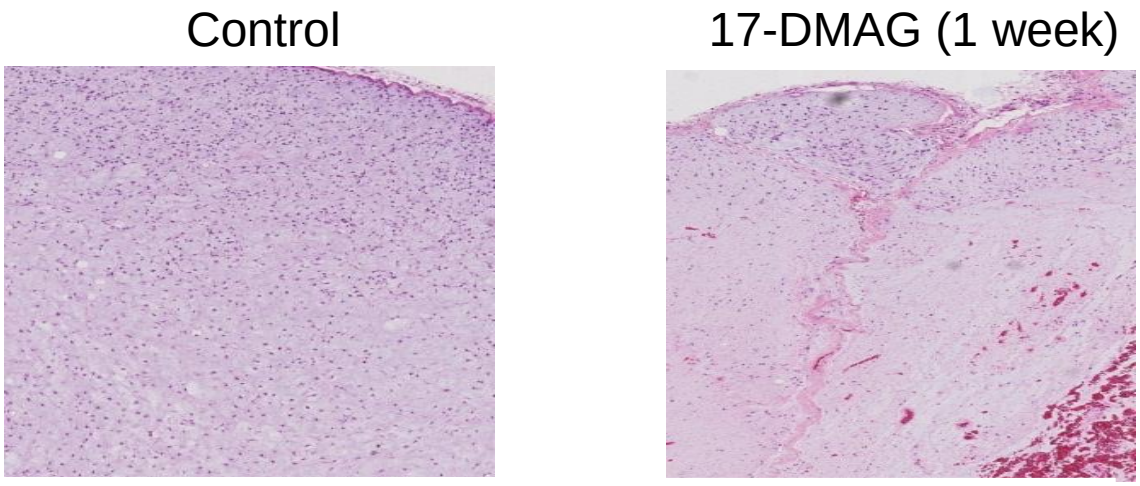


Fig.4.Histological examination of tumors from xenograft mice show reduced cell number, apoptosis, necrosis and vessel rupture after only one week of 17-DMAG treatment (Unpublished material from Åman group.)

2.8 Aim

The aim of this project was to elucidate the effect of 17-DMAG on MLS cells and its connection to expression and function of tyrosine kinase receptors RET, ErbB3 and EGFR.

3. Material and methods

3.1 Summary of data collection and methods of analysis

Myxoid liposarcoma cells have been provided from cell culturing. Cells have been analyzed according to expression of target proteins, RNA level, viability and proliferative capacity.

17-DMAG (Alvespimycin) is produced by Bristol-Myers. It is water soluble and upon arrival dissolved in physiological sodium-, chloride- and phosphate buffer to 1 mg/ml (1,62 mM). For the experiments, the drug has been diluted in cell medium, with or without serum, to a final concentration of 10 or 30 nM.

3.2 Methods

3.2.1 Cell culture

6 cell lines have been used; MLS cell lines Avory, 1765, 402 and DL221, fibrosarcoma cell line HT1080 and fibroblast cell line F470. The cell lines Avory, 1765, 402 and HT1080 are fast growing cell lines, whereas DL221 and F470 grow slowly. All cells have been cultured in RPMI 1640 GlutaMAX, with addition of 5 % fetal bovine serum and 1 % PEST (to a final concentration of 100 U/ml of penicillin and 100 µg/ml streptomycin). The cell medium contains 2 mM of L-Alanyl-Glutamine and phenol Red as a pH-indicator.

3.2.2 Cell proliferation assay (Xcelligence)

To examine cell viability and proliferation after addition of 17-DMAG, Xcelligence cell proliferation assay was used. This system measures the impedance of the wells, which is correlated to number of cells. When cells die, they round up, hence covering a smaller area.

In this experiment, cell medium was added to two 16-well E-plates and analyzed in RTCA DP culture hood analyzer, to measure background impedance. Then, duplicates of the MLS cell lines and the fibroblast cell line were added and incubated in the RTCA DP culture hood for 24 hours. After that, 10 or 30 nM of 17-DMAG was added and the plates were put back in the

RTCA DP culture hood for 160 hours. Impedance measurements were made once every 30 minutes. For the analysis, data was normalized in excel and the average expression of MLS cell lines and the fibroblast control cell line were analyzed in three categories; controls, 10 nM and 30 nM of 17-DMAG. Time points from approximately every 12 hours were taken and the difference between average cell indexes in the different groups was analyzed using Mann-Whitney U non-parametric test. Each treatment group consists of a total of 8 cell replicates from 4 MLS cell lines.

3.2.3 Manual drug assay analysis

Cells were cultured in a 96 well plate. After 24 hours, 17-DMAG was added, with or without additional factors. After 72 additional hours, the wells were washed with PBS and fixated with 4 % formic acid. Evan's blue, diluted in PBS, was added to all wells to color the cell membranes. The wells were then washed with PBS and photographed with a light microscope, using the Axiovision software.

3.2.4 Cell proliferation kit (CyQUANT)

To measure relative DNA quantity, corresponding to cell numbers, a CyQUANT cell proliferation assay kit (Life Technologies) was used. The cells were cultured and treated in the same steps as above. After 72 hours, the cell medium was removed and wells were washed with PBS. The plate was frozen down in -80^{o}C for 25 minutes, thawed at 37 ^{o}C for 15 minutes and repeated three times. After that, cell lysis buffer and nucleic acid binding CyQUANT GR dye were added and light absorption of the plate was analyzed in a microplate reader with emission at 485 nM and detection at 530 nM. The values, corresponding to the DNA content, were exported to Excel and normalized in comparison to controls.

3.2.5 Immunofluorescence

To visualize expression of target proteins and localization in the cell, immunofluorescence technique was used. Cells were transferred to a 4-well objective glass, then different concentrations of 17-DMAG were added at appropriate time points. Cells were washed using

phosphate buffered saline (PBS), then fixated cells for 5 minutes using 4 % formic acid. After additional washes with PBS, primary antibody diluted in block AB-buffer (1 % BSA, 0,5 % TritonX in PBS) was added.

After one hour incubation, wells were washed in PBS and secondary antibody (directed against primary antibody), containing a green GFP fluorophore, was added diluted in AB-buffer with 0,4 % phalloidin (red cytoskeleton color). The cells were washed with PBS and blue DAPI DNA stain was added as the cells were fixated on a microscope slide. The slides were analyzed using a Zeiss Fluorescence microscope and Axiovision 4.3.2 software. Filters for DAPI (blue), Texas red (Red) and GFP (green) were used. The same exposure time was used for different wells to be able to quantify the results.

3.2.6 Western blot

To further investigate expression of target proteins, Western blot technique was used. Cell samples were cultured and treated with 17-DMAG in 6-well plates. Cell samples were scraped off using RIPA-buffer with EDTA and phosphatase-inhibitor. Samples were centrifuged; supernatant was separated from pellet and then mixed with 4X LDS sample buffer. Protein concentration was measured in undiluted samples using BioRad protein Assay.

Samples were then mixed with Sample reducing agent and the same amounts of samples were loaded onto a NuPage 4-12 % Bis-Tris 12 well gel, together with ladder. Electrophoresis was done at 200 V for one hour, separating the proteins. Then, a transfer was done at 30 V for 75 minutes to transfer the proteins from the gel to a PVDF membrane. The membrane was washed in Tris-buffered Saline and Tween (TBS-T) and then put in block solution (5 % Skim milk powder in TBS-T or 5 % BSA in TBS-T). Primary antibodies were added and the membrane was incubated for one hour in room temperature or overnight at 4 degrees. The membrane was washed with TBST, and then secondary peroxidase-linked antibody was added diluted in block solution. After one hour of incubation, the membrane was washed in

TBST and put in West Femto Extended duration substrate. Bands were then detected using a GE LAS-4000.

To detect other target proteins, the membranes were stripped using stripping buffer and incubated with new antibodies. GAPDH was used as a loading control to be able to verify the total amount of loaded protein.

To quantify band intensity, Multi-Gauge software was used. Bands were calibrated against background and quantified in comparison to loading control. Then samples were normalized in comparison to untreated controls to investigate effect of 17-DMAG treatment. Calculations were done in Excel.

3.3 Statistical analysis

Data are shown as mean values of replicates unless stated differently. Up- and downregulation are shown in percent, with p-values when applicable. Analyses were done on Microsoft Excel ® 2013, IBM SPSS statistics 22 and GraphPad Prism 6. Differences between groups were determined using non-parametric Mann-Whitney U and p-values ≤ 0.05 were considered significant.

4. Results

4.1 Expression of tyrosine kinase receptor ErbB3 in untreated cells

Previous experiments made by Åman group have shown that MLS cell lines contain RET and EGFR, whereas fibroblasts do not. To verify the results for ErbB3 a western blot with antibodies directed against ErbB3 was made.

The results show that expression of ErbB3 is higher in MLS cell lines than in fibroblasts (Fig.6).

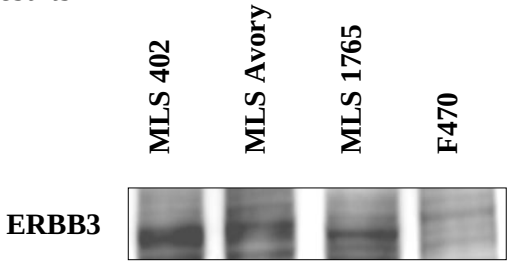


Fig.6 Western blot image of ERBB3 expression in three MLS cell lines and a fibroblast control cell line. F70, The fibroblast cell line has a lower expression of ErbB3

4.2 Cell proliferation assay on cells treated with 17-DMAG

To see the effect of 17-DMAG (diluted in serum free cell medium) in MLS cell lines, the cell index (referring to cell proliferation) was measured every 30 minutes during 56 hours after addition of 17-DMAG. Four MLS cell lines and a fibroblast cell line as control were treated in duplicates with 10 and 30 nM of 17-DMAG and compared to untreated controls. Decrease in cell proliferation was observed in MLS cell lines Avory and 1765, treated with the drug. In contrast, the proliferation rate was unchanged in treated MLS cell lines DL-221, 402 and the fibroblast control cell line F470 (Fig.7.).

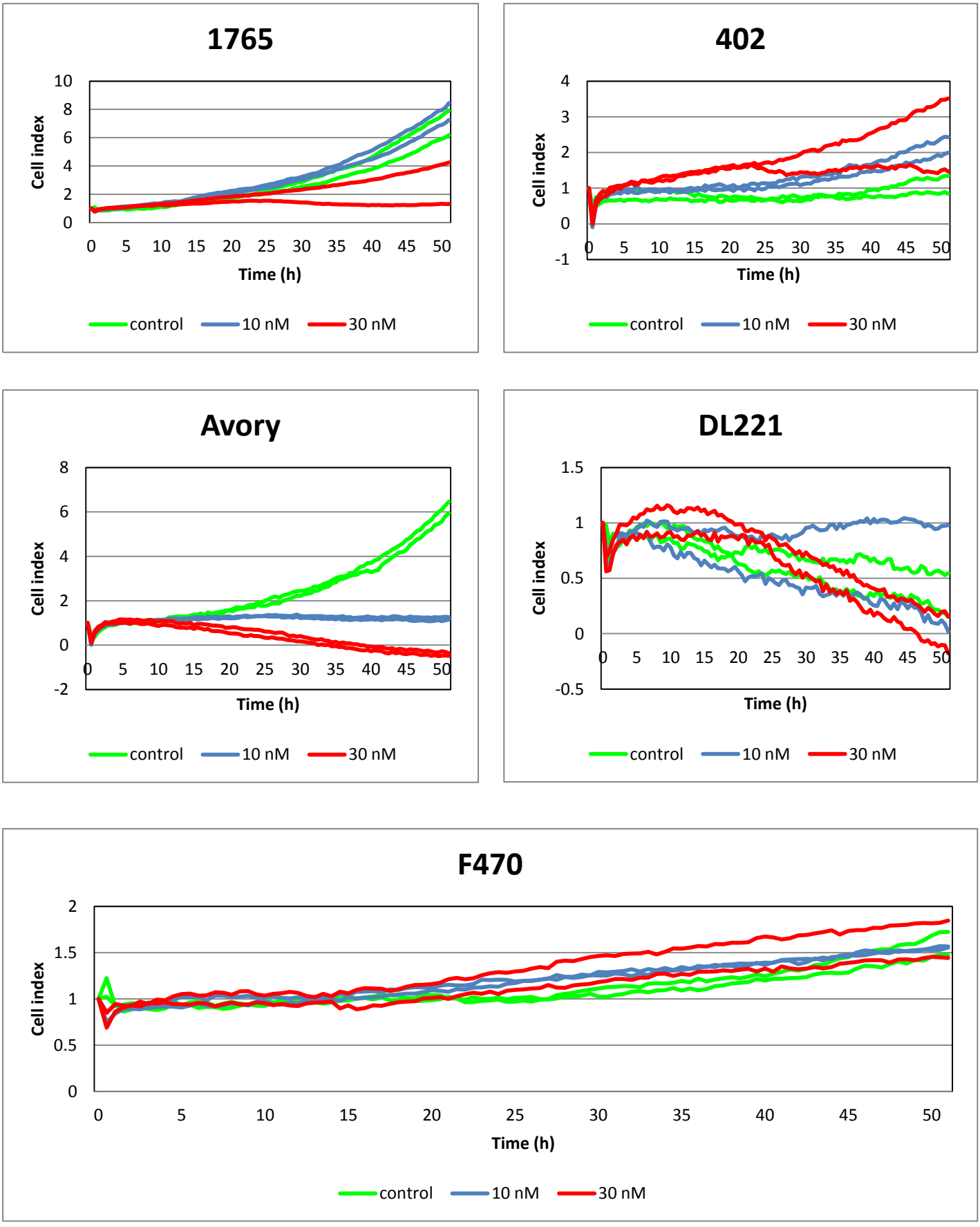


Fig.7. Cell proliferation assay in four MLS cell lines and a fibrosarcoma cell line. In Avory and 1765 we can see a clear effect of the treatment. In DL221, 402 and F470 the drug does not seem to affect the cells. Only data after drug has been added is shown. Each sample has been set up in duplicates.

4.3 Exploring target proteins using immunofluorescence and Western blot

To detect effect on target proteins DDIT3, ErbB3, EGFR and RET upon treatment with 17-DMAG, immunofluorescence was performed. MLS cell line Avory treated with 30 nM 17-DMAG for 4 and 6 hours was compared with untreated cells.

All target proteins are detectable to some extent but it is hard to detect any significant difference between the control groups and the treated groups (fig.8a-b)

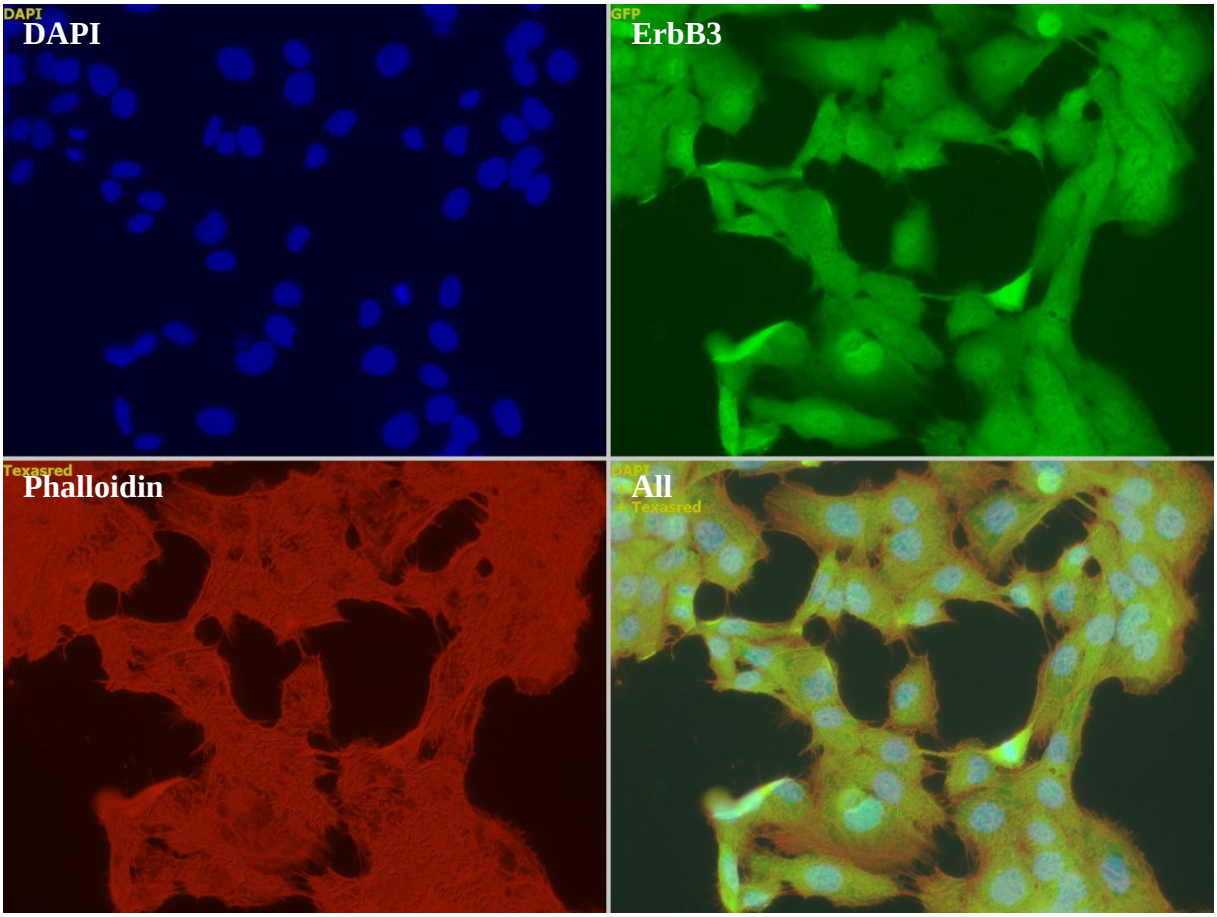


Fig.8a Complete image of an immunofluorescence of MLS cell line Avory treated with 30 nM 17-DMAG for 6 hours. Top left corner shows blue DAPI nuclear color. Bottom left corner shows red phalloidin actine color. Top right corner shows green GFP target protein color (in this case ErbB3) and bottom right corner shows a combined picture of all colors.

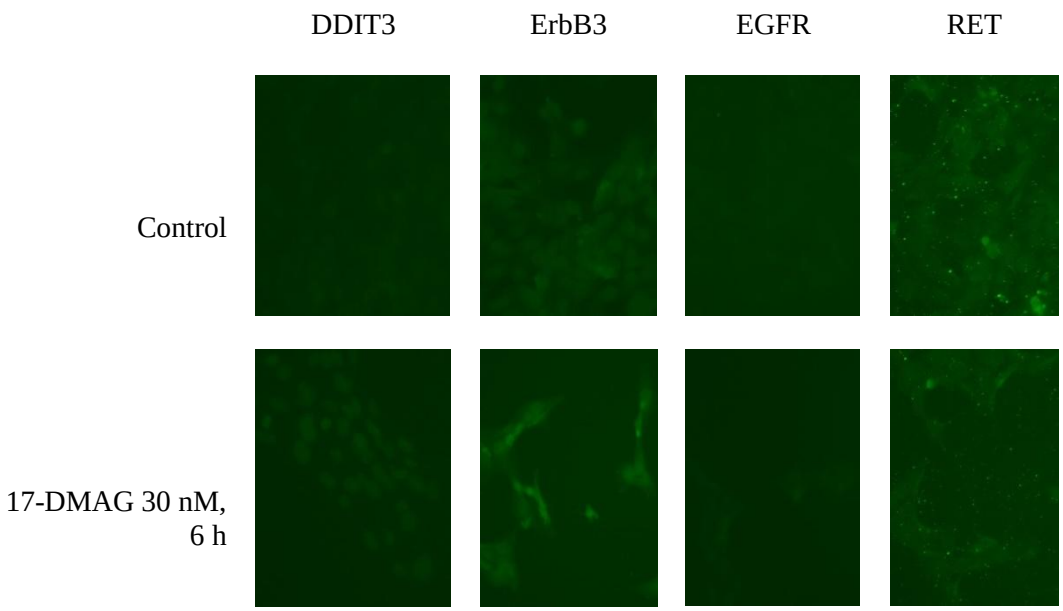


Fig.8b Immunofluorescence of MLS cell line Avory treated with 30 nM 17-DMAG for 6 hours and compared to controls. No difference can be noticed between samples treated for 4 or 6 hours, so only results for 6 hours are presented.Target proteins are DDIT3, ERBB3, EGFR and RET.

To ensure that the effect on target proteins did not occur later, two new plates of the MLS cell line Avory, treated with 30 nM of 17-DMAG (diluted in serum free cell medium), were set up. Antibodies were directed against ErbB3 and HSP90, the direct target protein of 17-DMAG. No significant difference between treated groups and untreated controls was found (fig.9).

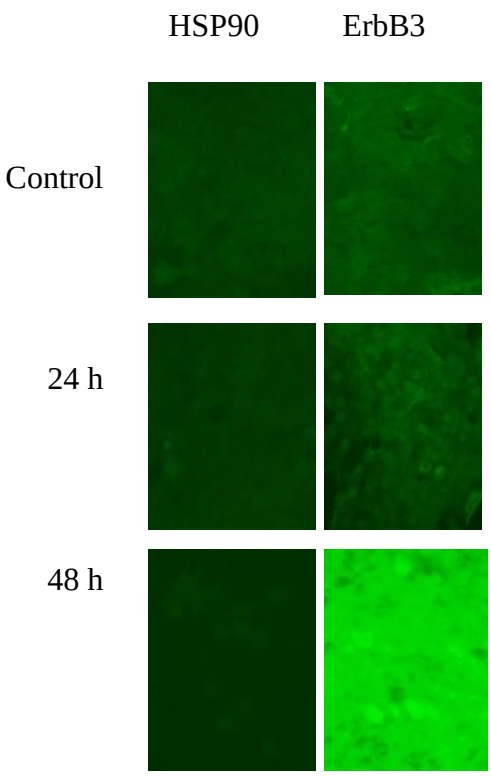


Fig.9 Immunofluorescence of MLS cell line Avory treated with 30 nM 17-DMAG for 24 and 48 hours and compared to controls. Target proteins are HSP90 and ErbB3. The cell amount after 48 hours is extremely high, so results are hard to interpret.

Since no difference between treated and untreated groups could be seen, one last immunofluorescence with antibodies directed against phosphorylated ErbB3 and RET was done. Phosphorylation of these proteins corresponds to activation, and a downregulation of phosphorylation would correspond to fewer activated tyrosine kinase receptors. The cells were treated for 16, 24 and 48 hours and compared to untreated control. There was no difference found between treated and untreated groups and results are not presented.

To be able to quantify the protein levels more precisely, a Western blot was performed. Samples were made of MLS cell line Avory treated with 30 nM of 17-DMAG for 4 and 6 hours and compared to untreated control. The drug was dissolved in cell medium and antibodies were directed against ErbB3. No difference could be seen between treated samples and untreated controls and bands have not been quantified (fig.10)

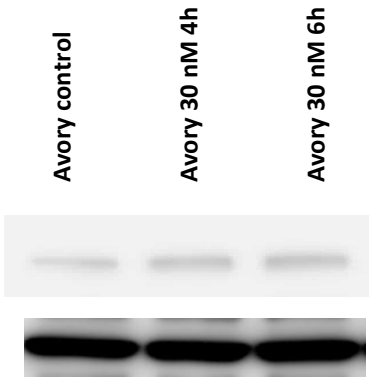


Fig.10 Western blot of MLS cell lines Avory treated with 17-DMAG for 4 and 6 hours. Top band is ErbB3 (148 kDa), bottom band is GAPDH (36 kDa) as loading control. No difference can be observed between the different samples.

4.4 Serum seems to mediate 17-DMAG effect

The absence of drug effect, though animal experiments and previous results have shown its presumed existence led us to further investigations of the previous protocols to see if anything differed between previous and new experiments. The major difference was that in previous experiments the drug had been diluted in cell medium containing 10 % fetal bovine serum. Fetal bovine serum contains a high content of proteins, including albumin, growth factors and other signal proteins that could mediate an effect. It was also possible that continuous freezing and thawing of the drug stock denaturized the drug.

To test these hypotheses, a 96-well plate with the MLS cell line Avory was set up. Different treatment concentrations of 17-DMAG, with and without serum was tested both with the old stock solution of 17-DMAG and a stock solution that had never been thawed. Cell confluence was observed daily in a light microscope. After 72 hours the cells were fixated, stained with nuclear staining and photographed. The results show that serum seems to be a necessary factor for the drug to affect the cells (Fig.11). 17-DMAG dissolved in cell medium still does not show any significant difference between treated and untreated group. 10 nM of 17-DMAG diluted in cell medium with 5 % FBS does not have any effect on the cells whereas 30 nM of the drug diluted in cell medium with 5 % FBS causes a decrease in cell density. The results were reproducible for both the old and unthawed batch of drug, disproving the theory of any difference between the stock solutions.

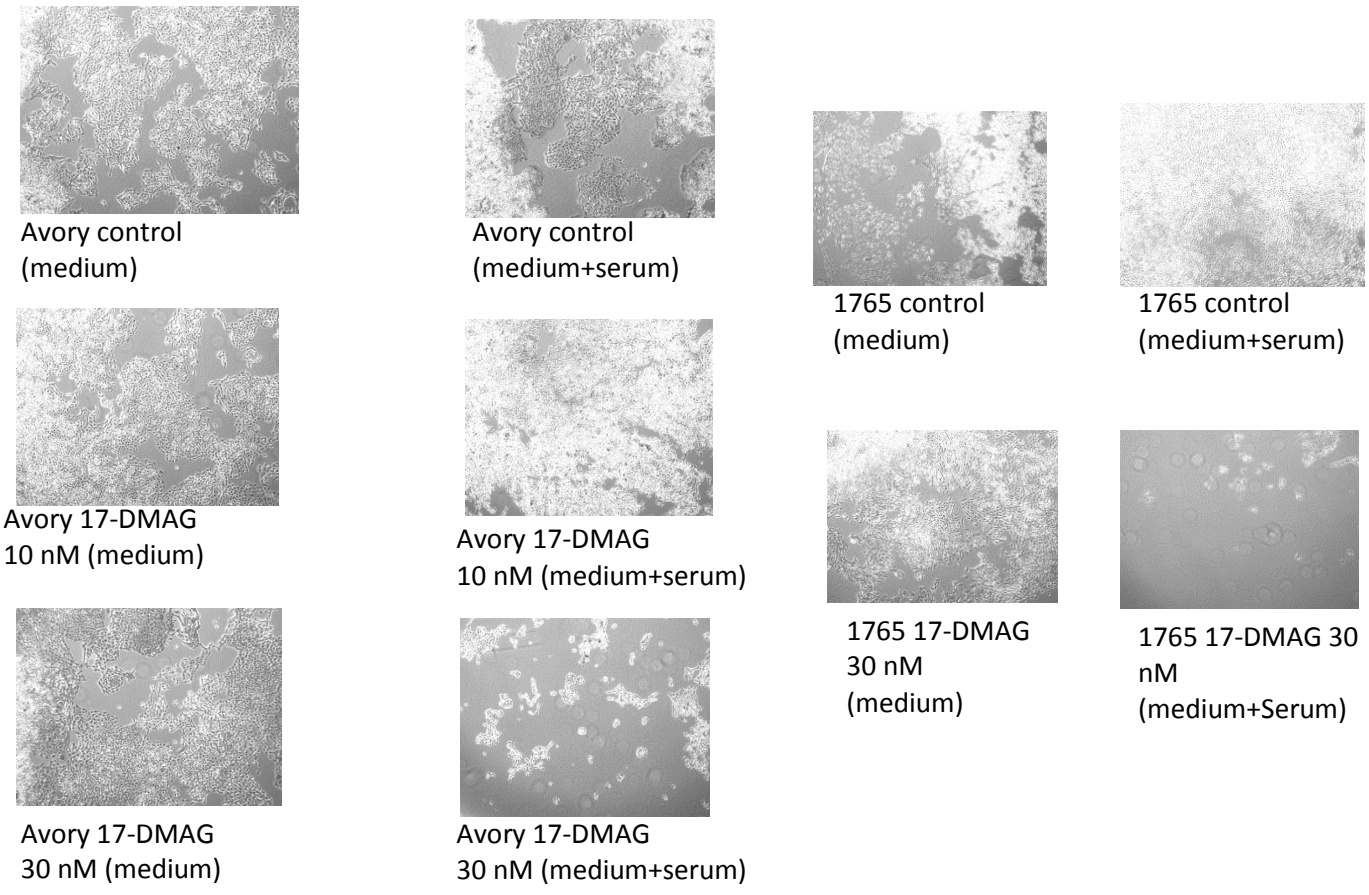


Fig.11 Images of tumor cells treated with 17-DMAG diluted in medium or medium with 5 % Fetal bovine serum. It seems that 17-DMAG diluted in medium does not mediate any effect whereas 30 nM of 17-DMAG diluted in serum does. Results are reproducible for both the tested cell lines and for both the unthawed and old batches of drug (results not shown).

Since serum seems to be an essential factor for 17-DMAG to have effect some of the previous experiments were rerun with 17-DMAG dissolved in cell medium with 5 % FBS.

4.5 New cell proliferation assay

A new cell proliferation assay was made to see how the cells were affected by 17-DMAG diluted in cell medium with serum. Decrease in cell proliferation was now observed in all MLS cell lines, whereas the fibroblast cell line was not affected by the drug (Fig.12a-c).

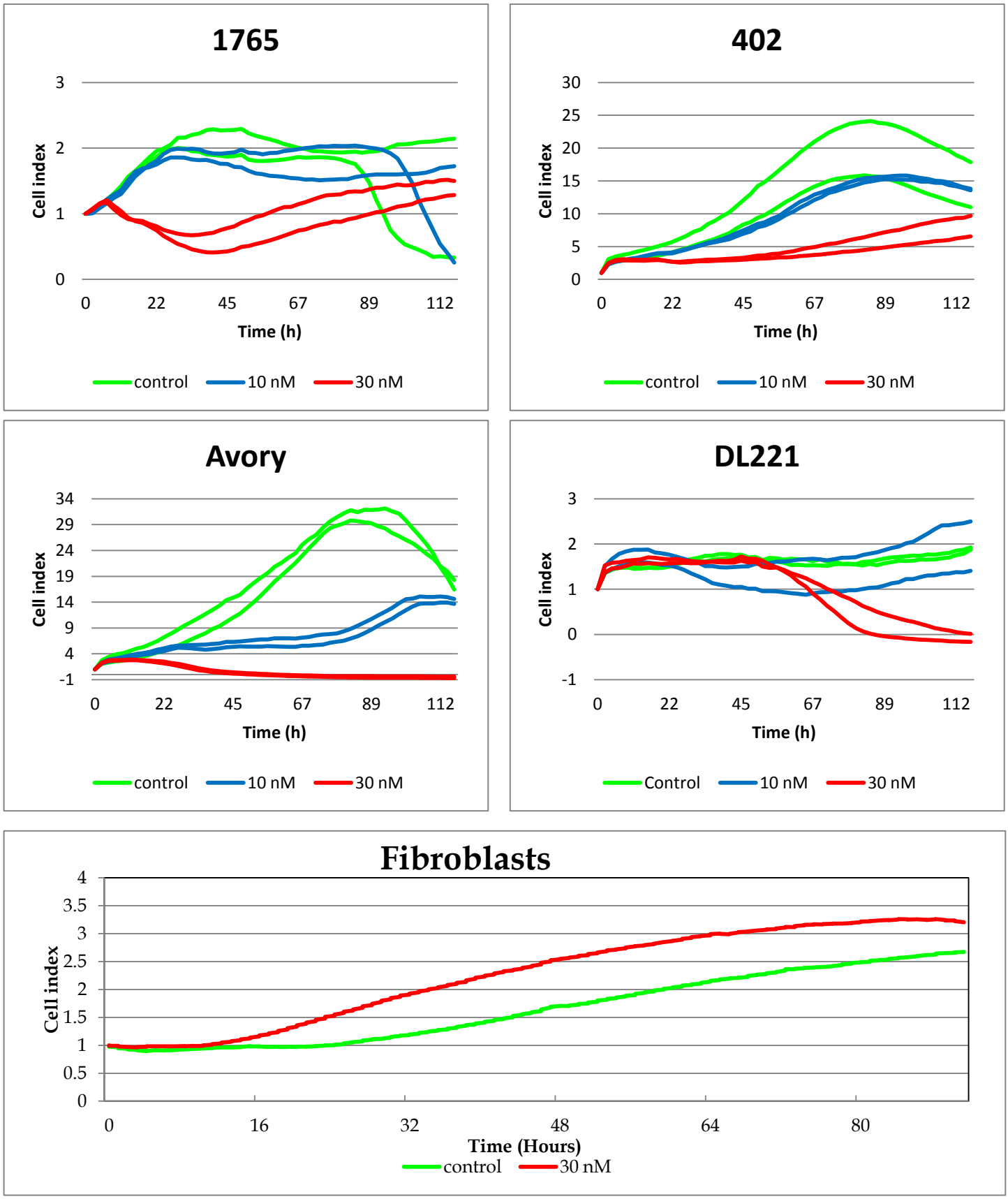


Fig.12a. Cell proliferation assay on 4 MLS cell lines and a fibroblast cell line indicates that all MLS cell lines are sensitive to the drug. Fibroblasts have only been run for 96 hours after addition of the drug.

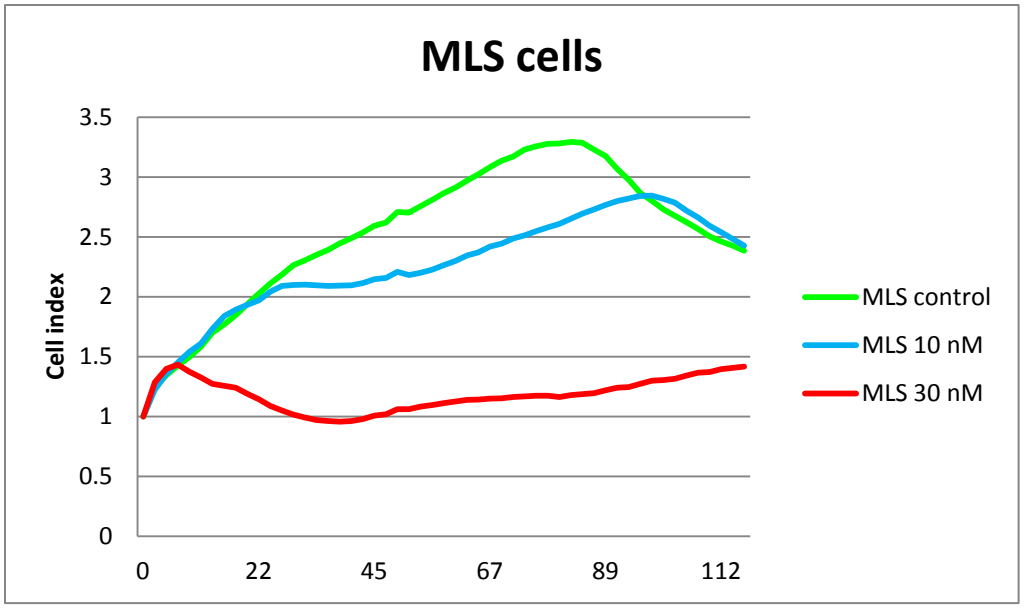


Fig.12b Average of MLS cells treated with 30 nM 17-DMAG have decreased cell index, whereas 10 nM of 17-DMAG does not affect the cells.

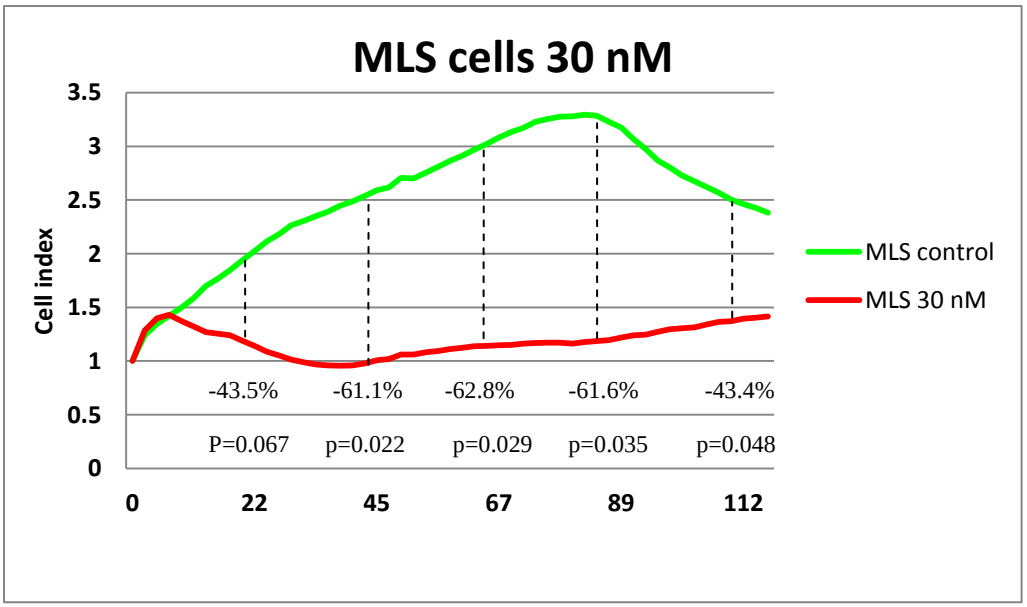


Fig.12c Control MLS cells and cells treated with 30 nM 17-DMAG. Percentage reduction in 30 nM samples indicated below time points. P-values calculated with Mann-Whitney U for each time point are highlighted below.

4.6 Immunofluorescence on cells treated with 30 nM 17-DMAG

A new immunofluorescence was made to detect any effect on target proteins. The cell proliferation data suggested that MLS cell lines Avory and 1765 responded strongest to 17-DMAG and showed a great decline in cell viability. Therefore, MLS cell line Avory was treated for 48 hours and compared to control. Antibodies were used against the target proteins

RET and ErbB3. The results are hard to interpret since control wells are extremely confluent and few cells have survived 48 hours of treatment. Only Avory controls and cells treated for 48 hours are presented since additional data did not contribute to the results (fig.13). Neither a difference in location, nor a downregulation of target proteins could be verified.

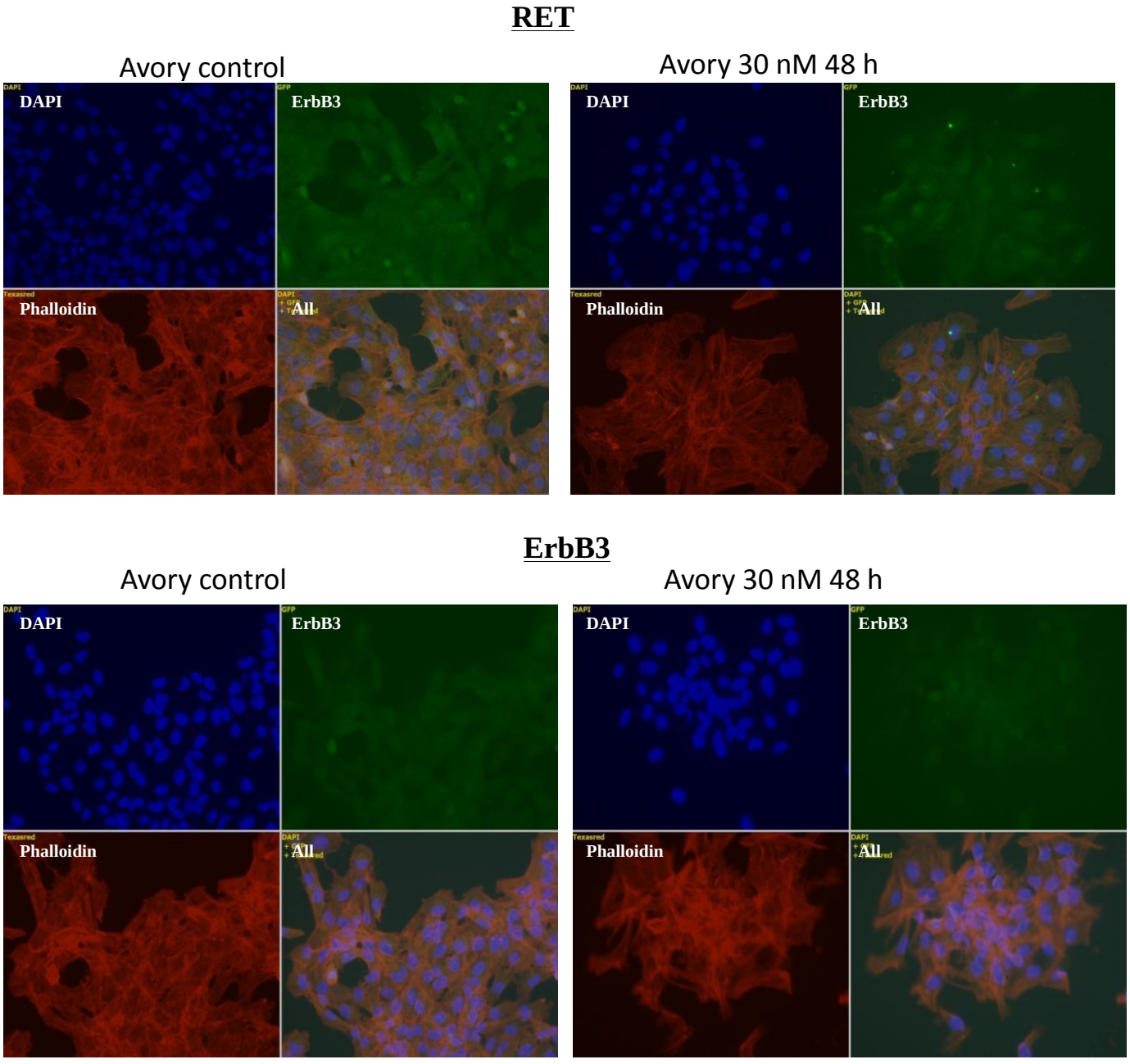


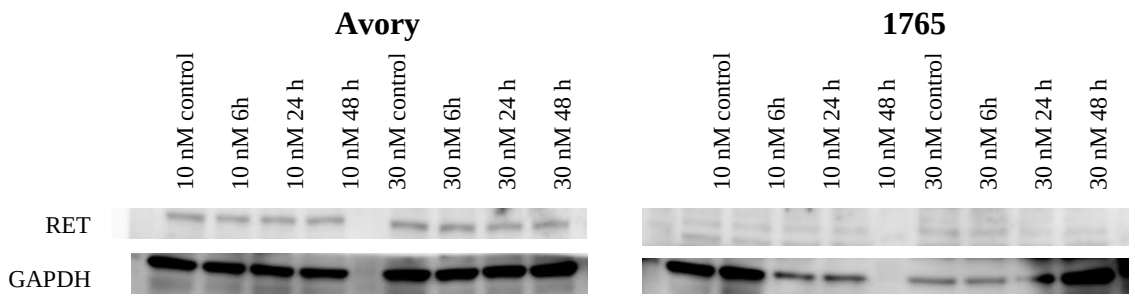
Fig.13 Immunofluorescence of MLS cell line Avory treated for 48 hours and compared to control. Reduction of target protein expression or change of cellular location cannot be detected.

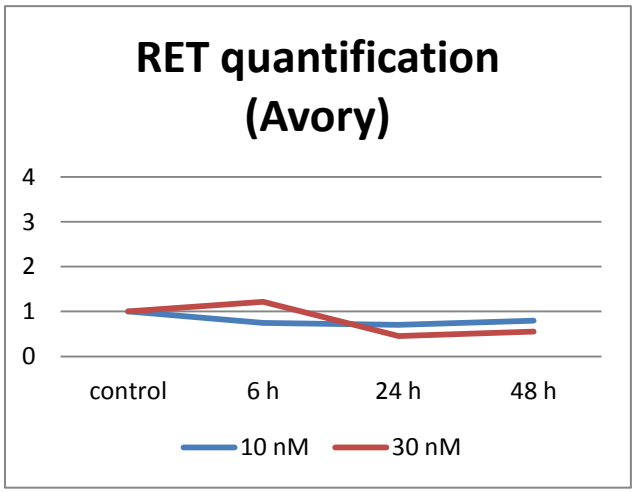
4.7 Western blot on target protein after addition of 30 nM 17-DMAG

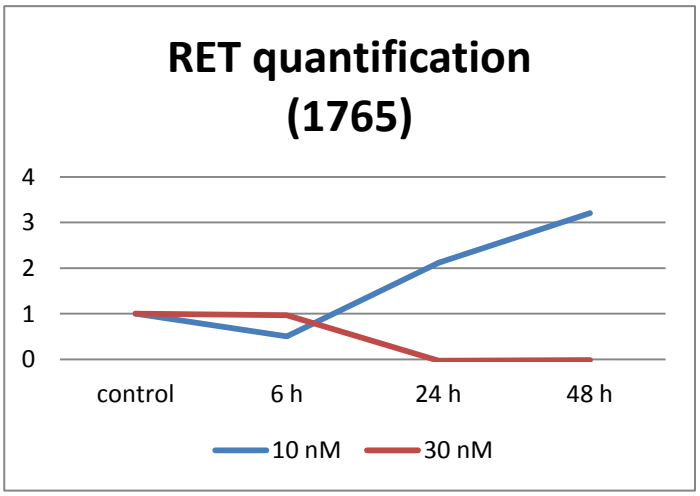
To be able to verify a reduction of target proteins, a Western blot with similar samples was performed. Antibodies against EGFR, ErbB3, RET, pEGFR, pErbB3 and pRET have been tested (fig.14). Band signal ratio between target protein and loading control was measured. (table.2). Downregulation of pRET and pErbB3 was observed after addition of 30 nM 17-DMAG. RET and ErbB3 were possibly downregulated, whereas EGFR and pEGFR seemed to be slightly upregulated (Fig.14).

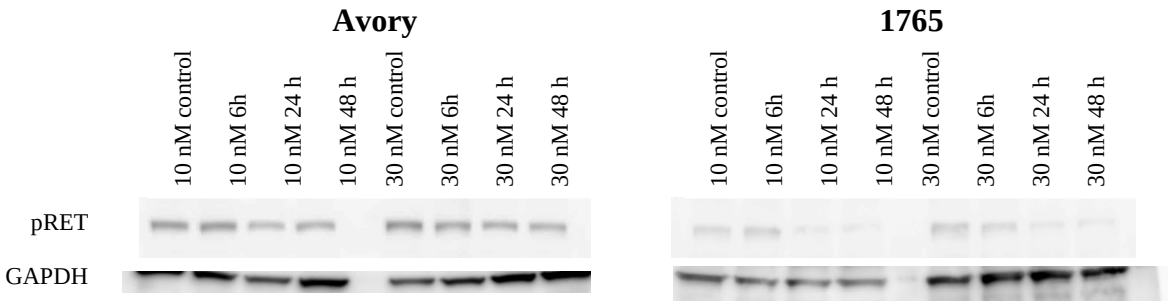
Cell index at different time points in comparison to controls																
Cell type	***Avory***								***1765***							
Conc.	***10 nM***				***30 nM***				***10 nM***				***30 nM***			
Time	c	6h	24h	48h	c	6h	24h	48h	c	6h	24h	48h	c	6h	24h	48h
RET	1	1.00	1.13	1.24	1	0.75	0.56	0.77	1	0.75	1.44	2.91	1	0.77	0.08	0.03
pRET	1	2.47	1.10	2.42	1	0.53	0.32	0.21	1	1.43	0.34	0.20	1	0.52	0.20	0.13
ErbB3	1	2.63	2.22	2.10	1	0.60	0.55	0.29	1	0.89	1.65	1.59	1	0.68	0.93	0.30
pErbB3	1	2.02	0.45	0.38	1	0.84	0.40	0.22	-	-	-	-	-	-	-	-
EGFR	1	0.72	1.33	0.69	1	0.76	1.06	0.92	-	-	-	-	-	-	-	-
pEGFR	1	1.23	0.70	0.56	1	0.95	1.01	1.29	1	1.20	0.60	0.59	1	1.65	1.43	2.87

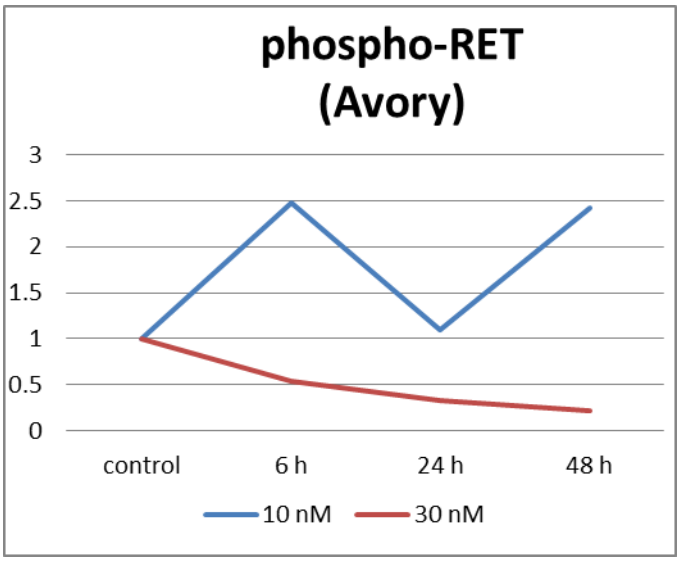
Table.2. Quantification index of MLS cells Avory and 1765 treated with 17-DMAG. Results are based on quantification data. EGFR and pErbB3 have only been tested with Avory membrane.

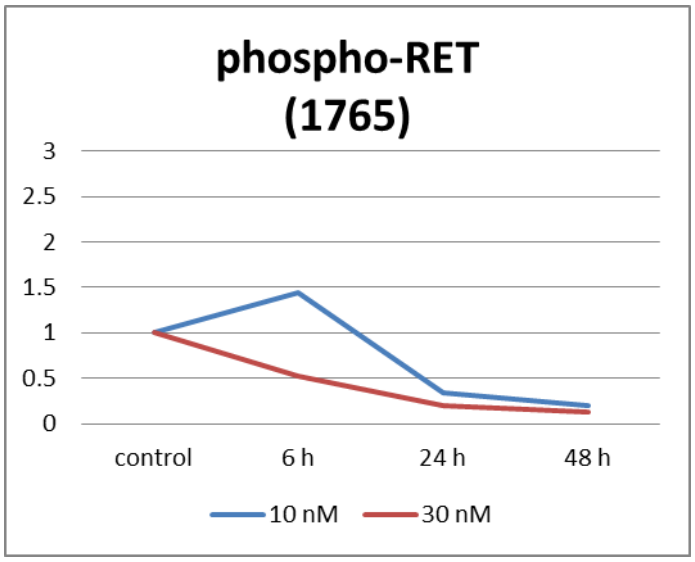

Avory
1765
10 nM control
10 nM 6h
10 nM 24 h
10 nM 48 h
30 nM control
30 nM 6h
30 nM 24 h
30 nM 48 h
RET
GAPDH

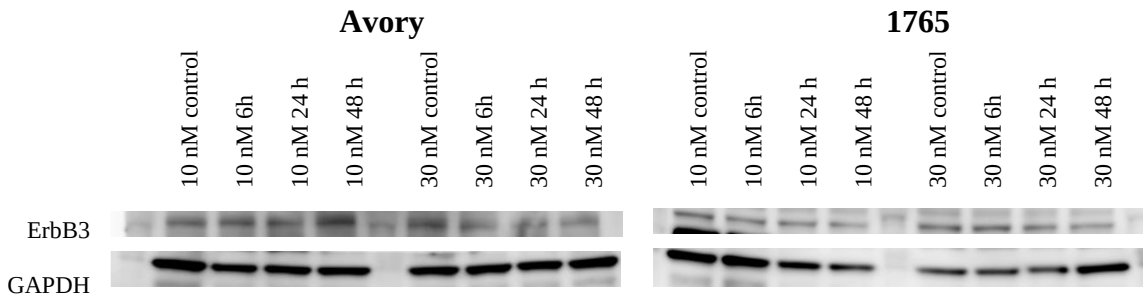

RET quantification (Avory)
4
3
2
1
0
control
6 h
24 h
48 h
10 nM
30 nM

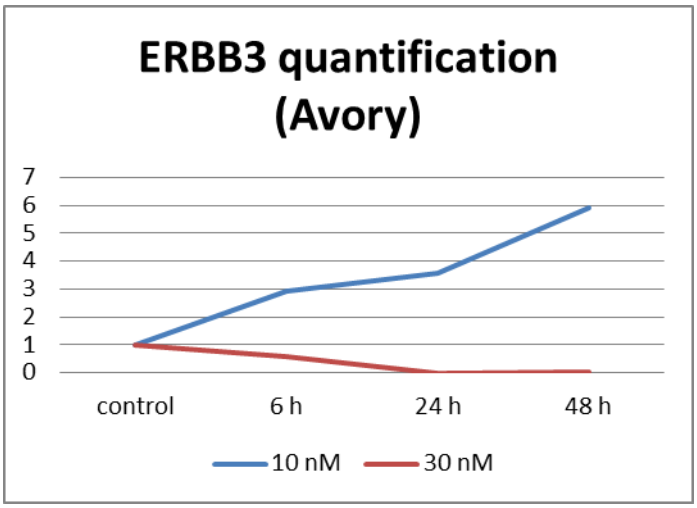

RET quantification (1765)
4
3
2
1
0
control
6 h
24 h
48 h
10 nM
30 nM

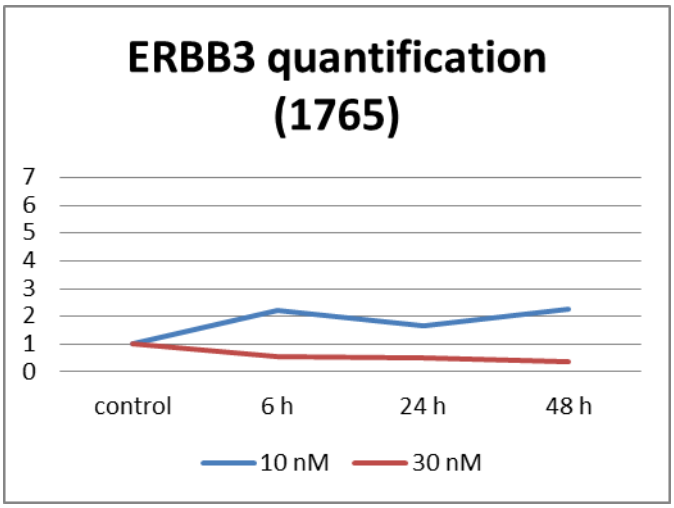

Avory
1765
10 nM control
10 nM 6h
10 nM 24 h
10 nM 48 h
30 nM control
30 nM 6h
30 nM 24 h
30 nM 48 h
pRET
GAPDH

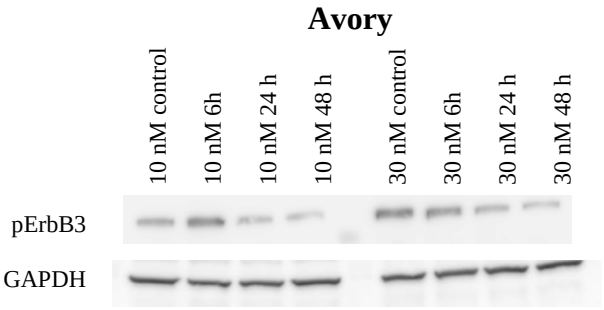

phospho-RET (Avory)
3
2.5
2
1.5
1
0.5
0
control
6 h
24 h
48 h
10 nM
30 nM

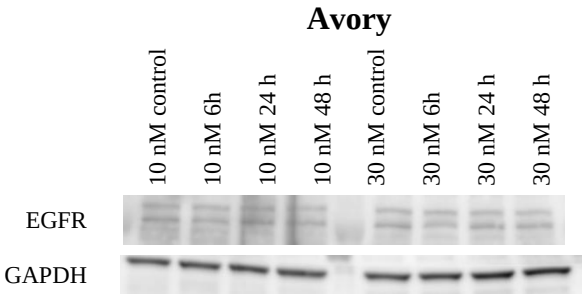

phospho-RET (1765)
3
2.5
2
1.5
1
0.5
0
control
6 h
24 h
48 h
10 nM
30 nM

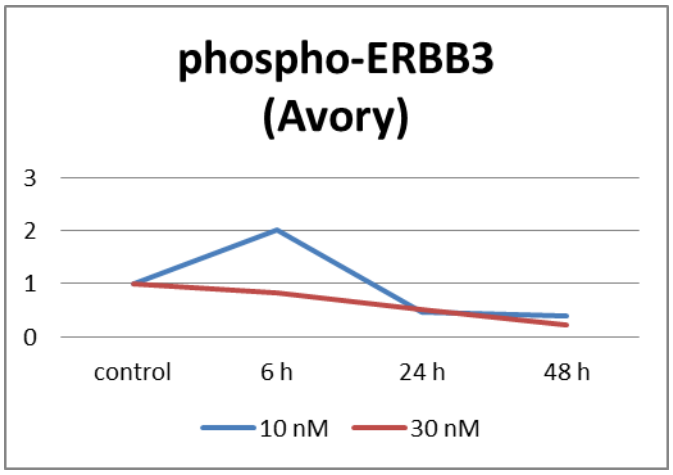

Avory
1765
10 nM control
10 nM 6h
10 nM 24 h
10 nM 48 h
30 nM control
30 nM 6h
30 nM 24 h
30 nM 48 h
ErbB3
GAPDH

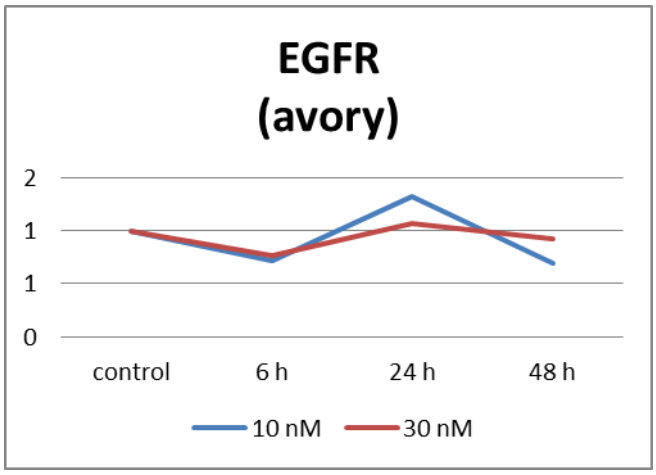

ERBB3 quantification (Avory)
7
6
5
4
3
2
1
0
control
6 h
24 h
48 h
10 nM
30 nM


ERBB3 quantification (1765)
7
6
5
4
3
2
1
0
control
6 h
24 h
48 h
10 nM
30 nM


Avory
10 nM control
10 nM 6h
10 nM 24 h
10 nM 48 h
30 nM control
30 nM 6h
30 nM 24 h
30 nM 48 h
pErbB3
GAPDH


Avory
10 nM control
10 nM 6h
10 nM 24 h
10 nM 48 h
30 nM control
30 nM 6h
30 nM 24 h
30 nM 48 h
EGFR
GAPDH


phospho-ERBB3 (Avory)
3
2
1
0
control
6 h
24 h
48 h
10 nM
30 nM


EGFR (avory)
2
1
1
0
control
6 h
24 h
48 h
10 nM
30 nM

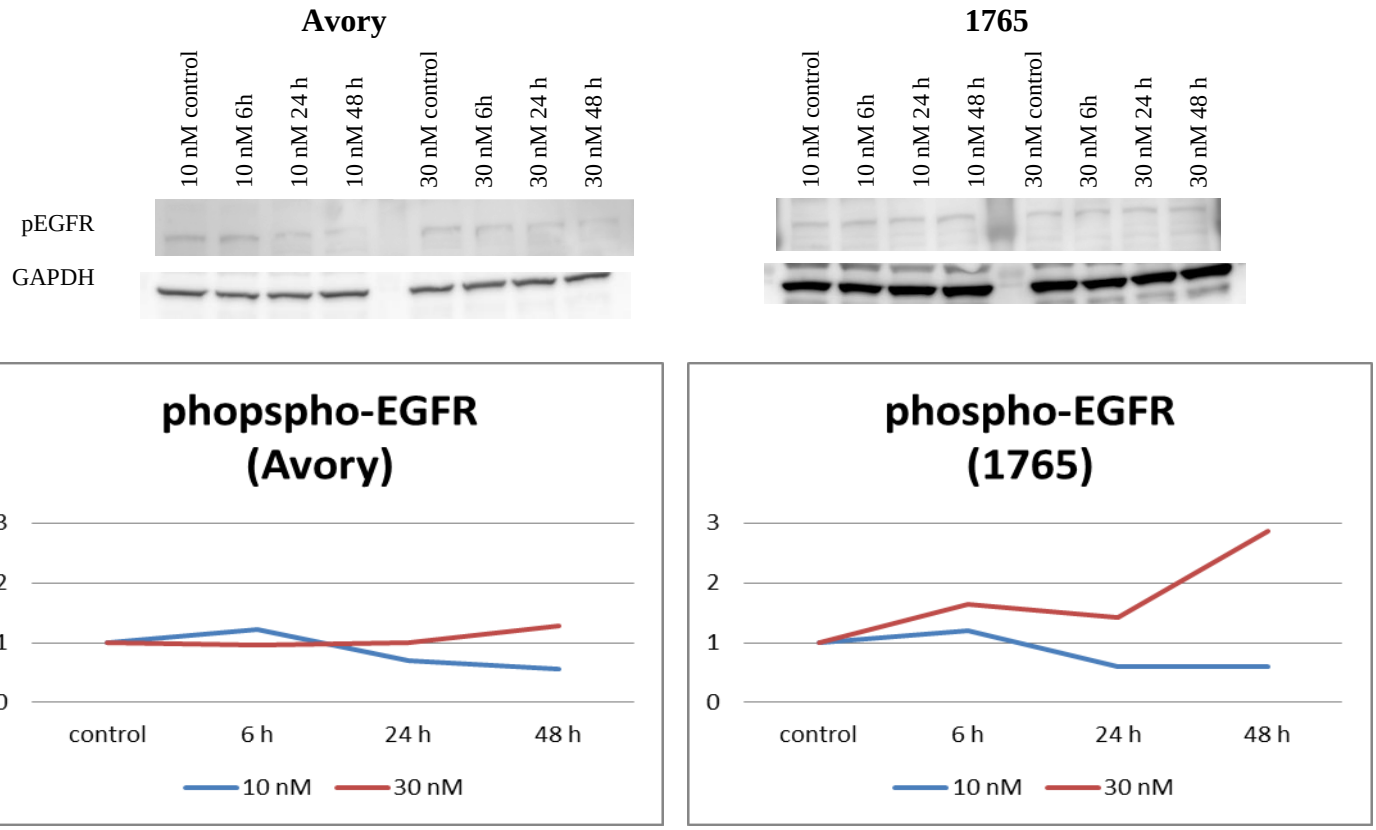
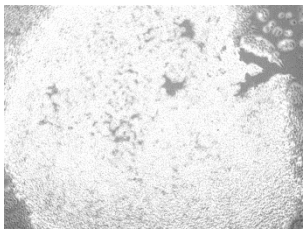


Fig.14. Western blot membranes and quantification in comparison to loading control (GAPDH) and normalized to control sample. Tested target proteins are RET (128 kDa), pRET (175 kDa), ErbB3 (148 kDa), pErbB3 (210 kDa), EGFR (134 kDa) and pEGFR (175 kDa). Most target proteins are tested on both Avory and 1765 membranes, pErbB3 and EGFR are only tested on Avory membrane. Target proteins pRET and pErbB3 seem to be downregulated by 30 nM 17-DMAG. RET and ErbB3 are possibly downregulated, whereas EGFR and pEGFR seem to be slightly upregulated.

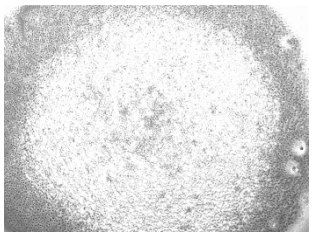
4.8 17-DMAG in cells cultured with growth factors and interleukins

Since serum contains high levels of growth factors, cytokines and other proteins, we wanted to examine if any of these proteins were the reason for the drug to have effect when diluted in serum.

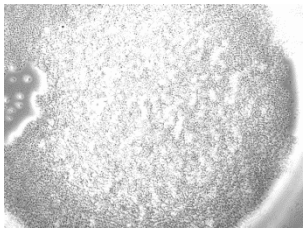
To examine this, cells were cultured in serum-free conditions in a 96-well plate and different combinations of factors with or without 30 nM 17-DMAG were added. Some of the factors are known to affect pathways regulated by HSP90, such as EGF (MAPK- and PI3K-pathway) (34), IL6, oncostatin, TGF-ß (NFkB and JAK/STAT-pathway) (35) and IGF-1 (PI3K-pathways) (35). Some others are highly expressed in serum, such as FGF, NGF, HGF and VEGF. This experiment indicated that some of the factors might have a small effect on cellular morphology, but these differences are very subtle and rather subjective. The effects on viability and growth arrest are easier to objectify visually. In this experiment it seems that IL6, IGF-1 and TGF-ß have an effect on the cells and cause reduced cell proliferation (fig.15).



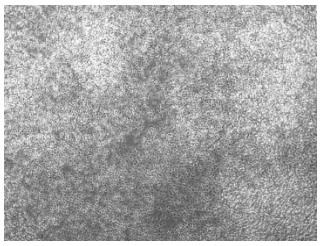
1765 FGF control



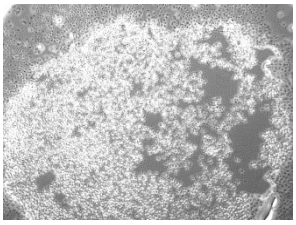
1765 EGF control



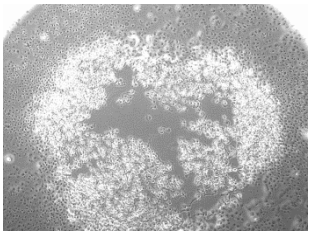
1765 HGF control



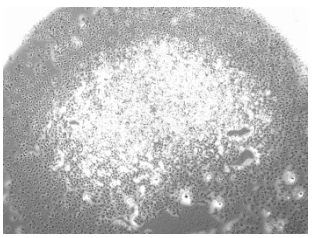
1765 IL6 control



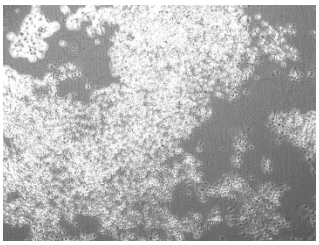
1765 FGF 30 nM



1765 EGF 30 nM



1765 HGF 30 nM



1765 IL6 30 nM

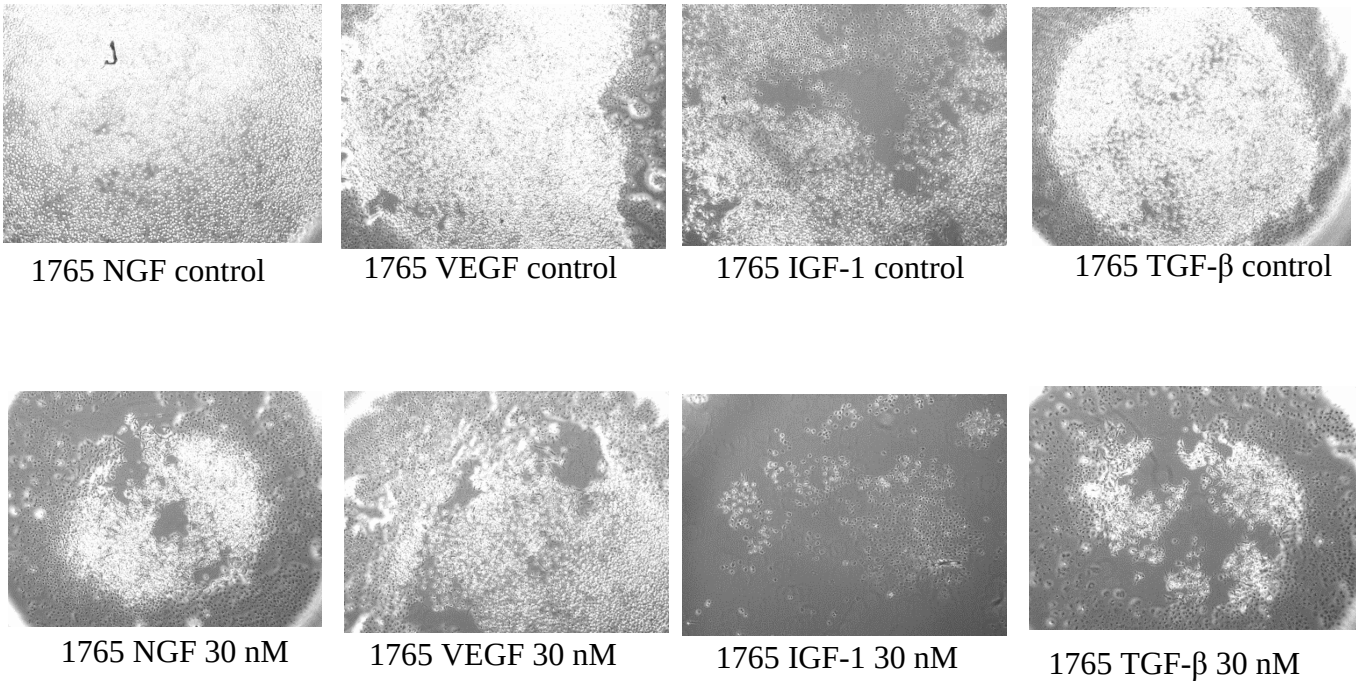
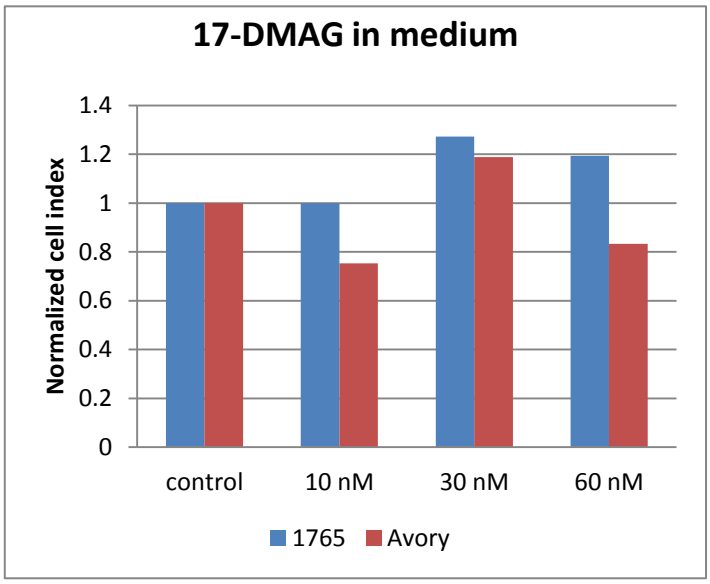


Fig.15 Cultured cells treated with different factors, with or without 17-DMAG. IL6, IGF-1 and TGF-β seem to potentiate the effect of 17-DMAG.

4.9 Using a CyQUANT assay to verify the effect of serum and growth factors

To verify and quantify these results, a cell proliferation assay was used. Cell lines Avory and 1765 were cultured and treated with 17-DMAG in different conditions (Fig.16a-b). The results verify that serum seems to be required for an effect and that IGF-1 and IL6 with 30 nM 17-DMAG decreased the total number of cells in both cell lines. TGF-β with 30 nM 17-DMAG also reduced the cell number, but to a lower extent.



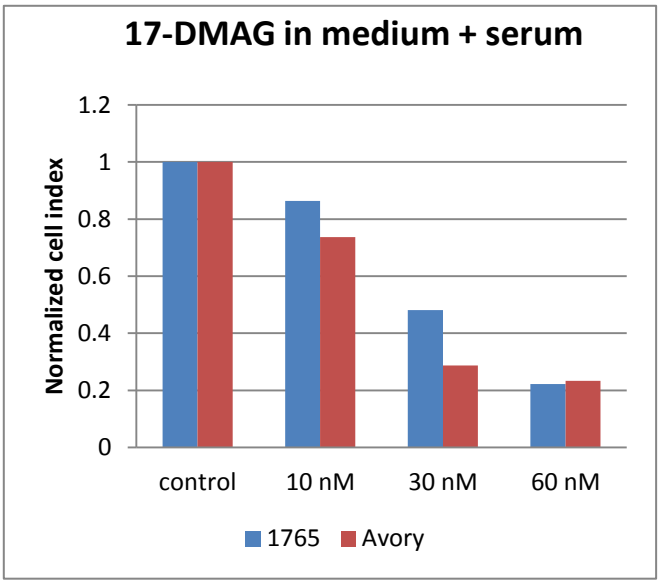


Fig.16a Measuring DNA content in cultured wells using a CyQUANT assay indicates that 17-DMAG diluted in cell medium with serum is required for the drug to have an effect. Data is normalized to controls.

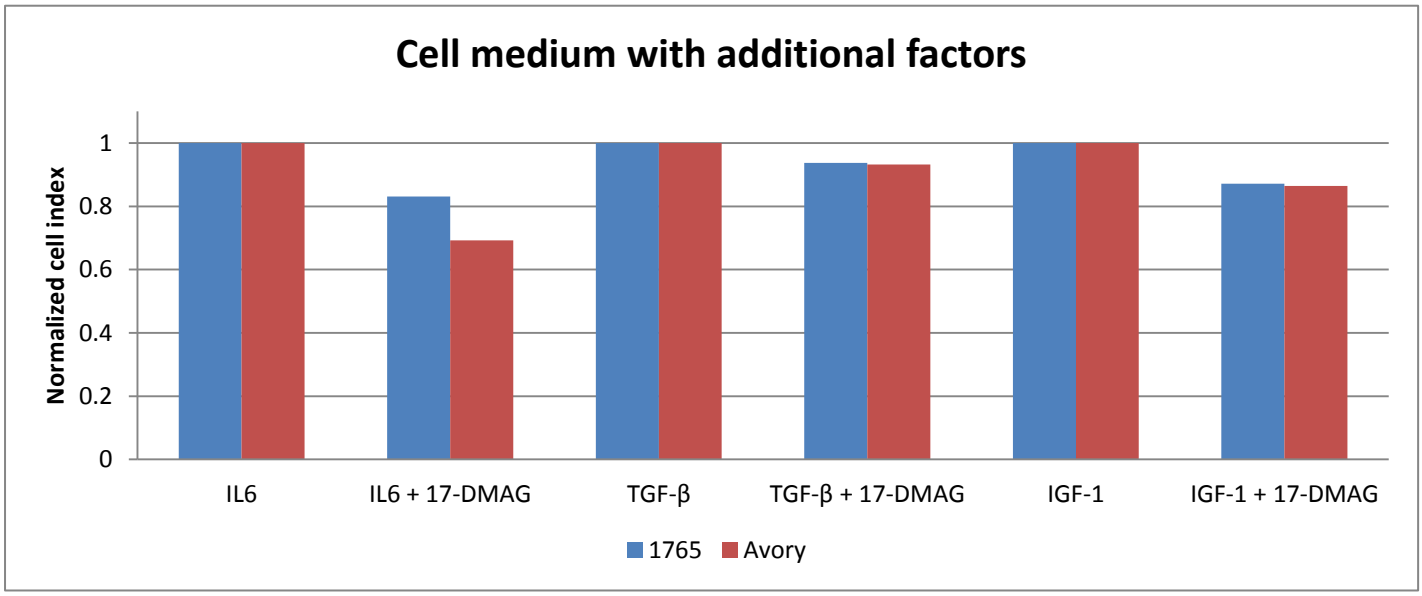


Fig.16b Measuring DNA content in cultured wells using a CyQUANT assay indicates that IL6 and IGF-1 in combination with 30 nM 17-DMAG decrease cell numbers in both Avory and 1765. TGF-β decreases cell number to a smaller extent. Data has been normalized to control cells of each factor.

5. Discussion

Inhibiting HSP90 is a quite novel anti-tumor strategy, where in rare cases effect has been reported on castration resistant prostate cancer. Phase 2 trials with different HSP90 inhibitors have been performed, but have not in general shown any major effects (31, 36).

When it comes to myxoid liposarcoma and other soft-tissue tumors, treatment with chemotherapy is sparse and mainly used when metastases have occurred. Only three drugs, doxorubicin, ifosfamid and trabectidin are registered for MLS and their effect is limited (12). This indicates a great need for novel treatment strategies, where HSP90 inhibition seems to be an interesting option.

In this paper, data has been presented indicating that 17-DMAG has potential treatment effect against cultured MLS cell lines. I have showed that some important target proteins of HSP90 and important signal pathways for maintaining a proliferative capacity in MLS are affected by the drug. The most prominent effect of HSP90 inhibition is the decrease in RET and ErbB3 receptor tyrosine kinase activation.

MLS cell lines Avory, 402 and 1765 are sensitive to HSP90 inhibition, showed by rapid decrease in cell proliferation after addition of the drug. Concerning MLS cell line DL221, HSP90 inhibition seems to have a delayed effect. What is unique with DL221 is that it grows slower that the other cell lines. DL221 also has a different isoform of the tumor specific fusion protein FUSDDIT3, and a p53 mutation, with a loss of function of the tumor suppressor attributes of this protein, causing multiple secondary mutations. Any of these differences might explain the delayed drug effect but more experiments are needed to verify this.

The MLS cell lines 402 and 1765 started to proliferate again after a couple of days' drug exposition (fig.12a). We hypothesized that this was either because of development of drug

resistance or because the drug had been metabolized. After 100 hours, we added more serum and 17-DMAG in the wells to see how the tumor cells would react (results not shown). The cells quickly started to die, indicating that they were still sensitive to the drug.

It seems that cell lines that express tyrosine kinases are sensitive to HSP90 inhibition. MLS cell lines 1765, 402 and Avory that contain high levels of RET, ErbB3 and EGFR are responsive to the drug, whereas fibroblasts with a low expression of tyrosine kinases are more resistant. Previous studies have reported that cell lines that are signaling through pathways affected by HSP90 are much more sensitive to HSP90 inhibition (28, 35), and it is likely that tyrosine kinase dependency is a key factor in HSP90 sensitivity. It would be interesting to see if MLS cell line DL221 expresses RET, ErbB3 and EGFR. If not, it is possible that signaling through tyrosine kinases is not the major tumor driving factor in DL221, explaining the lower drug effect.

Interestingly, serum seems to be necessary for the efficiency of 17-DMAG. An explanation to this could be the presence of different growth factors and interleukins in serum, which might be needed for the effect. The fact that HSP90 has increased effect when stimulating signaling pathways targeted by HPS90 has been confirmed in multiple myeloma by Lin et al, 2012 (35). Finding and activating pathways that are regulated by HSP90 might consequently be a key to successful HSP90 treatment (35). Results from this study indicated that IL6, IGF-1and to a lesser extent TGF-β, potentiated 17-DMAG efficiency in serum free conditions.

Another potential theory about the serum effect might be that serum contains ions that interacts with 17-DMAG, causing a precipitate that falls down to the bottom of the well, closer to the cultured cells. That is less likely since we have noticed an in-vivo effect of 17-DMAG in xenograft mice.

The molecular mechanism of HSP90 is largely unknown. However, it has been showed that HSP90 scaffolds different tyrosine kinase receptors to promote a process of heterodimerization and autophosphorylation. It is also possible that HSP90 itself helps phosphorylating tyrosine kinases, or prevents breakdown of these proteins once activated. Previous research has shown that all tyrosine kinase receptors tested in this paper are target proteins of HSP90 (37-39). It has been shown that RET and EGFR can dimerize (27) and experiments from Åman group using a PLA assay show that RET and ErbB3 are co-localized in MLS cell lines. It is hard to prove how HSP90 acts in detail, but one mode of action proposed in this study, could be that HSP90 inhibition leads to a reduction of phosphorylated pRET and pErbB3. Lower pRET and pErbB3 suggest a lower tyrosine kinase signaling, which in turn results in lower proliferation signals. RET and ErbB3 seem to be slightly downregulated as well, but more experiments need to be done to confirm this. On the other hand, both EGFR and pEGFR seem to be slightly upregulated in my results, which does not correspond to previous research on the HSP90 inhibitor Geldanamycin (38). The upregulation of EGFR and pEGFR could possibly indicate some kind of negative feedback process, where EGFR and pEGFR are upregulated to try to sustain a pathway necessary for tumor cell vitality. EGFR and pEGFR are said to be highly expressed in MLS cell lines, but in my experiments it seems that they are lowly expressed. To verify the effect of EGFR and pEGFR, we should do more experiments with other antibodies.

5.1 On-going experiments

To more precisely examine the genes that are up- and downregulated in different cell lines when treated with 17-DMAG I have collected samples for treated and untreated cells and sent them for a micro-array, which is a mass-analysis of what gene pathways that are active. With this material we plan to analyze the gene profile of different cell lines with and without the drug and see if there are some activated genes that may cause a mechanism for lower drug

effect in MLS cell line DL-221. This information will be used for further experiments where we will use single-cell PCR to try to find subpopulations within a cell population. Analyzing single-cells would give a better understanding on why some cells are more aggressive than others and why some cells survive different kinds of therapy. In the future, this may give us information on how to individualize cancer treatment depending on the cell population and active molecular pathways in a patient's tumor cells.

5.2 Methodological considerations

My results are based on a variety of different methods. This makes it possible to show the effect of HSP90 inhibition from different aspects. The disadvantage of this is that my results are not extremely statistically significant.

Research on cultured tumor cells is tricky since cultured cells also do not behave like tumor cells in our body. Concerning our MLS cell lines, they have been immortalized using a SV-40 virus that might affect the cellular response. Also, since you put the drug directly in the medium, you are sure that almost all the cells are exposed to the drug. In the human body, the bioavailability of the drug to the cells is lower and other factors such as drug metabolism in the liver, tumor suppressing effect of the immune system and a higher degree of drug resistance will affect the treatment effect. Therefore, these experiments cannot verify any treatment effect in vivo.

From my findings, I cannot tell if the target proteins change location from the nucleus to the cytoplasm. Immunofluorescence results were in general hard to interpret so my conclusions are drawn from my results on Western blot. Western blot is a very good method to see if proteins are expressed or not, but unfortunately it is not a very secure method to quantify expression of target proteins. As my quantification results show, RET, ErbB3, pRet and pErbB3 are downregulated by 17-DMAG treatment. When looking at the membranes, it is

clear that pRET and pErbB3 are downregulated, whereas the effect of RET and ErbB3 can be contributed to the loading control. To verify these results, more Western blots should be done.

The Xcelligence system is a great system to measure cell proliferation and drug response. Already in the first experiments, MLS cell lines Avory and 1765 were sensitive to 17-DMAG, although diluted in cell medium. This is probably because we mistakenly only used one tenth of the cells recommended for this experiment. In lower cell numbers it seems that cells might be sensitive to 17-DMAG, even if serum is not added.

Since all MLS cell lines were affected to some extent in the last Xcelligence assay, I decided to merge the data and to analyze all the MLS cell lines in three groups. Doing this gave me more replicates in each group to analyze, making it possible to show statistical correlation in between different treatment groups. The difference in cell index between groups could then be analyzed using a non-parametric Whitney Mann-U test. This might not be a correct method of comparing samples, since all MLS cell lines respond individually to the drug. A better method would be to run new experiments with more replicates of each treatment group for each cell line and analyze each cell line individually. Unfortunately, this would have been too time-consuming for this project.

Previous results have indicated that adding an HSP90 inhibitor with different factors can potentiate the drug effect. The results are not always coherent, and no results have been presented about MLS cell lines. Culturing cells and taking images is not the best method to show how many cells remain after addition of drug with different factors, since only one part of one of three replicates is shown in the image. Using a CyQUANT assay to measure DNA amount in treated and untreated wells made it possible to quantify the average drug effect in the different replicates. I have normalized each factor group with the untreated control of

respective factor, which I believe is the best way of showing how the drug is potentiated by the factor.

5.3 Limitations

Even though 17-DMAG seems to be a potential MLS treatment, it is hard to draw any conclusion from experiments on cultured cell lines and Xenograft cells. Side effects, different response in human and mice, pharmacokinetic complications and drug resistance are only a few of the problems that one may face in human tumor therapy. An example of this is the drug 17-AAG that showed great potential in cultured tumor cells, but problems with poor water solubility, drug administration, liver toxicity and drug resistance were only a few of the complications. Toxicity problems have occurred with 17-DMAG as well, but to a smaller extent (30). Recently, some new HSP90 inhibitors have been released on the market. They are so called small molecule inhibitors and have a more effective blockage of different isoforms of HSP90. Two new HSP90 inhibitors called NVP-AUY922 and NVP-HSP990 have shown great potential against prostatic cancer (30, 40).

In this paper I have only studied the effect on three tyrosine kinase receptors. There are approximately 20 tyrosine kinase classes with plenty of receptors that need to be further investigated. One receptor that has already shown some interesting results in MLS is the Flt1-receptor, responding to VEGF-β. We also need to further examine the expression of ligands, since it is known that the tumors can produce their own ligands, activating themselves in autocrine loops. By mapping the tyrosine kinase receptors and ligands that are active in the different tumors, one can get a better clue of the autocrine loops that are present.

6. Conclusion and implications

Tyrosine kinase signaling is an important oncogenic factor in MLS, suggesting that HSP90 inhibition is a possible method for treatment. Our results indicate that 30 nM of 17-DMAG specifically affects cells dependent on tyrosine kinase signaling, decreasing proliferation and causing cell death

Even if 17-DMAG does not become a new therapy for MLS, the principle of inhibiting HSP90 is a very promising way of treating tumors. In this paper I have briefly demonstrated the effect of HSP90 inhibition in MLS cells and further investigated the mechanism of HSP90 inhibition. A lot of questions remain and further experiments are needed to get a better overview of how HSP90 mediates its effect.

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8. Populärvetenskaplig sammanfattning

Cancer drabbar över 57 000 personer årligen i Sverige och är ett stort hälsoproblem i dagens samhälle. Det är den näst vanligaste dödsorsaken efter hjärtkärlsjukdomar och mellan var tredje och varannan person kommer att drabbas av cancer under sin livstid (1, 2). Cancer är ett samlingsbegrepp för maligna (elakartade) tumörer, det vill säga celler som börjat tillväxa ohämmat. Dessa tumörer tillväxer lokalt, men kan även skicka ut så kallade metastaser (dottersvulster) som sprider sig till andra delar av kroppen.

De vanligaste cancertyperna i vårt samhälle är bröstcancer, prostatacancer, lungcancer, olika hudcancrar och tjocktarmscancer. Alla dessa maligna tumörer hör till en grupp som heter epiteliala cancrar (carcinom). En mer ovanlig grupp av cancrar är maligna tumörer som utgår från mjukvävnad, så som muskler, brosk och fett. Den här gruppen kallas för sarkom.

Sarkom utgör mindre än 1 % av alla maligna tumörer. Det vanligaste sarkomet heter Liposarkom och är en tumör som är rik på fettceller. Myxoid liposarkom är den vanligaste varianten av Liposarkom och kännetecknas av små fettliknande tumörceller som ligger utspridda bland bindväv som ser slemaktig ut. ”Myxoid” kommer från grekiskan och betyder just ”slem”.

De här tumörerna drabbar personer i medelåldern, tillväxer mycket långsamt och har en medelgod prognos. Femårsöverlevnaden efter diagnos ligger på i snitt 70 %, vilket kan jämföras med 90 % för bröstcancer och 6 % för bukspottskörtelcancer. Myxoida liposarkom behandlas främst med kirurgi och strålning. Mer aggressiva tumörer och tumörer som spritt sig i kroppen behandlas med cellgifter. Det finns tre cellgifter registrerade för Myxoida liposarkom, men inget av dem har särskilt god effekt.

På senare år har en ny typ av cellgift kommit ut på marknaden. Det bygger på att hämma ett protein i kroppen som heter Heat shock protein 90 (HSP90). HSP90 är ett protein som är

viktigt för att cellerna ska hantera olika former av stress. Har cellerna producerat felaktiga proteiner på grund av till exempel överhettning eller giftiga ämnen (toxiner), så hjälper HSP90 till att rätta till dem. I cancer kan dock tumörceller utnyttja det här systemet till att bibehålla signaler för celltillväxt som normalt inte ska vara aktiverade.

En typ av proteiner som heter receptortyrosinkinaser kan mediera celltillväxtstimulerande signaler. Det finns en stor andel receptortyrosinkinaser i myxoida liposarkom och de anses vara en viktig tumördrivande faktor. Som ett försök att behandla myxoida liposarkom har man försökt att blockera dessa receptorer, men med föga resultat. Många receptortyosinkinaser är reglerade av HSP90 och försök i andra tumörer än Myxoida liposarkom har visat att receptortyrosinkinaser nedregleras av HSP90-hämning.

Försök har visat att HSP90-hämning dödar tumörceller från myxoida liposarkom. Vi ville se om receptortyrosinkinaser nedreglerades även i myxoida liposarkom och om detta kan vara en orsak till att tumörcellerna dör.

Vi har analyserat effekten på tumörceller i cellodling vid tillsats av HSP90-hämmaren 17-DMAG. Vi har undersökt hur snabbt cellerna tillväxer med och utan 17-DMAG genom att odla dem i plattor som kontinuerligt registrerar cellantalet. Vi har registerat mängden av olika receptortyrosinkinaser med och utan 17-DMAG genom en metod som heter Western blot. Vi har dessutom försökt lokalisera var i cellerna receptortyrosinkinaserna är uttryckta genom en metod som heter immunofluorescens. För att se ifall 17-DMAG orsakar några konsekvenser på gen-nivå har vi framställt prover som ska skickas på en stor screening av tumörernas gener. Genom att analysera detta hoppas vi hitta olika mönster av gener som förklarar varför olika tumörceller reagerar på olika sätt vid tillsats av 17-DMAG.

Våra resultat visar att celler som har högt uttryck av receptortyrosinkinaser är extra känsliga för hämning av HSP90. Två av tre receptortyrosinkinaser som vi har analyserats nedregleras

vid tillsats av 17-DMAG. Dessutom verkar det som att man kan potentiera denna effekt genom att tillföra proteiner som aktiverar andra signaleringsvägar som regleras av HSP90, samtidigt som man tillför 17-DMAG.

Icke-tumörceller innehåller väldigt små mängder av tyrosinkinaser. Det verkar inte som att dessa celler påverkas av 17-DMAG utan fortsätter att dela sig som vanligt, vilket är bra ur en biverkningssynpunkt.

Detta indikerar på att uttryck av receptortyrosinkinaser är en nödvändig faktor för att 17-DMAG ska ha effekt. Att hämma HSP90 minskar uttryck av receptortyrosinkinaser och dödar tumörceller. Hämning av HSP90 kan vara ett sätt att blockera tillväxten i receptortyrosinkinas-beroende tumörer som inte svarar på hämmare av receptortyrosinkinaser.

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