Chapter 3

Treatment of the follicular thyroid carcinoma cell-line FTC-133 with Troglitazone and Lovastatin synergistically increases expression of G1 transition inhibitors and induces re-differentiation

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ABSTRACT

Studies have shown that thiazolidinediones e.g. troglitazone and statins e.g. lovastatin are, in addition to their primary indication, also effective inhibitors of growth and invasion of tumor cells of various origins. Recently it was demonstrated that a combination of clinically achievable concentrations of lovastatin and troglitazone can produce a dramatic synergistic effect on growth in human glioblastoma and CL1-0 human lung cancer cells lines in vitro. The exact mechanism is still unclear but it was demonstrated that p27kip1 protein was significantly elevated and the phosphorylation status of Rb was reduced. A possible mechanism for this cell cycle arrest and apoptosis by both thiazolidinediones and statins may result from PTEN upregulation.

In addition to anti-cancer effects, thiazolidinediones and statins have been shown to have redifferentiating effects in DTC. This re-differentiating effect may be highly beneficial in patients with differentiated thyroid cancer as iodine uptake can be lost in DTC metastases due to de-differentiation. Once NIS is lost, treatment becomes problematic as I-uptake via NIS is vital for successful treatment with radioactive iodine.

We decided to further explore the beneficial in vitro effects of a combination of lovastatin and troglitazone in the follicular thyroid carcinoma cell-line FTC-133 on growth and apoptosis. After exposing the cells to Troglitazone and/or Lovastatin treatments for up to 4 days we tested for cell-growth and induction of apoptosis by MTS-assay and FACS analysis. To further elucidate the mechanisms leading to cell-cycle arrest we tested the expression levels of inhibitors of CDK4/6 cyclin complex assembly (p15, p16 and p27) by RT-PCR. In addition, we also evaluated the beneficial in vitro effects of a combination of lovastatin and troglitazone on the expression of the TSH receptor and NIS genes via RT-PCR.

In our study we found that in the human thyroid follicular cell-line FTC-133, the combination of lovastatin and troglitazone resulted in a remarkable synergistic effect on morphology and cell density. These effects coincide with redifferentiation as was demonstrated by an increase in TSH-receptor and NIS expression.
INTRODUCTION

The primary clinical indication of statins is in hypercholesterolemia and the prevention of myocardial infarction, whereas that of the thiazolidinediones is in improving insulin sensitivity in type 2 diabetes mellitus patients. Studies have shown that both classes of drugs are, in addition to their primary indication, also effective inhibitors of growth and invasion of tumor cells of various origins. The anticancer activity of statins was intensively studied and in vitro studies show an effect on growth and invasion of tumor cells e.g. anaplastic thyroid cancer, melanoma, prostate cancer and pancreatic cancer (118;119;218-220). In vitro beneficial effects of thiazolidinediones have been described in a number of malignancies e.g. breast cancer, hepatocellular carcinoma, pancreatic cancer, ovarian carcinoma, melanoma, lung carcinoma, and lymphoma cells (221-226). On the molecular level statins and thiazolidinediones have different cellular targets. Statins (e.g. lovastatin) are potent inhibitors of HMG-CoA reductase by binding to HMG-CoA reductase, the rate-limiting enzyme of the mevalonate (MVA) pathway, approximately 1000-fold more effective than the natural substrate (Wong,2002;Demierre,2005) whereas thiazolidinediones (e.g. troglitazone) are peroxisome proliferator-activated receptor (PPAR) agonists. Until recently statins and thiazolidinediones were only tested separately for anticancer effects. Recently, Yao et al. found that a combination of clinically achievable concentrations of lovastatin and troglitazone can produce a dramatic synergistic effect against human glioblastoma and CL1-0 human lung cancer cells lines in vitro at low concentrations. They found a significant elevation of p27kip1 protein and a reduced phosphorylation status of Rb (120). The exact mechanism is still unclear but it has been suggested that PTEN upregulation is a possible mechanism for cell cycle arrest and apoptosis by both thiazolidinediones and statins (227;228). In addition to growth related anti-cancer effects, thiazolidinediones and statins have been shown to have redifferentiating effects in DTC. Frohlich et al. investigated the effects of troglitazone, rosiglitazone and pioglitazone on differentiation in normal porcine thyrocytes and in follicular carcinoma cell-lines FTC-133 and FTC-238. Troglitazone was most effective of the tested thiazolidinediones in re-differentiating the carcinoma cell-lines as demonstrated by significantly increased radioiodine uptake and subsequent apoptosis (229). In a clinical study it was demonstrated that rosiglitazone was able to induce uptake of radioiodine in DTC in vivo (114). In the thyroid derived cell-lines FTC-133, FTC-238 and ARO an increase in differentiation has previously been shown. Wang et al. also found a significant effect of lovastatin on differentiation of the anaplastic thyroid cancer ARO cell-line. At a dose of 25 μM, lovastatin was able to significantly increase iodine uptake (118). This re-differentiating effect, which was observed at clinically achievable concentrations of lovastatin and troglitazone, may be highly beneficial in patients with differentiated thyroid cancer as iodine uptake can be lost in DTC metastases due to de-differentiation. Once NIS is lost treatment becomes problematic as l-uptake via NIS is vital for successful treatment with radioactive iodine.

We decided to further explore the beneficial in vitro effects of a combination of lovastatin and troglitazone in the follicular thyroid carcinoma cell-line FTC-133 on growth and apoptosis and to further elucidate the mechanisms leading to cell-cycle arrest by inhibitors
of CDK4/6 cyclin complex assembly (p15, p16 and p27). In addition, we also evaluated the beneficial in vitro effects of a combination of lovastatin and troglitazone on the expression of the TSH receptor and NIS genes.

**MATERIALS & METHODS**

**CELL CULTURE**

FTC-133 cells were cultured routinely in Ham’s F12 medium (Gibco BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 100 IU/ml penicillin (Life Technologies, Rockville, USA) and 100 µg/ml streptomycin (Life Technologies, Rockville, USA) in a humidified atmosphere of 5% CO2 and 95% O2 at 37°C. Cells were trypsinized and transferred (1:3) to new medium every 3-4 days. For experiments, the cells were seeded at a density of 1x10^4/cm² and the various treatments started after 24 h.

**TREATMENTS**

Troglitazone was purchased from the Cayman Chemical Company (Tallinn, Estonia), Lovastatin was purchased from Ag Scientific (San Diego, USA) and Geranylgeranyl transferase I (GGTI) purchased from Sigma (St.Louis, USA). Troglitazone and lovastatin were added to FTC-133 cells at a concentration of respectively 10µM and 1µM and the cells were exposed to this treatment for 2 days unless stated otherwise. GGTI (25µM) was used to selectively block the geranylgeranylation of proteins whereas lovastatin blocks both geranylgeranylation and farnesylation.

**CELLTITER 96® AQUEOUS ONE SOLUTION CELL PROLIFERATION ASSAY**

The effect of troglitazone, lovastatin or the combinational treatment for up to 4 days on growth (n=6) was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, USA) which is based on the conversion of a MTS tetrazolium compound (Owen’s reagent) by cells into a colored formazan product. In brief, 20µl of CellTiter 96® AQueous One Solution Reagent was added into each well of a 96-well assay plate containing the samples in 100µl of culture medium. The plate was incubated for 2 hours at 37°C in a humidified, 5% CO2 atmosphere. Absorbance was recorded at 490nm using the SPECTRMax GEMINI Microplate Spectrofluorometer plate reader (molecular devices, Sunnyvale, CA, USA).

**FACS EXPERIMENTS**

Annexin V-FITC (Bender MedSystems, Vienna, Austria) was used to detect phosphatidylserine on the outer leaflet of the cell membrane thus measuring initiation of apoptosis. In brief, after treatment with 10µM troglitazone and/or 1µM Lovastatin for 1 or 2 days, cells
were harvested by centrifugation, washed 1 time with ice-cold PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl2) at a concentration of 1 x 10^6 cells/ml. A total of 5 μl of Annexin V-FITC and 5 μl of 20 μg/ml Propidium iodide (PI) were added to 100 μl of cell suspension and incubated for 15 min in the dark before addition of 400 μl of binding buffer. Quantitative analysis of the apoptotic percentage of 10,000 cells was performed using the FACScan Analyzer (Becton Dickinson, Franklin Lakes, USA). Cell debris and PI positive cells were excluded by gating the cells and the shift in the percentage of Annexin positive cells was taken as a measure of apoptosis.

WESTERNBLOT

Whole cell extracts were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (0.45 μm) (Pierce, Rockford, IL, USA) all subsequent steps were performed in the presence of a commercial protease inhibitor cocktail in the recommended dose (Roche, Basel Switzerland). After 20 minutes of blocking at room temperature the membrane was incubated with the primary antibody GAPDH (sc-25778) (Santa Cruz Biotechnology, Inc, Santa Cruz, USA) at 4C O/N followed by a 1h incubation with horseradish peroxidase conjugated secondary antibody. The immune complexes were visualized using the Molecular Imager ChemiDoc XRS System (Biorad, Hercules, CA, USA). Following equalization of the amounts of protein by comparing using GAPDH expression, GAPDH, Rb and p-Rb expression was visualized using the primary antibodies Rb(4H1) mAb and phosho-Rb(Ser807/811) (cell signaling Technology, Danvers, USA) as described before.

cDNA

Total RNA was extracted by using Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) and RNA concentrations were determined by measuring the absorbance at 260 nm. Genomic DNA was eliminated and RNA was reversed transcribed using the RT2 first strand kit (Superarray bioscience corporation, Frederick, USA)

RT-PCR

Gene expression was examined using the RT2 system from superarray according to the manufacturers guidelines (Superarray bioscience corporation, Frederick, USA). In short, a 25μl mix was made by mixing 1μl primerset (NIS (SLC5A5), TSHr (TSHR) or P27 (CDKN1B)) with 11.5 μl H2O containing 25ng cDNA and 12.5μl RT2 Real-time sybr green/Fluorescein PCR Master MIX (Superarray bioscience corporation, Frederick, USA). All RT-PCR’s were performed in triplicate using a two-step cycling program (1 cycle 10’95C followed by 40 cycles 15”95C; 1’60C) on the BioRad iCycler (Biorad, Hercules, CA, USA) Results were expressed as fold induction compared to untreated cells:

gfold induction = 2^((control-beta)-(gene-beta))

Gene = Ct gene of interest in treated group
Beta = Ct beta-actin  
Control = Ct gene of interest in untreated group

STATISTICAL ANALYSES

Results are expressed as the mean plus or minus the standard error of mean. Student’s t-tests were used for all hypotheses testing. All statistical analysis was performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL). A p value of <0.05 was considered significant.

RESULTS

GROWTH

The follicular thyroid carcinoma cell-line, FTC-133 was exposed to Troglitazone and/or Lovastatin treatments for 2 days resulting in a remarkable synergistic effect on morphology and cell density when treatments were combined (Figure 1). Cells which received only one of the treatments appear normal whereas the troglitazone/lovastatin combination resulted in decreased growth and rounding up of cells. Similar to the troglitazone/lovastatin combination the geranylgeranylation blocker GGTL in combination with 10M troglitazone also resulted in decreased growth and rounding up of the cells whereas GGTL alone did not (Figure 1). Initially only the combination of troglitazone and lovastatin inhibited cell growth (89%, P=0.0019) whereas longer exposure times also resulted in impaired cell growth for the individual treatments (Figure 2). At day 2 the troglitazone and lovastatin treatment alone had no effect on growth while the combination resulted in a lower amount of viable cells (89% of the untreated cells). A 4 day exposure resulted in impaired growth of the cells which received treatment of troglitazone or lovastatin (90% troglitazone, P=0.0432; 68% lovastatin, P=0.0009) while the combination gave an additional effect (46%, P<0.0001) (Figure 2). After transfer to normal medium without lovastatin and troglitazone most cells were still viable and resumed normal growth and morphology (data not shown).

FACS ANALYSES

In order to test if impaired cell growth and detachment of the cells was due to apoptosis we performed a FACS analysis using ANNEXIN V which binds to the apoptotic marker phosphatidylserine (PS). None of the treatments resulted in increased binding of Annexin V to the cell surface of PI negative cells which received treatment for 1 or 2 days (Figure 3).

WESTERN-BLOT

As the phosphorylation state of the Rb protein plays a pivotal role in the negative regulation of the cell cycle we performed a western blot with antibodies specific for the state of phosphorylation (Figure 4). These data confirmed that the combination of troglitazone and
Treatment of the follicular thyroid carcinoma cell-line FTC-133 with Troglitazone and Lovastatin synergistically increases expression of G1 transition inhibitors and induces re-differentiation.

**FIGURE 1.** Effect of Troglitazone, Lovastatin and GGTI voluit treatments on FTC-133 cells. Cells were exposed to 10 μM Troglitazone, 1μM lovastatin, 25 μM GGTI or a combination for 2 days. The combination of troglitazone and lovastatin resulted in a remarkable effect on morphology and cell density, the cells appear to be less dense and rounded up (50x).

**FIGURE 2.** The effect of troglitazone, lovastatin or the combinational treatment for up to 4 days on growth was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay(Promega, Madison,USA) as described in materials and methods. Viable FTC-133 cells are depicted as percentage of untreated cells. After 2 days of treatment the troglitazone-lovastatin combination resulted in reduced growth vs. control of 89% (P=0.0019) Individual treatments with either troglitazone or lovastatin also resulted in impaired cell growth from day 3. Measurements were performed at least in triplicate. (troglitazone, 90%, P=0.0432; lovastatin, 68%, P=0.0009; T+L, 46%, P<0.0001)
Treatment of the follicular thyroid carcinoma cell-line FTC-133 with Troglitazone and Lovastatin synergistically increases expression of G1 transition inhibitors and induces re-differentiation.

FIGURE 3. FACS analyses for detection of apoptosis using the apoptosis marker phosphatidylserine as described in materials and methods. ANNEXIN V-FITC binding is depicted on the horizontal axis and PI on the vertical axis. None of the treatments resulted in an increased binding of Annexin V to the cell surface. There was a tendency for increased binding of annexin V after the combination of treatments for two days but this was not significant (P=0.06). Evaluation of the cell populations which were positive for AnnexinV/ and negative for PI staining are depicted below the histograms.

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Troglitazone</th>
<th>Lovastatin</th>
<th>Combination</th>
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<td>1 day treatment</td>
<td>6.81</td>
<td>7.6</td>
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<td></td>
<td>(p=0.63)</td>
<td>(p=0.60)</td>
<td>(p=0.53)</td>
<td>(p=0.056)</td>
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<tr>
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<td></td>
<td>(p=0.85)</td>
<td>(p=0.38)</td>
<td>(p=0.056)</td>
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Treatment of the follicular thyroid carcinoma cell-line FTC-133 with Troglitazone and Lovastatin synergistically increases expression of G1 transition inhibitors and induces redifferentiation.

**TABLE 1.** FTC-133 cells were treated with troglitazone (10 μM) and/orLovastatin (1μM) for 2 days. Fold-in- or decrease of P15, P16,P27, NIS and TSHr mRNA expression was compared to untreated cells (control) using RT-PCR as described in materials and methods. Fold in- or decrease was calculated using the formula: fold induction = $2^{((\text{control-beta})-(\text{gene-beta}))}$.

<table>
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<tr>
<th>Treatments</th>
<th>P15 fold difference</th>
<th>P15 p</th>
<th>P16 fold difference</th>
<th>P16 p</th>
<th>P27 fold difference</th>
<th>P27 p</th>
<th>TSHR fold difference</th>
<th>TSHR p</th>
<th>NIS fold difference</th>
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<td>3,16</td>
<td>0,02</td>
<td>10,1</td>
<td>0,005</td>
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**FIGURE 4.** Synergistic effects of Lovastatin and troglitazone on the phosphorylation status of Rb. Cells were exposed to 10 μM Troglitazone, 1μM Lovastatin or the combination for 2 days and cell lysates were analysed by western blot as described in materials and methods.
lovastatin induced a downregulation of phosphorylated Rb.

RT-PCR

In order to explore the mechanisms behind the negative effect on cell-cycle progression by troglitazone, lovastatin and the combination we tested the expression of P15, P16 and P27 which are known to play a role in cell-cycle progression using RT-PCR (Table 1). In addition, we studied the effects of troglitazone and/or lovastatin on cellular differentiation by a RT-PCR on the thyroid specific genes NIS and the TSH receptor. Treatment with troglitazone gave no rise in TSHr expression but induced NIS expression almost 3-fold (P=0.038). Lovastatin induced TSHr expression more than 5-fold (P<0.0001) while NIS was induced 12-fold (P=0.0005). When combined troglitazone and lovastatin treatments induced TSHr expression 3-fold (P=0.02) and NIS 10-fold (P=0.005).

DISCUSSION

The combination of a Troglitazone and/or Lovastatin treatment resulted in a remarkable synergistic effect on morphology and cell density in the human follicular thyroid carcinoma cell-line, FTC-133. An effect which was previously reported by Yao et al. in human glioblastoma and CL1-0 human lung cancer cells lines in vitro at similar low concentrations (120). They contributed the effects in part to the inhibition of the mevanolate pathway and tested this by counteracting the effects of the combined therapy with the addition of mevalonolactone (120). Blocking the mevanolate pathway will result in both inhibition of farnesylation and geranylgeranylation. Specific blockers can block either farnesylation (Farnesyl transferase inhibitor, FTI) which activates RAS proteins or geranylgeranylation (GGTI) which activates Rho proteins (230).

We could mimic the effect on cell growth and morphology of the troglitazone/lovastatin combination by the combination of the geranylgeranylation blocker GGTI with 10M troglitazone indicating that inhibition of geranylgeranylation in combination with troglitazone treatment is sufficient to induce the effects observed on growth and morphology. This points to a Rho related mechanism rather then Ras as GGTI inhibits geranylgeranylation of Rho (230).

In order to determine if the impaired cell growth and detachment of the cells was due to apoptosis or only to G1 cell arrest which was demonstrated by the phosphorylation state of Rb we performed a FACS analysis using ANNEXIN V. None of the treatments resulted in increased binding of ANNEXIN V to the cell surface. Additionally, most cells were still viable and resumed normal growth and morphology after transfer to normal medium showing that the cells appear to arrest rather than move into apoptosis after receiving the Troglitazone/Lovastatin combination treatment. Higher doses of lovastatin do appear to cause apoptosis as Wang et al. observed apoptosis in ARO-cells with a lovastatin dose of 50 μM (118).

One possible explanation for the observed growth inhibition may lay in Rho related inhibition via p27 an inhibitor of CDK4/6 cyclinD complex assembly. Geranylgeranylation of Rho
is essential for degradation of this inhibitor and facilitates progression of G1 to S phase (231). To initiate this degradation, Rho needs to be activated by geranylgeranylated during the G1 phase a process blocked by lovastatin and GGTI (232;233). Geranylgeranylation enables RhoA to be positioned at the inner face of the plasma membrane where it serves as a switch in cytoplasmic cascades by switching between an active (GTP) and inactive state (GDP)(232;233). Troglitazone also appears to have an effect on several cell cycle regulators including an increase of p21 and p27 levels and reduction in phospho-Rb in several cell lines such as the mRNA and protein level in rat and human hepatoma cells. Furthermore, forced expression of p27 results in G1 phase cell-cycle arrest in most cell-lines (234). On the protein level Yao et al. (120) observed this effect on p27 when using the combination treatment. In addition to the known effects on degradation of p27 via Rho we observed 12 fold increase in p16 expression and an almost 10 fold increase of P15 expression when the troglitazone and lovastatin treatment were combined.
P15INK4b and P16INK4a are members of INK4b-ARF-INK4 a tumor suppressor locus. An excess of these inhibitors can cause G1 cell-cycle arrest by blocking the assembly of the catalytical active CDK4/6 cyclinD complex which facilitates Rb phosphorylation (235). P15 and p16 are more primarily associated with growth arrest whereas p21 and p27 are more associated with apoptosis (236). This seems to correspond with our findings that the FTC-133 cells only experience growth arrest and no apoptosis after treatment. So an accumulation of these CDK inhibitors is likely to result in G1 phase cell-cycle arrest rather than the induction of apoptosis. The effects on p15 and p16 give at least a partial explanation for the inhibitory effects of the troglitazone/lovastatin treatment but multiple pathways may be involved.

Upregulation of PTEN expression via ppar-gamma has been suggested as a possible mechanism for cell cycle arrest and apoptosis by both glitazones and statins (227;228). Troglitazone is a well known PPAR-gamma agonist and statins have also been shown to activate PPAR gamma in a dose dependent manner by inhibiting the mevanolate pathway. Using double negative constructs Yano et al. determined that activation of ppar-gamma by statins seems to be regulated via RhoA rather than Ras or Rac (237). The hypothesis that PTEN expression could be involved in the induction of growth arrest via PPAR-gamma is supported by the presence of two PPAR-gamma response elements in the genomic sequence upstream of PTEN (227). Furthermore Teresi et al. showed that both lovastatin and the glitazone, rosiglitazone are able to increase PTEN expression through PPAR-gamma in a dose dependant manner. After 2 days a dose of 1-10μM of lovastatin proved to be most effective in stimulation PTEN expression. In our experiments we used the thyroid cancer cell-line FTC-133 which harbours a splice variant resulting in a PTEN negative phenotype (238). As we saw identical effects on growth compared to the effects described by Yao et al. we conclude that PTEN upregulation is not essential for the induction of growth arrest in cell-lines treated with troglitazone and lovastatin. However, PTEN expression may play a role in the induction of apoptosis after troglitazone and lovastatin treatment in cells which are capable of PTEN expression.

Besides reduced growth and invasion of tumors, both lovastatin and troglitazone have previously been shown to promote cellular differentiation in thyroid derived cell-lines(FTC-133, FTC-238 and ARO), a feature which may be beneficial in improving conventional RAI
therapy which is based on I131-uptake by NIS (118;121).

Using RT-PCR we observed an increase in NIS and TSHr expression when we combine d the lovastatin and troglitazone treatments. This redifferentiating effect can be largely account- ed for by the lovastatin treatment although the troglitazone treatment alone was able to upregulate NIS expression.

The effects of the combined troglitazone/lovastatin treatment we observed seems to be universal for cancer cell-lines as Yao et al. discovered similar effects in human glioblas-toma, lung-, prostate-, pancreatic- and cervical cancer cells lines (120). The effects on the CDK inhibitors give at least a partial explanation for the inhibitory effects of the troglita-zone/lovastatin treatment but multiple pathways may be involved. Although the synergism of troglitazone and lovastatin is dramatic in vitro its usefulness will still have to be proven in vivo. However, there is hope that the combination of troglitazone and lovastatin can induce the effects on growth and differentiation status in vivo, because the synergistic effects were found at clinically achievable concentrations in the human follicular thyroid carcinoma cell-line, FTC-133 (116;239;240). Therefore we think that a combined Troglita-zone/Lovastatin treatment may proof to be beneficial in patients with DTC as remarkable reduction of growth coincides with increased NIS expression.