Dual effects for lovastatin in anaplastic thyroid cancer: the pivotal effect of transketolase (TKT) on lovastatin and tumor proliferation

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Accepted 31 January 2018

To cite: Wang C-Y, Shui H-A, Chang T-C. *J Investig Med* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jim-2017-000634

ABSTRACT

This study tested the hypothesis that the effects of lovastatin on anaplastic thyroid cancer cell growth are mediated by upregulation of transketolase (TKT) expression. The effects of lovastatin on TKT protein levels in ARO cells were determined using western blot and proteomic analyses. After treatment with lovastatin and oxythiamine, the in vitro and in vivo growth of ARO cells was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assays and tumor xenografts in nude mice. TKT protein expression in the ARO tumors was assessed using immunohistochemistry analysis. Proteomic analysis revealed that 25 µM lovastatin upregulated TKT expression. Co-treatment of ARO cells with 1 µM lovastatin + 1 µM oxythiamine increased TKT protein expression compared with control levels; however, no differences were observed with 10 µM lovastatin + 1 µM oxythiamine. Furthermore, treatment with either oxythiamine or lovastatin alone reduced ARO tumor expression of TKT, as well as decreased ARO cell proliferation in vitro and tumor growth in vivo. However, mice treated with both lovastatin and oxythiamine at the same time had tumor volumes similar to that of the untreated control group. We conclude that either lovastatin or oxythiamine reduced ARO cell growth; however, the combination of these drugs resulted in antagonism of ARO tumor growth.

Significance of this study

What is already known about this subject?

- Oxythiamine, an irreversible inhibitor of transketolase (TKT), reduces tumor cell proliferation and enhances apoptosis in many in vitro and in vivo cancer models.
- Several studies have also reported the effects of lipid-lowering statins against tumor cells.
- Lovastatin, another lipid-lowering drug, showed dual effects, inducing neoplastic proliferation at low doses, but antineoplastic effects at high doses.
- In ARO cells, high concentrations (50 µM) of lovastatin induced apoptosis; however, redifferentiation was noted in cells treated with lower concentrations (25 µM).

What are the new findings?

- Lovastatin alone or with oxythiamine upregulated TKT expression in ARO cells.
- Treatment with either oxythiamine or lovastatin alone decreased ARO cell proliferation in vitro.
- Although treatment with either oxythiamine or lovastatin alone reduced tumor growth in vivo, mice co-treated with both lovastatin and oxythiamine had tumor volumes similar to that of the untreated control group.

How might these results change the focus of research or clinical practice?

- A combination of low-dose lovastatin and oxythiamine did not enhance the effects of either drug alone.
- High-dose lovastatin and oxythiamine combinations may be an important and reliable adjuvant therapy for anaplastic thyroid cancer.
- Further studies are necessary to determine the pathway of antagonism as well as the mechanism by which lovastatin influences TKT expression.

INTRODUCTION

Thyroid cancer is an important endocrine tumor with increasing incidence worldwide.[1] Depending on the histological definition, thyroid cancer can be categorized as well-differentiated cancer, poorly differentiated thyroid cancer (PDTC) or anaplastic cancer (ATC)[2]; differentiated thyroid cancer (DTC) is assumed to progress to PDTC and finally to ATC through accumulated gene mutation.[3] About 1.3–9.8 per cent of patients with thyroid cancer may progress to ATC with a poor response to the current therapies and a median survival of only 5 months.[4] ATC is a fatal malignancy and usually progresses rapidly with distant metastasis.[5]

Response to treatment is often correlated with the differentiation status of thyroid cancer[2] as DTC has a relatively better response than PDTC or ATC. Thus, several therapeutic approaches, termed redifferentiation therapy, have focused on the reversion of PDTC or ATC

to DTC.[2 6–8] In addition to retinoic acid, thiazolidinedione, histone deacetylase inhibitors 6 and lovastatin could induce ATC redifferentiation in vitro.[9] Our lab first reported cytomorphological evidence of redifferentiation of ATC cells after in vitro exposure to tumor necrosis factor-alpha (TNF-α).[10] We also demonstrated biochemical evidence of differentiation from ARO cell culture medium after treatment of TNF-α.[10] We also showed that 50 μM lovastatin, a lipid-lowering drug, induced apoptosis in ARO cells treated with higher concentrations; however, lower concentrations (25 μM) induced redifferentiation.

Statins are potent inhibitors of hydroxy-3-methyl-CoA reductase and lower blood lipid levels. However, several studies have reported their effects against tumor cells.[11–13] We previously showed that although 5 and 10 mg/kg/day lovastatin inhibited in vivo ATC growth, increased tumor growth was observing in mice treated with 1 mg/kg/day lovastatin relative to the untreated control group.[14] Thus, lovastatin may have 'dual effects', such that low doses increase neoplastic proliferation but high doses have an antineoplastic effect. In addition, our proteomic analysis revealed that upregulation of FLOT1 was pivotal in the redifferentiation of ATC cells.[15] Vascular endothelial growth factor (VEGF) may also play a role in governing the dual effects of lovastatin on tumor proliferation.[15]

In addition to FLOT1 and VEGF, we repeatedly observed increasing transketolase (TKT) protein expression in ATC cells treated with lovastatin. Transketolase is vital in the pentose phosphate pathway (PPP), inducing glucose degradation that is important for tumor survival and proliferation.[16] In various tumor types, TKT or transketolase-like 1 (TKTL1) expression appear to be valid biomarkers for tumor prognosis.[17–19] Furthermore, low-dose lovastatin (1 mg/kg/day) could increase tumor growth in nude mouse model.[14] Thus, we speculate that low-dose lovastatin can increase ATC growth via increasing TKT but not TKTL1 expression.

Our study aimed to investigate whether the effects of low-dose lovastatin on tumor proliferation were mediated via upregulation of TKT or TKTL1 expression using in vitro and in vivo studies. We examined the role of TKT in ATC cells using oxythiamine, an irreversible inhibitor of TKT, which reduces tumor cell proliferation and enhances apoptosis in in vitro and in vivo cancer models.[20 21] However, Frohlich *et al*[22] reported that oxythiamine showed only a weak effect on TKTL1-expressing thyroid carcinomas. Analysis of the mechanism by which lovastatin impacts tumor cell growth at various doses may be clinically relevant in the treatment of thyroid carcinoma.

MATERIALS AND METHODS

Cell culture

The ATC cell line, ARO cells were kindly provided by Dr Chen, S.D., Chang Gung Memorial Hospital, Taoyuan, Taiwan, and were maintained in RPMI 1640 medium supplemented with 10 per cent fetal calf serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 0.1 μg/mL of streptomycin (Life Technologies, Taiwan) at 37°C in a humidified atmosphere of 5 per cent CO_2.

The follicular thyroid cancer cell line, SW579 cells (Bioresource Collection and Research Center, Taiwan), were maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10 per cent fetal calf serum, 2 mM L-glutamine, 100 units/mL of penicillin, and 0.1 μg/mL of streptomycin at 37□ in a humidified atmosphere of 5 per cent CO_2.

Cell proliferation analysis

Cultured cells were seeded at a concentration of 4×10^3 cells/well in 100 μL of culture medium containing various amounts of lovastatin or oxythiamine into tissue culture grade 96-well microplates (flat bottom) for 48 hours at 37°C and 5 per cent CO_2. The cell proliferation reagent, WST-1 (10 μL/well; Roche, Germany), was added for 1 min on a shaker, then incubated for 4 hours at 37°C and 5 per cent CO_2. The absorbance of the samples against a background control as blank was measured using a microplate (ELISA) reader. The wavelength for measuring the absorbance of the formazan product was between 420 and 480 nm (max. absorption at about 440 nm) according to the filters available for the ELISA reader.

Western blot analysis

Protein samples (50 μg) were isolated from control and experimental ARO cells and were separated using 10 per cent SDS-PAGE. After the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bio-Sciences, Piscataway Township, New Jersey USA), the membrane was blocked with blocking buffer consisting of Tris–buffered saline, pH 8.0, containing 0.05 per cent Tween-20 (TBST) and 5 per cent skim milk at room temperature for 1 hour. After incubating the membranes with rabbit anti-caspase 3 (Upstate, Millipore, Temecula, California, USA), rabbit anti-FLOT1 (Upstate), rabbit anti-ANXA5 (Life Span Biosciences, Seattle, Washington, USA), rabbit anti-TKT (H50; Santa Cruz Biotechnology, Santa Cruz, California, USA), or mouse anti-:Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Upstate) primary antibodies at room temperature for 1 hour, they were incubated with either goat antirabbit or rabbit antimouse secondary antibodies for 1 hour at room temperature. The bands were visualized via an ECL detection kit, after which quantification of protein intensity was performed by densitometry. The intensities of the target bands were normalized by the intensity of the internal control, GAPDH.

Proteomic investigation of ARO cells treated with lovastatin

For the proteomic analysis of ARO cells, 2×10^5 cells/mL were cultured in 10 cm cell culture plates for 24 hours after which they were treated with 25 μM lovastatin (MERCK, Taipei, Taiwan); untreated cells served as the negative control. After 48 hours, the cells were washed twice in phosphate buffered saline (PBS), lysed in lysis buffer (2 M thiourea, 8 M urea, 10 mM Tris-HCl, pH 8.5), and homogenized using a 25G needle six times. Insoluble material was removed by centrifugation (14,000 rpm for 20 min at 10°C). Protein concentration was determined using the Sypro-ruby protein assay reagent (Invitrogen, Carlsbad, California, USA) and a Typhoon Trio laser scanner (GE Healthcare Bio-Sciences).

2D gel electrophoresis and gel imaging

Immobilized non-linear pH gradient (IPG) strips (pH 3–10) were rehydrated overnight in the presence of the samples in the dark at room temperature. Isoelectric focusing was carried out using a Multiphor apparatus for 80 kV-h at 20°C. After the IPG strips were incubated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 per cent glycerol, and 1 per cent SDS containing 65 mM dithiothreitol for 15 min followed by another incubation in the same buffer containing 240 mM iodoacetamide for 15 min, they were placed onto 12 per cent uniform polyacrylamide gels poured between low fluorescence glass plates. The strips were overlaid with 0.5 per cent low melting point agarose in running buffer.

Gels were fixed in 30 per cent methanol and 7.5 per cent acetic acid overnight. After washing in water, total protein was stained at room temperature using SYPRO Ruby fluorescent for 3 hours. Following two washes in water, spots were visualized with a 2D-Imager with Image Master 2D platinum software (GE Healthcare Bio-Sciences) for spot detection, gel matching, and spot quantification. All gels were also stained with silver. Images were analyzed using Melanie III (Swiss Institute of Bioinformatics, Geneva, Switzerland). Differences between the treated and untreated samples were identified by directly overlaying the images with Adobe Photoshop (Adobe Systems Incorporated).

Protein identification by MALDI-TOF

Image Master 2D platinum software (GE Healthcare Bio-Sciences) was used for spot detection and spot quantification as previously described.[15] Spots with significant differences between the control and experimental groups in percentage spot volume underwent in-gel digestion followed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis and subsequent identification.

Spots were washed twice in 25 mM ammonium bicarbonate in 50 per cent acetonitrile followed by drying for 10 min. After reducing the samples in 10 mM dithiothreitol and 25 mM AmBic for 45 min at 50°C and alkylating in 50 mM iodoacetic acid for 1 hour at room temperature in the dark, the spots were washed in 50 per cent acetonitrile two times and vacuum-dried. After the proteins were proteolyzed with 30 ng of modified trypsin (Life Technologies) overnight at 37°C, the peptides were isolated using 5 per cent trifluoroacetic acid and 50 per cent acetonitrile, vacuum-dried, and analyzed by MALDI-TOF MS (Autoflex II, Bruker Daltonics, Bremen, Germany) following suspension in water. All mass spectra were internally calibrated with trypsin autolysis peaks.

For peptide mass fingerprinting, each mass spectrum was obtained from signals generated from at least 500 laser shots. The mass spectra were processed without smoothing using Flexanalysis 2.2 software (Bruker Daltonics) and mass (monoisotopic mass) lists were obtained by Biotools V.3.0 software (Bruker Daltonics). The UniProt database (http://www.pir.uniprot.org) was searched using the MS-Fit database searching engine (http://prospector.ucsf.edu/mshome.htm) with a mass tolerance set at 100 ppm and one missed tryptic cleavage permitted.

Tumor xenografts

The mice were maintained in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. All experimental mice received appropriate anesthetics and cared to minimize pain and discomfort during all procedures. The Committee on Animal Care of the Far Eastern Memorial Hospital approved the protocol. The health of the animals was properly monitored during the experimental period.

Twenty-four 7-week-old male nude mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). After 1-week acclimation, all mice were implanted with 1×106 ARO cells suspended in PBS by direct subcutaneous injection (0.25 mL, 23G needle) at a single dorsal site (day 1).

Animals were randomly assigned to the following treatment groups: control, lovastatin, oxythiamine, and lovastatin together with oxythiamine. Nude mice were treated with lovastatin (1 mg/kg/day) or oxythiamine (80 mg/kg/day) or both diluted in ddH_2O through oral gavage as previously described.[23] The dose of oxythiamine was selected based on a previously described protocol by Zhao *et al*.[24] Mice received treatments from day 18 to 30. Mice in the control group received oral gavages of ddH_2O.

The bodyweights of the mice were measured every three days from day 7; the tumor size from day 18 after inoculation was measured every three days. At day 30, the mice were anesthetized by using inhaled diethyl ether after which the tumors were harvested. Tumor volumes were measured using the following equation: L x S2/2 (L, largest dimension of the tumor, S, shortest dimension of the tumor, detected by electronic vernier caliper in two dimensions).

Immunohistochemistry (IHC) analysis

Tumor tissues were cut into 5 µm sections, placed on slides, and fixed with 4 per cent paraformaldehyde for IHC of TKTL1 expression. Briefly, the slides were hydrated by rinsing them in decreasing concentrations of ethanol. For epitope retrieval, the tissues were heated to 65°C in 10 mM sodium citrate buffer (pH 6.0) for 5 min. After rinsing in distilled H_2O, endogenous peroxidase was inhibited with one 10 min incubation in 3 per cent H_2O_2. Slides were washed with PBS, and non-specific antibody binding was inhibited by incubating the tissue sections in 3 per cent bovine serum albumin in PBS for 15 min. The tissue sections were next incubated with a mouse monoclonal anti-TKTL1 antibody (clone JFC12T10; Zytomed Systems, Berlin, Germany)[25] at a concentration of 4 µg/mL for 60 min in a humidified chamber at room temperature. After the slides were washed with PBS, the tissues were incubated with biotinylated anti-mouse IgG (Biotinylated Link, LSAB+-kit, Dako Cytomation, Hamburg, Germany) for 25 min, washed with PBS, and incubated with streptavidin-peroxidase (Streptavidin-HRP, LSAB+-kit, Dako Cytomation) for 25 min. The tissue sections were incubated with 3-3′-diaminobenzidine (DAB with Chromogen, Dako Cytomation) for 20 min at room temperature as previously described.[17] Stained tissues were mounted either without nuclear counterstaining or

were counterstained with the nuclear counterstain, haematoxylin, for better visualization of the tissue morphology. Pathological visual scoring of the immunohistochemistry results were performed as previously described.[26]

Statistical analysis

Data were represented as mean±SD. Differences among and within groups (ie, among time points or various doses of lovastatin and oxythiamine) were compared using one-way analysis of variance with post-hoc Bonferroni pairwise comparisons. Independent two-samples t-tests were employed to compare the percentage spot volume (per cent Vol) of proteins isolated from control and lovastatin-treated cells. For repeated measurements of tumor volume in nude mice in the four treatment groups (control, lovastatin, oxythiamine, and lovastatin together with oxythiamine), a linear mixed model analysis was performed to compare the difference between treatment groups. All statistical assessments were two-tailed, and P-values<0.05 were considered significantly different. Statistical analyses were performed using SPSS V.15.0 statistics software.

RESULTS

Proteomic changes in ARO cells treated with 25 µM lovastatin

Spot analysis identified differential protein expression in the lovastatin treatment group compared with untreated controls. The proteomic studies were carried out in duplicate. Subsequent MALDI-TOF analysis revealed that annexin II, keratin 8, keratin 19, transcription elongation factor A(SII)-like 4, and TKT levels were significantly increased, and the expression of heat shock 70 kDa protein (heat shock protein 70) and ubiquinol-cytochrome c reductase complex core protein I was significantly decreased with lovastatin (figure 1).

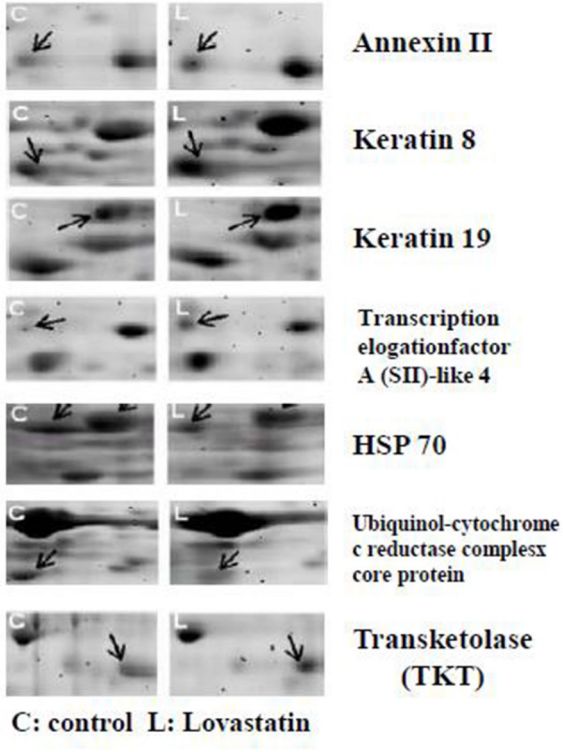
Lovastatin increases TKT expression

Low-dose lovastatin upregulated TKT expression in ARO cells (figure 1). Similarly, treatment with both 1 µM lovastatin and 1 µM oxythiamine for 48 hours upregulated TKT expression (figure 2). However, with 10 µM lovastatin and 1 µM oxythiamine, TKT expression was similar to that of untreated cells.

We next analyzed the effects of increasing lovastatin treatment at 3, 6, and 9 hours. As shown in figure 3, no significant differences were observed between the four doses of lovastatin at each time point. When the lovastatin concentration was fixed at 5 µM, TKT expression was significantly decreased after 6 hours compared with the 3 (P=0.007) and 9 hour (P=0.045) time points (figure 3). No significant difference between treatment time points was observed when the lovastatin concentration was fixed at 1, 8, and 10 µM.

Either lovastatin or oxythiamine inhibit ATC cell proliferation

The effects of various concentrations of lovastatin and oxythiamine on ARO cell proliferation were determined using an 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay after 1, 3, and 5 days. As



Protein Name	Expression
Annexin II	Increase ↑
Keratin 8	Increase ↑
Keratin 19	Increase ↑
Transcription elogationfactor A (SII)-like 4	Increase ↑
HSP 70	Decrease ↓
Ubiquinol-cytochrome c reductase complesx core protein	Decrease ↓
Transketolase (TKT)	Increase ↑

Figure 1 Proteomic analysis of differential protein levels in response to lovastatin. ARO cells were treated with and without 25 µM lovastatin for 48 hours followed by 2D-gel separation and matrix-assisted laser desorption/ionization time of flight analysis (n=5 per group).

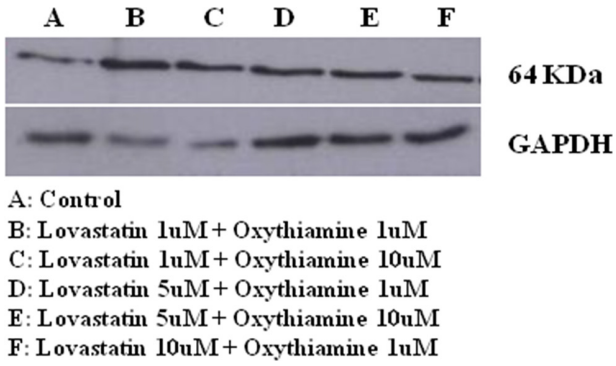


Figure 2 Transketolase (TKT) expression in response to varying concentrations of lovastatin and oxythiamine. ARO cells were treated with the indicated concentrations of lovastatin and oxythiamine for 48 hours. TKT expression was determined by western blot analysis.

shown in figure 4A a dose-dependent decrease in cell number was observed with lovastatin after 1, 3, and 5 days. In addition, the cell number was also decreased with 10 µM oxythiamine on day 3 and 25 µM oxythiamine on day 5 (figure 4B). Although low-dose lovastatin (1 mg/kg/day) promoted tumor cell growth in vivo, these results suggest that either lovastatin or oxythiamine inhibit ARO cell proliferation in vitro.

Figure 5 shows that ARO and SW579 cell proliferation decreased with increasing oxythiamine dose. Specifically, cells treated with 2 and 3 µM oxythiamine had significantly lower cell proliferation compared with the untreated control group and those treated with 0.5 and 1 µM oxythiamine ($P<0.001$ for ARO, $P\leq0.003$ for SW579). ARO cell proliferation was significantly lower with 3 µM oxythiamine compared with cells treated with 2 µM oxythiamine ($P=0.001$).

Oxythiamine-induced tumor growth inhibition was eliminated by lovastatin

In mice treated with oxythiamine (80 mg/kg/day) alone, the tumor volumes were lower than those of the untreated control group ($P<0.001$; figure 6). Although tumor volumes in the lovastatin group were lower than the controls, they did not reach statistical significance. The bodyweights of the mice were also similar (table 1). Furthermore, mice co-treated with both lovastatin and oxythiamine had significantly larger tumor volumes compared with the mice treated with oxythiamine alone ($P=0.004$). No significant difference in tumor volume was observed between the mice treated with both lovastatin and oxythiamine compared with the untreated control mice (figure 6). These results suggest an antagonism between the drugs.

Effects of lovastatin and oxythiamine on in vivo TKTL1 expression in tumors

The in vivo tumor expression of TKTL1 was next analyzed by immunohistochemistry. As shown in figure 7, TKTL1 expression was reduced in mice treated with oxythiamine alone (figure 7C) and further decreased with co-treatment with lovastatin and oxythiamine (figure 7D). However, TKTL1 expression was not correlated with tumor size as the biggest tumor in this study (1966 mm^3) only expressed moderate TKTL1 levels, and a medium-sized tumor of 770 mm^3 expressed the highest level of TKTL1 (figure 7B).

DISCUSSION

In vitro studies indicate that lovastatin can induce apoptosis and differentiation in ARO cells.[9] Additionally, dual effects of lovastatin on ARO tumor growth have been noted in vivo.[14] The present study sought to determine if the effects of lovastatin on ATC growth are mediated by TKT. Using in vitro proteomic analysis performed in duplicate, we found that TKT is upregulated after treatment of lovastatin (25 µM) for 24 hours, and the TKT inhibitor, oxythiamine, suppressed both the growth of ARO and SW579 cells. The

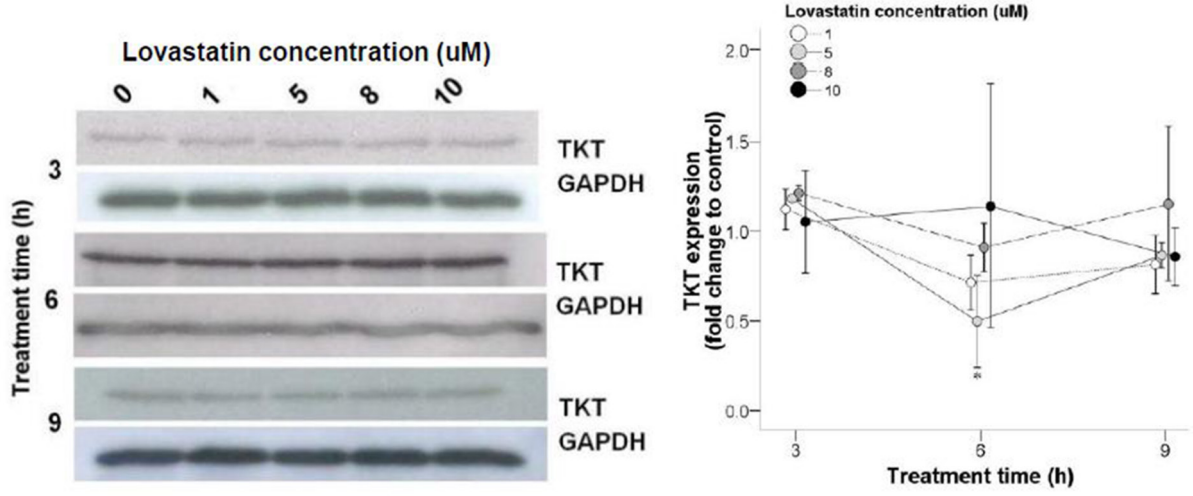


Figure 3 Concentration-dependent changes in transketolase (TKT) expression induced by lovastatin over time by ARO cells. ARO cells were treated with the indicated concentrations of lovastatin for 48 hours. TKT expression was determined by western blot analysis. Data were represented as mean and SD. *A significant difference from the 3 and 9 hour time points.

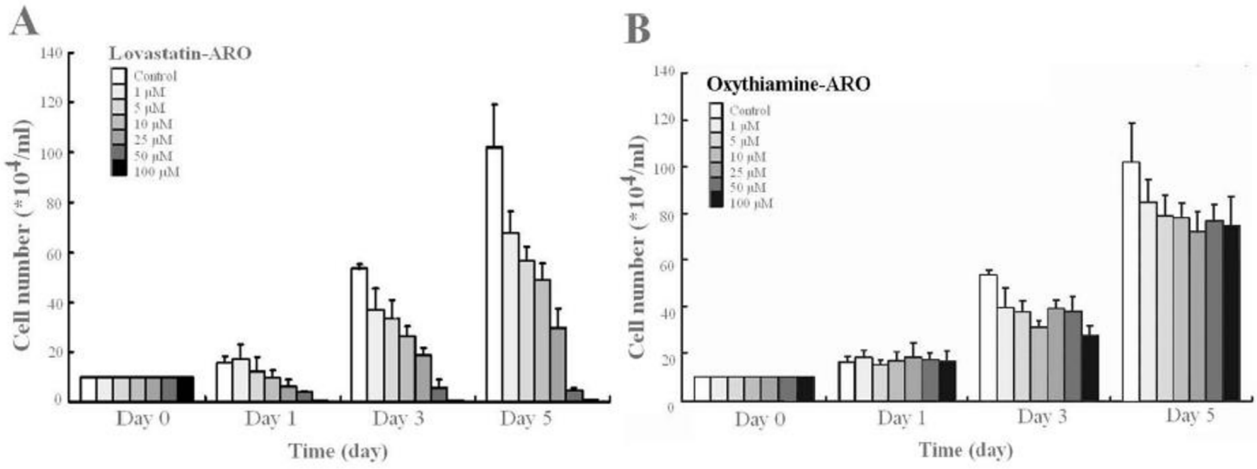


Figure 4 Both lovastatin and oxythiamine inhibit ARO cell proliferation. ARO cells were treated with the indicated concentrations of lovastatin or oxythiamine. Cell proliferation was determined after 1, 3, and 5 days using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

present in vivo studies also show that oxythiamine inhibited ARO tumor growth; however, these effects were ameliorated by lovastatin. The combination of low-dose lovastatin and oxythiamine resulted in antagonism that affected ARO tumor growth.

We previously reported that 5 and 10 mg/kg/day lovastatin inhibited ATC growth in nude mice while 1 mg/kg/day lovastatin actually induced significantly greater tumor growth compared with the untreated control tumors.[4] As lovastatin upregulated the expression of TKT, which is important for anaerobic tumor growth,[6] we hypothesized that the growth induced by lovastatin was mediated through TKT or TKTL1. Although we demonstrate here that lovastatin can induce TKT expression in cell culture, no such acceleration of tumor growth was observed with lovastatin. This discrepancy could be due to the initial smaller implanted tumor volume in the lovastatin group. However, mice co-treated with both lovastatin and oxythiamine had tumor volumes similar to that of the untreated control group showing prominent tumor growth, suggesting oxythiamine-induced tumor growth inhibition was eliminated by lovastatin.

Overexpression of TKT and TKTL1 proteins is associated with local colorectal cancer progression and lymph node involvement[17] as well as aggressiveness and poor prognosis in patients with colon, urothelial and breast cancer.[27 28] In thyroid cancer cell lines, the highest TKTL1 expression has been observed in those derived from invasive tumors.[22] Furthermore, shRNA-mediated TKTL1 silencing induces apoptosis and reduces the growth of gastric[29] and colon carcinoma[30] cells in vivo. In papillary thyroid carcinomas

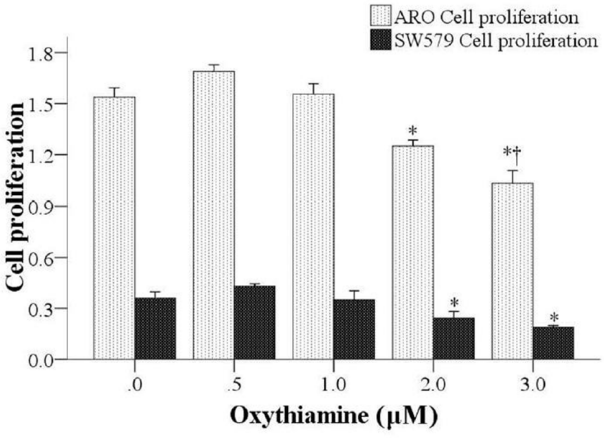


Figure 5 Oxythiamine inhibition of ARO and SW579 cell growth. ARO and SW579 cells were treated with the indicated concentrations of oxythiamine for 48 hours. Cell proliferation was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data were represented as mean and SD. *A significant difference compared with the three groups treated with low oxythiamine doses (0, 0.5, and 1 μM). †A significant difference compared with the 2 μM group.

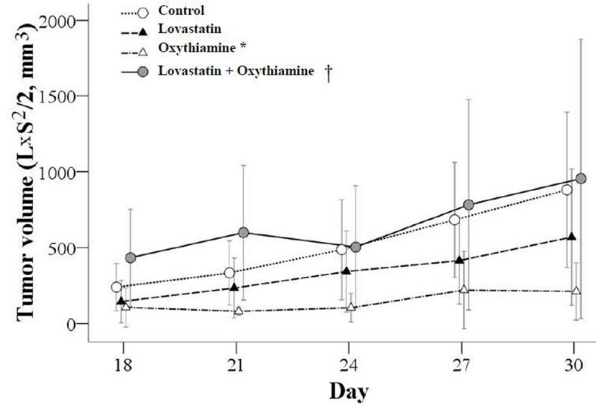


Figure 6 Effects of lovastatin and oxythiamine on ARO tumor volume in nude mice. ARO tumor volume was determined from day 18 to 30 following inoculation with ARO cells. Data were represented as mean and SD. *A significant difference compared with the control and lovastatin groups. A significant difference compared with the oxythiamine group.

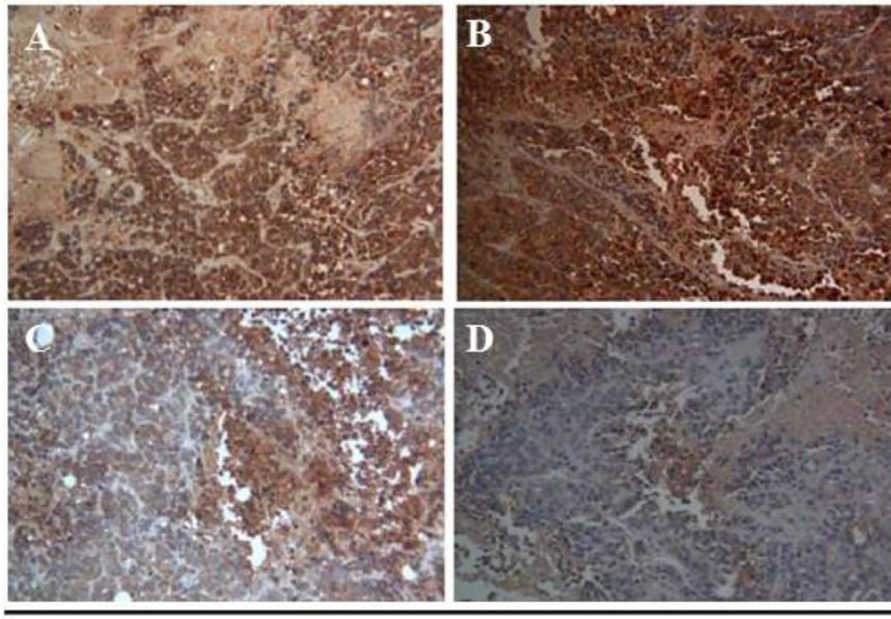
Table 1 Summary of bodyweight (g)

Time (days)	Group A Control	Group B Lovastatin	Group C Oxythiamine	Group D* Lovastatin + oxythiamine	P value† between groups
18	22.28±1.17	22.03±2.03	21.58±1.61	22.80±1.46	0.828
21	21.53±0.92	21.65±1.78	20.63±1.34	20.62±1.88	0.646
24	21.08±0.57	21.18±1.44	19.93±1.81	19.20±1.75	0.075
27	21.28±0.43	21.53±0.92	19.95±2.38	17.83±2.75	0.039
30	21.28±0.32	21.63±1.22	19.10±2.28	18.83±3.27	0.016
P va1ue† within-groups	0.237	0.956	0.477	0.038	

Data were summarized as mean±SD.
*One mouse at day 24 and two mice at day 27 in group D were not available due to death.
†P values were derived using one-way analysis of variance test.

<1.5 cm, overexpression of TKTL1 is associated with lymph node metastasis.[2] However, we observe increased TKT expression in ARO cells cultured with 25 µM lovastatin. In addition, mice treated with lovastatin have increased TKTL1 tumor expression, which was reduced with oxythiamine.

Lovastatin reduces ATC proliferation in a dose-dependent manner, which is similar to that reported by Chung



Mouse ID	Tumor Vol (mm^3)	TKTL1 Expression	Mouse ID	Tumor Vol (mm^3)	TKTL1 Expression
A1	1105	+++	C1	148	++
A2	4018	+++	C2	54	++
A3	1217	+++	C3	160	++
B1	770	++++	D1	732	++
B2	356	++++	D2	1966	++
			D3	163	+

Figure 7 Effects of lovastatin and oxythiamine on ARO tumor expression of transketolase-like 1 (TKTL1). (A) TKTL1 protein levels were determined at the end of the study period (day 30) using immunohistochemistry. Group A, untreated control group; group B, 1 mg/kg/day lovastatin; group C, 80 mg/kg/day oxythiamine; and group D, 1 mg/kg/day lovastatin + 80 mg/kg/day oxythiamine. (B) The pathological visual score of the immunohistochemistry results. D4, D5 were lovastatin+oxythiamine.

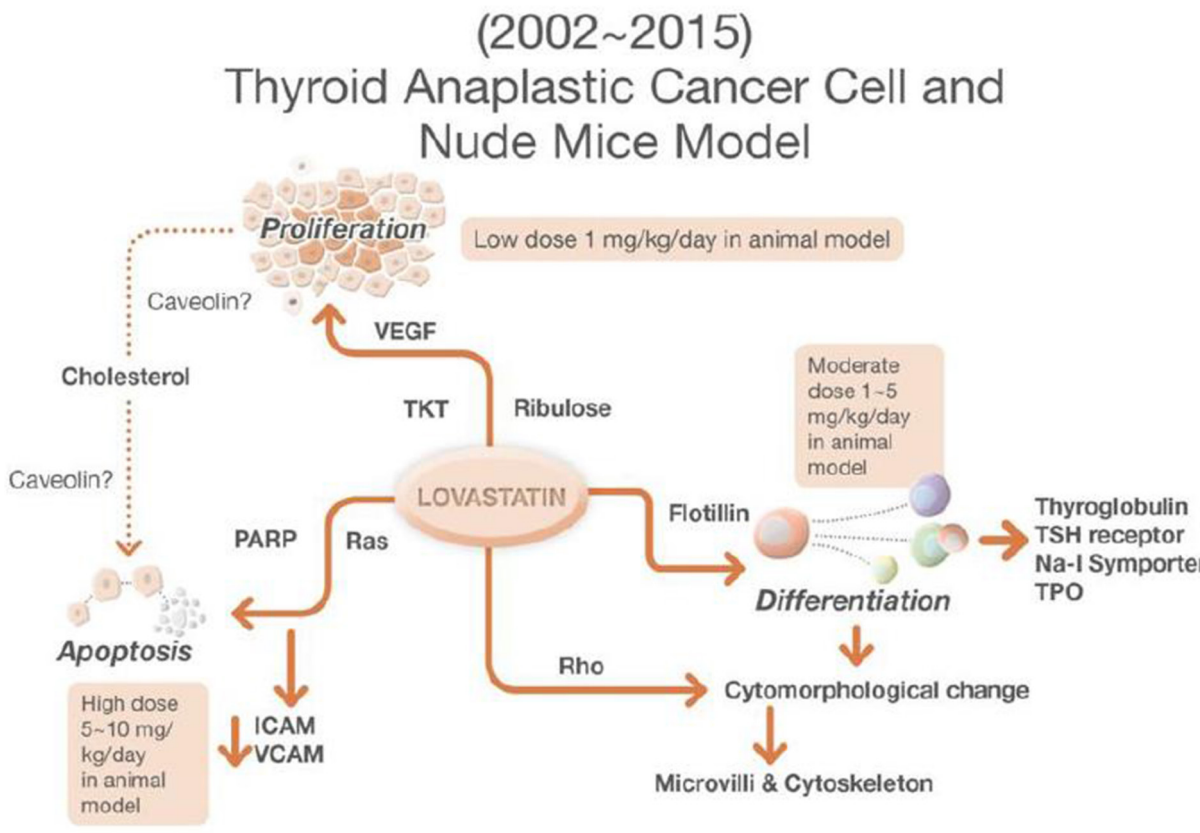


Figure 8 Lovastatin could induce apoptosis, proliferation, and redifferentiation in anaplastic cancer cells via different mechanisms and at various concentrations in a nude mice model.[9–11 14 15 32] ICAM, intercellular adhesion molecule; TKT, transketolase; TSH, thyrotropin; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor.

et al[31] in 8505C and BHT-101 ATC cells. Meanwhile, the mechanism by which lovastatin suppresses ATC growth and whether it is dependent on cell differentiation has been partially elucidated in our prior studies (figure 8).[9–11 14 15 32] In ATC cells, lovastatin-induced growth inhibition is mediated by suppression of Rho/ROCK signaling, thereby upregulating p27.[32] In addition, a role for RhoA GTPase in antineoplastic effects of lovastatin[9 33] as well as inhibition of Ras farnesylation[11 34] have been reported. Further studies using shRNA directed against TKT will be undertaken to determine if this protein mediates the effects of lovastatin on tumor growth.

In Lewis lung carcinoma cells, oxythiamine inhibited in vitro invasion and migration as well as repressed tumor metastasis in vivo.[35] In the present study, oxythiamine suppressed the in vitro growth of both ARO and SW579 cells and reduced TKT levels. In addition, oxythiamine significantly inhibited ARO tumor growth. Contrary to our expectations, an antagonistic effect with respect to tumor growth was observed in the group treated with both lovastatin and oxythiamine. This is similar to the antagonism reported for lovastatin and paclitaxel in ATC cells.[31] On the other hand, Fröhlich *et al*[22] reported oxythiamine to be less effective in thyroid cancer cell lines with high TKTL1 expression even at higher concentrations. Therefore, it is possible that the increased TKT expression by lovastatin reduced the activity of oxythiamine. As lovastatin can alter the differentiation status of ARO cells, oxythiamine may be rendered inactive in cells differentiated by lovastatin. It is also possible that the effects of lovastatin are mediated by factors other than TKT in the absence of TKT activity. In addition to TKT, lovastatin also upregulated annexin II, keratin 8, keratin 19, and transcription elongation factor A(SII)-like 4 expression and downregulated heat shock 70 kDa protein and ubiquinol-cytochrome c reductase complex core protein I, which is similar to our previous report.[15] Further studies will assess the possible role of these proteins in mediating the effects of lovastatin.

TKT (chromosome 3p21.1)[36] and TKTL1 (chromosome Xq28)[37] are two distinct and separately expressed proteins within the PPP system. In our study, TKTL1 expression was reduced in mice treated with oxythiamine alone and further decreased with co-treatment with lovastatin and oxythiamine. However, TKTL1 expression was not correlated with tumor size. The biggest tumor revealed only moderate TKTL1 level, and a medium-sized tumor expressed the highest level of TKTL1. Meanwhile, no significant difference in tumor volume was observed between the mice treated with both lovastatin and oxythiamine compared with the untreated mice. These results suggest an antagonism between the drugs, and that lovastatin influences ATC tumor growth via TKT but not TKTL1.

The present study has limitations that warrant further discussion. The mechanism by which lovastatin influences TKT levels has not been determined. Further studies will assess the signaling pathways that regulate the effects of

lovastatin in ATC. In addition, 40 per cent of the animals receiving both lovastatin and oxythiamine died prior to the end of the study. The tumor volumes are small, suggesting that the treatment is toxic and the deaths are not due to tumor-related complications. Further studies using different concentrations and perhaps different TKT inhibitors are warranted to address the therapeutic value of targeting TKT. Meanwhile, these results show a dual effect of lovastatin on ATC cellular proliferation that correlates with modulation in TKT expression; however, it may not have direct functional relevance in the observed effects of lovastatin on ATC cells.

Since conventional therapy showed no obviously therapeutic effect in patients with ATC, we need more novel therapeutic strategies to treat such a fatal disease.[38] Although high-dose lovastatin[14] and oxythiamine inhibit ARO cell proliferation and tumor growth, a combination of low-dose lovastatin and oxythiamine did not enhance the effects of either drug alone. We assume that high-dose lovastatin and oxythiamine related medications could be considered to play an important and reliable adjuvant role together with target therapy in treating this fatal anaplastic thyroid cancer. Further studies are necessary to determine the pathway of antagonism as well as the mechanism by which lovastatin influences TKT expression.

Contributors C-YW: study concepts, study design, data analysis and manuscript preparation. H-AS: study concepts, study design, data analysis, data acquisition and data analysis. T-CC: study concepts and manuscript review.

Competing interests None declared.

Provenance and peer review Not commissioned; externally peer reviewed.

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