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## Comparative investigation of antitumoral effectiveness of Rho-kinase inhibitor Y-27632, pravastatin and atorvastatin in anaplastic thyroid cancer cell culture

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## ABSTRACT

Anaplastic thyroid cancer is an aggressive malignancy with a poor prognosis. In metastatic cases instead of treatment alternatives including surgery, radiotherapy, and chemotherapeutic regimens, targeted treatments should be sought for.

Statins are 3-hydroxy-3 methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, and inhibit conversion of HMG-CoA into mevalonate. Inhibition of mevalonate pathway Ras prenylation, can also inhibit tumoral growth. Rho/Rho kinase pathway has an important role in tumoral proliferation, and metastasis in which activity of ROCK increases leading to tumoral invasion. Herein we investigated antitumoral effectiveness of two HMG-CoA reductase inhibitor statins namely pravastatin, and atorvastatin, and Rho-kinase inhibitor Y-27632 in anaplastic thyroid cancer cell cultures through suppression of cellular proliferation. Various concentrations of pravastatin (20, and 60  $\mu\text{M}$ ), and atorvastatin (10, and 30  $\mu\text{M}$ ), Y-27632 (10, and 30  $\mu\text{M}$ ), and their combinations including pravastatin -Y-27632 (20  $\mu\text{M}$  + 10  $\mu\text{M}$ ; 60  $\mu\text{M}$  + 30  $\mu\text{M}$ ), atorvastatin - Y-27632 (10  $\mu\text{M}$  + 10  $\mu\text{M}$ , and 30  $\mu\text{M}$  + 30  $\mu\text{M}$ ) solutions were prepared. Anaplastic thyroid cancer cell culture media were treated with these more water-soluble drug solutions of pravastatin which induced lower dose-, and time-dependent decreases in cellular indices relative to more lipid-soluble atorvastatin which also markedly suppressed cellular proliferation. Y-27632 also decreased cell indices in a dose-, and time-dependent manner. Combination of Y-27632 with pravastatin, and atorvastatin did not demonstrate additive, synergic or antagonistic interactions.

HMG-CoA reductase, and also Rho-kinase inhibitors are promising treatment alternatives of anaplastic thyroid cancers. Further in vivo, and clinical studies are needed on this issue.

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### 1. Introduction and objective

Anaplastic thyroid cancer is among types of cancer with a poor prognosis with a median survival time of 2–6 months.<sup>1,2</sup> In some patients, despite implementation of surgical treatment, chemotherapy, and radiotherapy, survival rates do not change.<sup>3,4</sup> Therefore, novel treatment approaches are needed.

Statins are HMG-CoA reductase inhibitors which are used in the treatment of hypercholesterolemia.<sup>5</sup> Atorvastatin is lipid-, and pravastatin water-soluble statins. Atorvastatin, and pravastatin competitively inhibit HMG-CoA reductase. As a consequence, conversion of HMG-CoA into mevalonate is inhibited. Inhibition of mevalonate decreases blood cholesterol level, and also reduces concentrations of nonsteroid isoprenoids including farnesyl pyrophosphate (FPP), and geranylgeranyl-pyrophosphate (GGPP).<sup>6,7</sup> Isoprenoids play a role in the posttranslational modification of small GTPase proteins as Ras and Rho. Membrane translocation is very important in the activation of Ras, and Rho families.<sup>8,9</sup> Farnesylation, and inhibition of geranylgeranylation cause inactivation of Ras, and Rho.<sup>10</sup> It is already known that Rho proteins play roles in

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many stages of the development, and progression of cancer cells including gene expression, and apoptosis. ROCK proteins are Rho-kinase proteins which function as a sub-effector of Rho. Rho-ROCK signal pathway play a role in the motility, and angiogenic activity of cancer cells. Activation of ROCK signal pathway induces proliferation of tumour cells.<sup>11–13</sup> Y-27632 is a selective ROCK inhibitor, and inhibits kinase activity of ROCK via binding its kinase domain in competition with ATP. Thereby, it inhibits ROCK protein, and so Rho-ROCK signal pathway with resultant proliferation, and growth of cancer cells.<sup>14–16</sup>

Herein, we aimed to demonstrate antitumoral activity of atorvastatin, pravastatin, and Y-27632 solutions singly or in combination.

## 2. Materials and method

In our study, anaplastic thyroid cancer cell culture (8305C) procured from The American Type Culture Collection (ATCC) was used. Frozen cells were thawed in laminar flow cell culture cabinet at 38–39 °C for 1–2 min under room temperature. Then bovine serum (FBS) (10%), glutamine (1%), penicilline (1%) (10.000 U/ml) + streptomycin (10.000 mg/ml), and RPMI (Rosswell Park Memorial Institute Media) were added to this culture to prepare a medium. The thawed cells were placed in 15 cc flacons, and prepared medium was added to complete to 15 cc. Then the flacon was centrifuged at 2100 rpm under 37 °C for 10 min. At the termination of centrifuging, the supernatant was poured out. The remaining 1–2 cc portion was transferred into 25 cm-cell culture flask, and 5 cc culture solution was added on this portion. Cell culture medium was observed for 2 days in a room humidified with 5% carbon dioxide, and warmed up to 37 °C. Then, 70–80% confluent cells were subjected to trepanization procedure before passaging. All culture media containing cells which reached confluency during passaging in 25 cm-flasks was poured out. A small amount (0.5 cc) of trypsin was pipetted into the flask for the activation of the remaining medium, and then poured out. Afterwards 1 cc was again pipetted into the flask, and waited for 10 min for the detachment of cells. The detached cells were observed under microscope, and the cells in the flask were transferred into a 15 cc-flacon, and completed to 15 cc with culture medium. This flacon was centrifuged at 2100 rpm for 10 min under 37 °C ambient temperature. Supernatant formed at the end of the centrifuging was poured off, and 1–2 cc portion of the solution was transferred into a 75 cm-flask, and 15 cc culture medium was added on it. The number of cells increased with passaging. Pravastatin sodium, atorvastatin calcium, and Y-27632 (Tocris, USA) were used. These active ingredients were dissolved in 32% pure alcohol, and pravastatin sodium (20, and 60 µg), atorvastatin sodium (10, and 30 µm), Y-27632 (10–30 µg) solutions were prepared in indicated concentrations. For the study, a total of 12 groups were determined. Control group contained only cell, and culture media. In the alcohol group cells, medium, and 32% pure alcohol were used. Besides 20, and 60 µM pravastatin sodium, 10, and 30 µM atorvastatin sodium, 10 and 30 µM Y-27632 groups were prepared. Finally four combination groups were constructed as follows: 20 µM pravastatin sodium + 10 µM Y-27632; 60 µM pravastatin sodium + 30 µM Y-27632; 10 µM atorvastatin + 10 µM Y-27632, and 30 µM atorvastatin + 30 µM Y-27632. Previously proliferated anaplastic thyroid cancer cells were seeded onto well-plates with e-plates containing microelectrode sensors at the bottom of each well. Twelve thousand cells were seeded onto each well. Inoculation was performed such that each well contained equal number of cells. The control group contained only cells, and medium, while alcohol group comprised cells, medium, and 32% pure alcohol. Then

previously prepared drugs were seeded onto the wells of all groups. The prepared e-plates were placed in real-time cell analyzer (xCELLigence RTCA, ACEA Biosciences Inc.) Electrical impedance was measured with microelectrodes placed at the bottom of e-plates of cell culture media, and quantitative information was gathered about the biological condition of the cells including their counts, vitality, and motility. During six days cell indices which indicate real-time vitality of the cells were continually measured with RTCA, and the results were recorded.

### 2.1. Statistical analysis

Numerical data obtained as an outcome of the experiment were expressed as mean ± standard deviation (SD). For statistical comparisons ANOVA (Analysis of variance), and an appropriate post-hoc test (with Dunnet or Bonferroni correction) were used. P values less than 0.05 were considered as statistically significant.

## 3. Results

Our study consisted of 12 groups including control group, alcohol group, 20 µM, and 60 µM pravastatin sodium, 10 µM, and 30 µM atorvastatin calcium, 10 µM, and 30 µM Y-27632 groups, and combination groups (pravastatin sodium 20 µM + Y-27632 10 µM; pravastatin sodium 60 µM + Y-27632 30 µM; atorvastatin 10 µM + Y-27632 10 µM and atorvastatin 30 µM + Y-27632 30 µM). For 6 days (nearly 160 h) proliferation of anaplastic thyroid cancer cells was continually supervised using RTCA device, and cell indices which demonstrate real-time vitality of the cells were recorded. Temporal cell index graphics of all groups are shown in Fig. 1. When alcohol group was compared with the control group, decrease in cell indices of both groups was not statistically significant. ( $p > 0.05$ ). Therefore, since the drugs were prepared with alcohol, comparisons were made between alcohol, and other groups. Solutions of pravastatin sodium (20 µM, and 60 µM) which is relatively more soluble in water were evaluated as for their effects on cell indices, and in the 20 µM group decrease in cell indices was not statistically significant ( $p > 0.05$ ), while this decrease was found to be statistically significant in the 60 µM group which increased with time ( $p < 0.05$ ) (Fig. 2). Pravastatin sodium demonstrated dose-dependent decrease in the cell index. However its effectiveness appears to be lower when compared with atorvastatin which is more lipid soluble. Solutions of atorvastatin calcium prepared in 10 µM, and 30 µM concentrations achieved statistically significant decreases in cell indices ( $p < 0.01$ ). Starting from the 2. Day of their application, proliferation of great majority of cells was suppressed (Fig. 3).

When two prepared solutions of Y-27632 in 10 µM, and 30 µM concentrations were compared with the alcohol group, 10 µM Y-27632 did not decrease cell index statistically significantly ( $p > 0.05$ ), however when its dose was increased (ie. 30 µM Y-27632 group) it depressed cell index markedly relative to the alcohol group ( $p < 0.01$ ). This decrease became more prominent with time. In comparisons between combination groups, Y-27632 – pravastatin, and Y-27632- atorvastatin calcium combinations were evaluated. Y-27632 10 µM + pravastatin sodium 20 µM combination group was compared with 30 µM Y27632, and 60 µM pravastatin sodium groups one by one without any change in cell indices. Although an additive or synergistic effect was not observed between Y-27632, and pravastatin sodium, an antagonistic interaction was not detected either. Similarly cell indices were evaluated for Y-27632 - atorvastatin calcium combinations. Also 10 µM Y-27632 + 10 µM atorvastatin calcium and 30 µM Y-27632 + 30 µM atorvastatin calcium combination groups were also analyzed. Y-

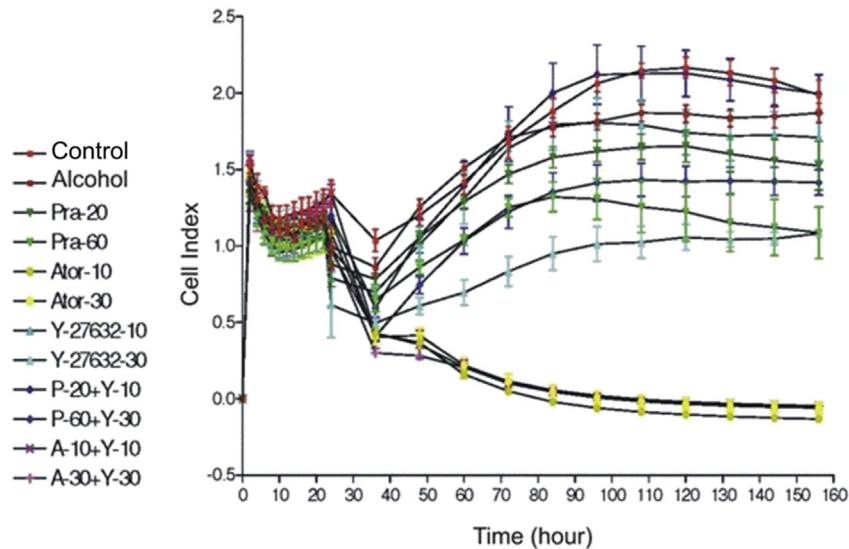


Fig. 1. Temporal changes in cell index graphics of all groups.

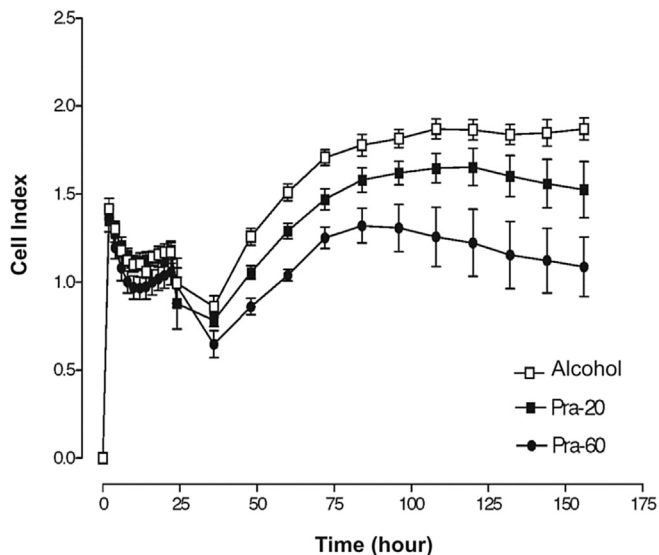


Fig. 2. Comparative effects of 20  $\mu\text{M}$ , and 60  $\mu\text{M}$  pravastatin groups, and alcohol group on cell indices.

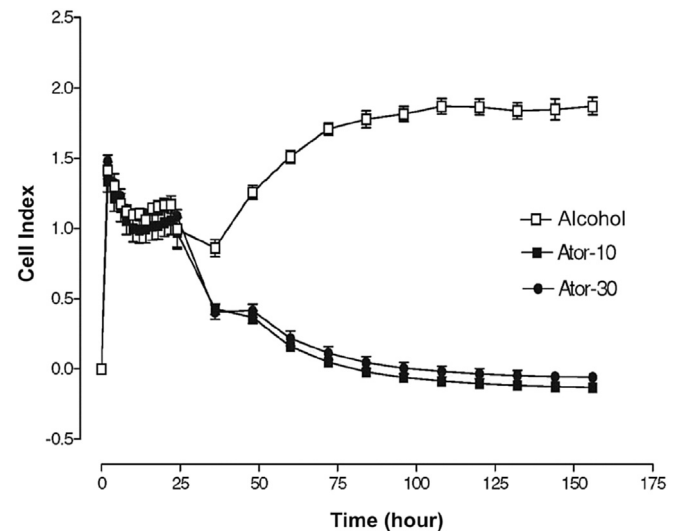


Fig. 3. Comparative effects of 10  $\mu\text{M}$ , and 30  $\mu\text{M}$  atorvastatin groups, and the alcohol group on cell indices.

27632 (10  $\mu\text{M}$ ) + 10  $\mu\text{M}$  atorvastatin calcium group, 10  $\mu\text{M}$  Y-27632 and 10  $\mu\text{M}$  atorvastatin calcium group, and on the other hand 30  $\mu\text{M}$  Y-27632 30  $\mu\text{M}$  + 30  $\mu\text{g}$  atorvastatin calcium groups were analyzed separately. Despite lack of any additive or synergistic interaction between Y-27632, and atorvastatin calcium groups, any antagonistic intergroup interaction was not also observed.

Water-soluble pravastatin decreased cell index in a dose-dependent manner. This effect became more prominent with time. A statistically significant difference was not observed between alcohol, and 20  $\mu\text{M}$  pravastatin groups. (NS, nonsignificant). However with increasing doses pravastatin statistically significantly decreased cell index relative to the alcohol group ( $p < 0,05$ ).

Lipid-soluble atorvastatin decreased cell index. Two days after its administration, proliferation of most of the cells was suppressed. Both atorvastatin groups (10  $\mu\text{M}$ , and 30  $\mu\text{M}$ ) decreased cell index statistically significantly when compared with the alcohol group ( $p < 0.01$ ).

#### 4. Discussion

Anaplastic thyroid cancer is a poorly differentiated tumour originating from follicular epithelium, and consists of 2–5% of all thyroid cancers. In most of the patients, since the disease is advanced at the time of diagnosis, surgical treatment is not recommended. For locally-advanced inoperable disease combined radiotherapy, and chemotherapy is advised.<sup>17–19</sup> Despite various treatment approaches, an effective therapy for anaplastic thyroid cancer is not available. Therefore new treatment approaches are needed. To this end, in their study Wunderlich et al. detected that combined use of bortezomib which is an ubiquitin-proteasome inhibitor, and an aurora kinase inhibitor (MLN8054) has exerted antitumoral activity in 3 anaplastic thyroid cancer cell –lines through suppression of cell growth, and induction of apoptosis.<sup>20</sup>

Our aim was to investigate in vitro anaplastic thyroid cancer cell culture media the antiproliferative effects of statins, more precisely, water soluble pravastatin sodium, and lipid soluble

atorvastatin calcium, as well as Rho-kinase inhibitor Y-27632 on cancer cells. Many studies have been performed about antitumoral effects of statins. According to simvastatin, and pravastatin study carried out by Menter et al. simvastatin which is more lipid-soluble was found to be more effective on suppression of proliferation in tumour cell cultures when compared with pravastatin which is more water-soluble.<sup>21</sup> HMG-CoA reductase inhibitors prevent formation of mevalonate from HMG-CoA. Inhibition of synthesis of mevalonate, also prevents synthesis of nonsteroid isoprenoids as FPP, and GGPP. Isoprenoids play a role in the posttranslational modification of many proteins including small GTPases as Ras, and Rho. Posttranslational prenylation, and membrane translocation have crucial importance in the activation of Ras, and Rho families. Inhibition of farnesylation, and geranylgeranylation induce inactivation of Ras, and Rho.<sup>22–24</sup> According to the study performed by Duncan et al. the authors demonstrated that mevalonate contributes to the proliferation, and growth of human breast cancer cells in mice.<sup>25</sup> Based on in vitro lovastatin study by Wen-Bin Zhong et al. lovastatin observedly decreased anaplastic thyroid cancer cells in a concentration-dependent manner. As noted by authors, lovastatin inhibited cell growth at the G1 phase of the cell cycle. In the same study, it was observed that mevalonate, and GGPP had blocked cellular inhibition induced by lovastatin, and in turn lovastatin had inhibited membrane translocation of Rho family. In cells treated with lovastatin, levels of Rho A, and Rac 1 protein observedly decreased at a rate of 55%, and 65%, respectively. As another outcome of the study, the authors detected that inhibition of RhoA/ROCK signal pathway increased levels, and reinforced stability of p27 protein. Lovastatin inhibits CDK2 activity, and interrupts cell cycle at the G1 phase thus blocking proliferation of cancer cells. At this stage of the study, both lovastatin, and selective ROCK inhibitor Y-27632 were used, and the authors observed that Y-27632 per se had increased levels of p27, and inhibited cell growth at a rate of 25 percent<sup>11</sup>. Various studies have demonstrated that statins block growth of prostate, breast, and liver cancer cells at G1 phase, and mean survival rates of the patients with hepatocellular cancer who received daily doses of 40 mg pravastatin prolonged 2-fold<sup>26–29</sup> In our study we compared lipid-, and water-soluble statins, and observed the impact of Rho-kinase inhibitor on anaplastic thyroid cancer cell cultures. In solutions prepared with lower, and higher concentrations of atorvastatin calcium, only higher concentrations of pravastatin sodium, and Y-27632, statistically significantly decreased cell index of anaplastic thyroid cancer cells, in other words they suppressed cellular proliferation. In our study, pravastatin sodium - Y-27632, and atorvastatin calcium - Y-27632 combination groups were compared. Any additive, synergistic or antagonistic effects of these combinations were not observed. In a study on prostate cancer, it was demonstrated that lovastatin, fluvastatin, and simvastatin blocked proliferation of prostate cancer cells in the culture media at G1 phase of the cell cycle, however 200-fold higher pravastatin concentration was required to see the same effect.<sup>30</sup> Still in our study, we didn't observe a statistically significant decrease in cell indices with low concentrations of pravastatin sodium.

We performed our study using pravastatin, atorvastatin, and ROCK selective inhibitor so as to introduce new perspectives within this context. In cases with anaplastic thyroid cancer, and types of cancer without any established effective treatment modality, further in vivo, and clinical studies are needed.

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