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Atorvastatin inhibits RhoC function and limits head and neck cancer metastasis

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SUMMARY

Objective—RhoC oncogene is a well characterized marker of metastasis in a majority of invasive cancers, including HNSCC. Elevated RhoC expression has been found to be associated with distant metastasis. Statins are a class of drugs that are used to reduce cholesterol levels by inhibiting HMG-CoA reductase activity which in turns prevents mevalonate synthesis, which is a precursor for synthesis of cholesterol and prenylation. Interestingly, the proper function of Rho proteins depends on prenylation. Significantly, it has been reported that metastasis in human melanoma can be reduced by atorvastatin which inhibits RhoC activity by preventing its geranylgeranylation. Given that RhoC is a key oncogene involved in metastasis, we hypothesized Atorvastatin can reduce head and neck metastasis by inhibiting RhoC activity.

Methods—*In vitro* and *in vivo* studies were carried out to evaluate the ability of Atorvastatin to inhibit RhoC function and HNSCC metastasis. Cell motility, proliferation, cell invasion, and colony formation assays were performed according to the standard protocols.

Results—Atorvastatin treatment significantly reduced the active form of RhoC *in vitro* and diminished cell motility, invasion, proliferation and colony formation. Importantly, we observed a significant decrease in p-ERK1/2 and p-STAT3 in Atorvastatin treated cell lines. *In vivo* experiments revealed inhibition of angiogenesis and lung metastases with Atorvastatin therapy.

Conclusions—This study is the first of its kind to establish a potential role of Atorvastatin in head and neck cancer therapy. These findings suggest that Atorvastatin can be a potential low risk adjuvant therapy to minimize metastases in aggressive forms of HNSCC.

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Conflict of interest statement

The authors declare that they have no competing interests.

Keywords

Head and neck squamous cell carcinoma; RhoC; HMG-CoA reductase; Geranylation; Lung metastasis

Introduction

Head and neck cancer is the sixth leading cause of cancer related deaths^{1–3} with 5-year survival rates of only 50%. Furthermore, mortality is due not only to the recurrence of the local tumor, but more importantly, due to metastasis to vital organs including pulmonary,⁴ bone and liver metastases.⁵ Therefore, there is an urgent need to identify the molecular mechanisms involved in head and neck tumor metastasis so as to design more effective treatment modalities.

Important molecular and genetic research in the past decade has revealed that RhoC (a GTPase belonging to the Ras superfamily) is over-expressed in a wide range of invasive carcinomas.^{6,7} These studies have found a strong correlation between elevated RhoC expression and tumor progression, invasion and metastasis to distant body regions.⁸

Previous studies in our laboratory have shown that RhoC expression is elevated in tumors of patients with HNSCC when compared to normal squamous cell epithelium.⁸ More importantly, increased RhoC expression at the primary tumor site is strongly correlated with lymph node metastasis and can be used to identify a metastatic phenotype even in small primary tumors.⁸ We have also shown that inhibition of RhoC strongly reduces cell motility and invasion *in vitro*. In addition, there was a marked reduction in lung metastasis when RhoC inhibited tumor cell lines are injected through the tails of SCID mice.⁹ Altogether, these studies suggest that RhoC is an important oncogene that plays a major role in cell motility and in transforming non-invasive cells into invasive phenotypes.^{10,11} Therefore, it is of great interest to elucidate the biochemical pathways involved in RhoC activation in order to identify potential steps of the pathway that can be targeted to inhibit its function. Interestingly, studies have reported that Rho proteins including RhoA and RhoC require isoprenylation for their proper biological activity.¹² This is a process by which long chain fatty acid molecules are covalently attached near the C-terminus of a protein enabling it to anchor to membranes in the cell. The biochemical pathway used for cholesterol biosynthesis is shared with the biochemical process that leads to isoprenylation of Rho proteins. The rate limiting step in cholesterol biosynthesis is the conversion of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate which interestingly is also a substrate for isoprenoid biosynthesis. Atorvastatin belongs to the statin family of drugs that inhibits cholesterol biosynthesis by blocking the activity of HMG-CoA reductase and preventing the conversion of HMG-CoA to mevalonate. Consequently, this pathway also prevents the activity of Geranylgeranyl pyrophosphate (GGPP) which is responsible for prenylation or lipidation of Rho proteins, including RhoC, and is an essential step that is needed for their functional biological activity.¹² Therefore, statins are considered good candidates for not only inhibiting cholesterol biosynthesis but also preventing the prenylation of Rho proteins, including RhoC.

To date, very few studies have reported on the role of statins on RhoC activation in HNSCC. A diagram of the inhibitory mechanism of RhoC by the statin class of drugs is depicted in Fig. 1. At pharmacologically achievable concentrations, statins also inhibit tumor growth and induce apoptosis in leukemic and other malignant cells.¹³ It has also been shown that the mevalonate pathway of cholesterol synthesis may have a strong association with the growth of mammary tumors.¹⁴ Recent studies show a dramatic (<50%) risk reduction in metastasis in prostate cancer among statin users.¹⁵ Moreover, statins have also been shown to act as inhibitors of cell proliferation by inducing apoptosis in several cancer types¹⁶ and also prevent cell invasion and metastasis in various cancers including breast cancer.¹² These studies strongly suggest the possibility of using statins as potent anti-cancer drugs.

The findings presented in this study demonstrate that Atorvastatin plays an important role in modulating RhoC function *in vitro* by decreasing cell motility, invasiveness, stress fiber integrity, proliferation, and anchorage dependent colony formation and also by depleting the phosphorylation of ERK1/2 and STAT3. Furthermore, *in vivo* studies show a marked reduction in neo-vascularization and distant lung metastasis in SCID mice. Therefore, elucidating the molecular mechanisms by which statins regulate RhoC activation will be an important step towards a more effective treatment of head and neck cancer.

Materials and methods

Cell culture

University of Michigan squamous cell carcinoma cell lines (UM-SCC) -1 and -47 are well established cell lines derived from patients with T2N0 of floor of the mouth and T3N1 of the tongue respectively.^{17,18} The cell lines were grown as described in our earlier studies.¹⁹

Determination of RhoC [GTP]

The effect of Atorvastatin on RhoC [GTP] in UM-SCC-1 and -47 was determined by G-LISA using G-LISA kit (Cytoskeleton, Denver, CO,) with slight modification using RhoC primary antibody from Cell Signaling as per the manufacturer's protocol. Atorvastatin was procured from Toronto Research Chemicals, Toronto, ON, Canada.

Cell motility, invasion, stress fiber formation, proliferation and clonogenic survival assays

Motility assay

Cell motility assay was performed in 60 mm Petri dishes. At about 80% confluence, a fine scratch in the form of groove was made with the help of a pipette tip and immediately photographed. Next, cells were supplemented with DMEM containing 10% FBS and allowed to grow in the presence of solvent control or different concentrations of Atorvastatin. The width of the scratch was measured at 0 h and after 24 h to calculate the percentage of the gap covered by the cells in this time period.

Invasion assay

Cell invasion assay was performed as described previously⁹ in absence or in presence of Atorvastatin using BD Bio-Coat Matrigel Invasion Chamber. Matrigel invaded cells were counted microscopically at 100× magnification. *Colorimetric analysis*: membranes were carefully removed from the insert and dipped in 7% (v/v) acetic acid to extract the Toluidine dye from the invaded cells. The color intensity of the dye was read at 690 nm.

Confocal microscopy

The effects of Atorvastatin on stress fiber formation were performed by Phalloidin staining according to the manufacturer's protocol of Texas Red-x phalloidin (Life Technology). Cover slips containing fixed and stained cells were mounted on slide using prolong-Gold antifade reagent with DAPI (Life Technology) and stored at 4 °C. Confocal microscopy was performed using Olympus FV1000 microscope.

Cell proliferation assay

Cell proliferation assay in presence of solvent control or different concentrations of Atorvastatin was performed on xCELLigence according to the manufacturer's protocol (Roche Applied Sciences, Indianapolis, IN).

In vitro clonogenic survival assay

The clonogenic survival assay was performed according to Franken et al.²⁰ Briefly, 500–600 Atorvastatin pre-treated cells were allowed to grow in 60 mm Petri dishes for two weeks in presence of either Atorvastatin or solvent control. Colonies formed were stained with 0.5% (w/v) crystal violet prepared in 0.6% (v/v) glutaraldehyde solution.

Preparation of cytosolic and membrane fractions and Western blot analysis

Cytosolic and membrane fractions were prepared as described earlier.²¹ Briefly, Atorvastatin and or Geranylgeranyl pyrophosphate ammonium salt's (GGPP, 10 µM) pre-treated UM-SCC-1 and -47 were homogenized and centrifuged. The cytosolic (supernatant) and membrane fractions (pellet) were analyzed by 4–12% SDS–PAGE. Membranes were incubated overnight with either primary polyclonal RhoC, phospho-P^{44/42} (p-ERK^{1/2}) or P-STAT3^{ser727}. After incubation with primary antibodies, the membranes were blotted for 1 h with secondary HRP-conjugate goat anti-rabbit antibody. Proteins were visualized using ECL super signal system.

Animal xenograft

All animal studies were approved by the Ohio State University Animal Research Committee. Five animals per treatment were selected to receive Atorvastatin (10 mg/kg body weight). This is a pre-standardized and well tolerated dose for *in vivo* studies.¹² Furthermore, the dose administered to the mice was calculated based on a published study²² that mimics the amount of Atorvastatin in the range of pharmacological dose for human.²³ In parallel five mice were given a placebo by gavage every alternate day. Next, suspension of 1×10^6 UM-SCC-47 cells was injected in the flank region or through lateral tail vein of mice using 0.5-in., 27 gauge needles. At the end of the third week animals were euthanized

in CO₂ chamber. The lungs were dissected and fixed in formalin buffer thereafter transferred to 75% (v/v) methanol and then processed for H&E staining. For flank model study, animals were euthanized at the end of the sixth week and localized tumors and lungs were processed in the same way as described above for CD31 and H&E staining.

Statistical analysis

Statistical analyses were performed using Student's *t*-test. The mean was reported with Standard deviation ($\pm$ SD). Differences were considered to be statistically significant when *p* values were less than 0.05.

Results

RhoC [GTP] expression is greatly reduced in Atorvastatin treated head and neck squamous cell carcinoma cell lines

Active or GTP-bound RhoC was determined using G-LISA kit in lysates obtained after treating UM-SCC-1 and -47 cell lines with solvent control or 5 μ M Atorvastatin overnight. As shown in Fig. 2A, parental cell lines and those treated with solvent control show high levels of active RhoC (pg/ml). In contrast, there is 48% decrease in RhoC [GTP] in Atorvastatin treated UM-SCC-1. We obtained a similar reduction (52%) in the RhoC [GTP] expression in UM-SCC-47 (graph not shown). These data indicate that Atorvastatin decreases the expression of active of RhoC in head and neck squamous cell carcinoma.

Translocation of RhoC from cytosol to membrane

To investigate the translocation of RhoC from cytosol to membrane, sub-cellular fractions were prepared from parental lines, solvent control, and Atorvastatin treated cells, and the fractions were probed with anti-RhoC antibody. The total RhoC expression in control and Atorvastatin treated samples remained unchanged (Fig. 2B). Further, as shown in Fig. 2C, RhoC is predominantly detected in membrane fractions of solvent control cells. However, upon Atorvastatin treatment, RhoC is observed to be restricted to cytosolic fraction and very little if any was found in the membrane fraction. These data suggest that, upon Atorvastatin treatment of HNSCC cell lines, RhoC becomes associated with cytosolic fraction and its translocation to the membrane is prevented. For the rescue experiment, we treated the cells with Atorvastatin followed by 10 μ M GGPP. Cytosolic and membrane fractions were prepared as described above. Interestingly, a significant amount of RhoC was detected in the membrane fraction upon GGPP treatment (Fig. 2D). This further confirms that geranylation is required for the proper activation and translocation of RhoC from cytosol to the membrane.

Atorvastatin attenuates phosphorylation of ERK 1/2 and STAT3 in head and neck squamous cell carcinoma

As shown in Fig. 2E and F, treatment of cells with Atorvastatin results in a decrease in the phosphorylation of ERK1/2 (extracellular signal-regulated kinase) and ser⁷²⁷ STAT3 (signal transducer and activator of transcription 3). These data suggest that RhoC activates ERK1/2 and STAT3 pathways by regulating their phosphorylation in head and neck squamous cell carcinoma which are attenuated by the treatment of Atorvastatin.

Atorvastatin decreases cell motility, cell invasion, colony formation and stress fiber formation in head and neck cancer cell lines

Motility assay—Fig. 3A and C shows a perceptible decrease in cell motility in Atorvastatin treated cells when compared with solvent control lines. Furthermore, the dose response effect of Atorvastatin on cell motility is graphically presented in Fig. 3B and D. As evident, the decrease in motility is gradual and dose dependent. There is a 70–80% decrease in motility observed in UM-SCC-1 at 5 μ M and 7 μ M concentration of Atorvastatin, while a decrease of 60–65% was observed in UM-SCC-47 at the same concentration. Our study also observed a reduction in cell proliferation in Atorvastatin treated cells by about 30% at 5 μ M concentration (discussed in a later section). When this is taken into consideration, we still have about 50% reduction in cell motility after treatment with Atorvastatin at the same concentration.

Invasion assay—As seen in Fig. 4A, the number of cells that invaded the Matrigel are far less in the Atorvastatin treated cells when compared to DMSO control ones. Fig. 4B and C show the colorimetric analysis of the extent of invasion ($n = 3$; $p < 0.05$). Our colorimetric analysis shows a reduction in invasion by 60% and 42% in UM-SCC-1 and -47 respectively. This strong decrease cannot be due to reduced cell proliferation only, and therefore this suggests Atorvastatin treatment reduces cell invasion in HNSCC cell lines.

Confocal microscopy—Cells from UM-SCC-1 and -47 when treated with solvent control demonstrate distinct stress fiber formation, which is an important characteristic of Rho family GTPases.^{24,25} In contrast, treatment with Atorvastatin resulted in imprecise F-actin polymerization resulting in a significant disruption in the formation of stress fibers (Fig. 4D). This finding is in agreement with previously reported studies in which inhibition of geranylgeranylation prevented actin reorganization in human smooth muscle cells.²⁶

Cell proliferation assay—As shown in Fig. 5A, a significant decrease in cell proliferation in UM-SCC-1 upon 3 μ M and 5 μ M Atorvastatin treatment was observed. In UM-SCC-1 lines, a 28% decrease in cell proliferation after 24 h of treatment using 3 μ M of Atorvastatin was observed, while a decrease of 41% in proliferation was observed at 5 μ M concentration. Similarly, in UM-SCC-47 lines, 27% and 36% depletion in proliferation was detected when compared to control (for brevity, figure not shown). The contribution of cell proliferation in invasion and migration cannot be ruled out. This decrease in cell proliferation may contribute to reduced cell motility and invasion in these lines. However, these three experiments are independent in their setup and this is the reason why each experiment was normalized with its own controls.

In vitro clonogenic survival assay—As given in Fig. 5B and C, there is a remarkable decrease in colony formation in UM-SCC-1 and -47 after Atorvastatin treatments. The decrease was gradual and in a dose-dependent manner. The bar graphs show a decrease in colony formation by 70–80% in UM-SCC-1 while a 80–90% decrease was observed in UM-SCC-47 when treated with 5 μ M and 7 μ M Atorvastatin respectively.

These *in vitro* results provide ample supporting evidence that treating with Atorvastatin can be a useful tool for therapeutic measure in head and neck carcinoma.

Animal model

RhoC plays an important role in lung metastasis and microvessel density formation

Flank model: Our flank model, implanted with UM-SCC-47 cell line showed a small decrease in tumor growth in the Atorvastatin treated mice (gross tumor size, tumor growth curve, average tumor weight and tumor integrity) (Fig. 6A–D). Moreover, a microscopic study of CD31 antibody stained tumor revealed a remarkable difference in microvessels formation in Atorvastatin treated mice which were very small and poorly developed as compared to the corresponding control group (Fig. 6E). Fig 6F shows the average number of microvessels counted in five different fields. H&E staining of lungs show the patches of metastatic foci and highly inflamed blood vessels in the control group while the lungs were clear in the Atorvastatin treated group (Supplementary Fig. 2). It should be noted that we did not observe any weight loss or toxic effect of the drug during the course of treatments (Supplementary Fig. 1).

Tail vein model: UM-SCC-47 cell line administrated through tail vein shows large metastatic foci in the lungs of the control mice while few such metastatic lesions were seen in the lungs of the mice treated with Atorvastatin (Fig. 7A). The number of lesions per animal in control and Atorvastatin treated mice are depicted in graphic form in Fig. 7B.

Discussion

RhoC is a member of the well characterized Rho family of GTPases (belonging to the larger group of the Ras superfamily) that are involved in a wide range of cellular activities including intracellular signaling, cytoskeletal organization, cell proliferation, and gene expression.²⁴ The correlation between overexpressed RhoC and invasive carcinoma of various types are well established.^{27–29} Notably, RhoC has been shown to reorganize the actin cytoskeleton which is a critical step towards changing cells into motile and invasive forms.³⁰

Metastasis is a major cause of mortality in head and neck cancer patients. It involves numerous alterations in the extracellular matrix and in the actin based cytoskeleton of the cell, such that a locally growing tumor becomes a metastatic killer. DNA array analysis of highly metastatic cells has revealed that the small GTPase RhoC is overexpressed in metastatic cells, including head and neck squamous cell carcinoma.³¹ These proteins are modified post-translationally by a process known as prenylation. This involves the covalent addition of a 20 carbon geranylgeranyl isoprenoid to conserved cysteine residues at or near the C-terminus making the protein more lipophilic.³² Generally this modification results in the translocation of the protein from the cytosol to the membrane where it plays a major role in signal transduction downstream from membrane receptors. The isoprenoid units are derived from mevalonate, which apart from being the precursor for cholesterol synthesis also generates the long hydrophobic farnesyl and geranylgeranyl moieties. Since these

moieties are prerequisites for membrane anchoring of Ras/Rho proteins, any depletion should result in inhibition of Ras/Rho prenylation and subsequently the inhibition of their function at a more upstream level. Previous studies in our laboratory have shown that RhoC is constitutively active in head and neck cancer cell lines as well as in patient derived primary tumors.^{8,9} To investigate the effect of Atorvastatin on RhoC [GTP], we treated two separate cell lines with this compound and analyzed its activation by G-LISA, which only detects active RhoC. Interestingly, a significant decrease in active RhoC was observed in Atorvastatin treated cell lines (Fig. 2A). However, it should be noted that we did not observe a decrease in total RhoC expression upon Atorvastatin treatment, as evident by Western blot analysis (Fig. 2B). Furthermore, we have shown that Atorvastatin therapy inhibits the translocation of RhoC from cytosol to membrane, which occurs only after its prenylation (Fig. 2C). Indeed, the fact that RhoC prenylation is the mechanism by which membrane translocation occurs was confirmed by rescue experiments where, isoprenoids were added in the presence of Atorvastatin, resulting in a restoration of RhoC membrane localization (Fig. 2D). A similar set of observations has been reported for breast cancer³³ and melanoma.¹² Further consequences of this alteration in sub-cellular localization is a substantial decrease in motility, invasion, cell proliferation and anchorage dependent clonogenic survival in cell lines treated with this drug (Figs. 3–5). In addition, as shown in Fig. 4D, Atorvastatin disrupts stress fiber formation in UM-SCC-1 and -47 cell lines, suggesting that RhoC mediated cytoskeletal rearrangement is affected by this treatment. These findings are in agreement with previously published work on statins in other tumor types.^{34,35} Overexpressed RhoC leads to increased motility due to an increase in actin fiber formation and focal adhesion.³⁶ Therefore, based on our findings, Atorvastatin may control metastasis by decreasing the functional activity of RhoC [GTP], which results in the diminished directional movement of head and neck cancer cells and consequently the prevention of metastasis.

Several studies have reported that RhoC signals through ERK1/2 and other proteins to increase invasion and metastasis. Studies on breast cancer show that Rho proteins can activate the MAPK cascade resulting in increased invasion.³⁷ In addition, an antibody array analysis in prostate cancer cells revealed that Pyk2, FAK, MAPK and AKT were phosphorylated upon RhoC induction. They further showed that knocking down Pyk2 decreased phosphorylation of FAK, MAPK and AKT followed by suppression of MMP2 and MMP9, known to increase invasive ability of tumor cells.³⁸ To investigate the effect of Atorvastatin on downstream targets of RhoC we chose two independent key signaling molecules. ERK1/2 and STAT3, which are reported to be significantly involved in cancer metastasis.^{39,40} We found a significant down regulation of p-ERK1/2 in HNSCC cells after Atorvastatin treatment (Fig. 2E). While our findings are in accordance with previous published work on the inhibitory effect of statins on the MAPK signaling cascade,^{41,42} the exact mechanism of their action has yet to be characterized. Therefore, it can be postulated from our results that Atorvastatin may inhibit phosphorylation of ERK1/2 by decreasing the expression of active RhoC in HNSCC.

Next, we examined the effect of Atorvastatin on the phosphorylation of an important signaling molecule STAT3, which is found to be overexpressed in many forms of metastatic cancers including HNSCC.^{43,44} Our findings reveal a significant decrease in STAT3

phosphorylation in Atorvastatin treated HNSCC cell lines. Furthermore, studies in our laboratory show that by knocking down RhoC using shRNA, phosphorylation of STAT3 decreased significantly (un-published data). This strongly suggests that Atorvastatin decreases STAT3 phosphorylation by inhibiting RhoC GTPase activity (Fig. 2F). This novel finding also suggests a new downstream target of RhoC through which it can promote metastasis. Further studies are needed to identify the signaling molecules involved in the activation of STAT3 by RhoC. A possible candidate is IL-6, which is a known activator of the JAK-STAT3 signaling pathway. Its expression has recently been shown to be greatly elevated in the serum and tumors of patients with HNSCC,^{45,46} bone and breast cancer metastases.^{47,48} Interestingly, studies on the role of IL-6 in inflammation show that its levels are significantly down regulated when cells are treated with statins.^{49,50} Taken together it can be concluded that Atorvastatin could be used as a therapeutic drug to down regulate the activation of two important metastasis promoting signaling genes, ERK1/2 and STAT3 in HNSCC.

Aside from localized tumor, lung metastasis is a common occurrence in patients, with the lungs (93%) and bones (43%) being the primarily common sites of metastasis with HNSCC.^{51,52} To test our hypothesis that statin therapy would inhibit distant metastases, we designed two separate *in vivo* experiments. In the first, tumor was established in the flank of mice after two weeks of statin therapy. As shown in Fig. 6A–F, there was a small decrease in primary tumor growth between control and Atorvastatin treated animals. This is in contrast to previous studies including ours that have shown that RhoC does not affect tumor growth.^{53–55} Moreover, Ikoma et al. 2004 studied the effect of RhoC inhibition on tumor growth in lung cancer using the orthotopic model and reported that it does not affect tumor growth.¹¹ Our *in vivo* studies show a small decrease in tumor growth and furthermore, there was a marked decrease in the tumor vascularity and distant metastasis between control and Atorvastatin treated groups. This antiangiogenic property of Atorvastatin is in accordance with the work published by Dulak and Jozkowicz⁵⁶ and this is probably contributing to the small tumor sizes observed in our *in vivo* studies.

In addition, the role of RhoC in cancer metastasis is well documented. Our previous studies show that the inhibition of RhoC by siRNA reduces lung metastasis significantly in a mouse model.¹⁹ In this current study, we did not see metastatic foci in the lungs of Atorvastatin treated mice. This observation is in accordance with our previous findings which prove that inhibition of RhoC results in reduced distant metastasis (Supplementary Fig. 2).

To further illustrate the effect of Atorvastatin on distant metastases, in our second animal model, SCC injected through tail vein of mice were treated with placebo or Atorvastatin until the termination of the experiment. Similar to our earlier *in vivo* model, numerous metastatic foci were present in the lungs of the control group of mice, while the Atorvastatin treated mice showed significantly less number and very small metastatic lesions (Fig. 7A and B).

In précis, our *in vivo* results strongly suggest that Atorvastatin greatly reduces angiogenesis and metastasis in head and neck cancer. However, it should be noted that the tail vein model in mice represents only partial metastatic events and further studies using orthotopic as well

as genetically engineered models are needed to understand how Atorvastatin inhibits the metastatic potential of RhoC.

In summary, we show that an Atorvastatin treatment of HNSCC cell lines decreases invasion and migration. Statin treatment also decreases the membrane fraction of RhoC and limits the activation of ERK1/2 and STAT3 signaling cascades. Most importantly, in an *in vivo* animal model, RhoC inhibition resulted in an inhibition of metastases compared to placebo treated animals. In total, this work illustrates the potential promise of statin compounds as adjunct to standard therapies available for HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.oraloncology.2013.04.003>.

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Figure 1.

A sketch diagram showing the major steps involved in cholesterol biosynthesis from its precursor 3-hydroxy-3methylglutrayl CoA and the inhibition of HMG-CoA reductase by statins. Further, the diagram illustrates the steps in prenylation of proteins including that of RhoC.

Figure 2.

Effect of Atorvastatin on RhoC activation in UM-SCC-1 (A) determined by G-LISA. A significant decrease in RhoC activation was observed in Atorvastatin treated cells as compared to either parental or solvent control (sol. cont.) treated samples. *Statistically significant ($p < 0.05$). (B) Western blots analyses showing the effect of Atorvastatin on total RhoC expression. (C) Translocation of RhoC from cytosol to membrane. As shown, the expression of RhoC in membrane fraction is much higher in solvent control (DMSO) when compared to the cytosolic fractions. The pattern is reverse in Atorvastatin treated samples with the membrane fractions showing almost negligible expression of RhoC when compared with the cytosolic fractions. (D) Treatment of GGPP showing rescue of RhoC to the membrane in presence of Atorvastatin. (E and F) Phosphorylated ERK^{1/2} and STAT3^{ser727} decreased significantly in Atorvastatin treated cells respectively.

Figure 3. The effect of Atorvastatin on cell motility. The slow movements of cells were Atorvastatin dose dependent (after 24 h) as compared with solvent control (DMSO) treated in UM-SCC-1 (A) and -47 (C) respectively (magnification, $\times 20$). Columns (B&D), percentage of motility with the initial reference point as 0 h. *Statistically significant ($p < 0.05$).

Figure 4.

Cell invasion assay of control and Atorvastatin treated (A) UM-SCC-1 and -47 respectively (magnification $\times 40$). (B and C) colorimetric analysis of the dye extracted from the cells invaded through the matrigel and membranes ($p < 0.05$). (D) Confocal microscopy showing stress fiber formation is disrupted in Atorvastatin treated cells but is well defined in solvent control in UM-SCC-1 and 47.

Figure 5.

Cell proliferation assay in presence of Atorvastatin. (A) Significant decreases in proliferation were observed upon Atorvastatin treatment in UM-SCC-1. Colony formation in Atorvastatin treated cell lines decreases in a dose dependent manner. Bar graph showing the number of colonies in solvent control (DMSO), 1.0–7.0 μ M treated Atorvastatin samples (B&C) in UM-SCC-1 and -47 respectively. *Statistically significant $p < 0.05$.

Figure 6.

Flank model showing (A) Tumor formation in SCID mice upon Atorvastatin treatment. (B) Tumor growth curve of placebo and Atorvastatin treatment group of mice. (C) Bar graph showing the average weight of the tumors at the termination of experiments. (D) H&E staining of the tumor showing tumor integrity both in control and Atorvastatin treated group of mice. (E) Assessment of microvessel density in control and Atorvastatin treated mice using immunostaining with CD31 antibody show well-developed microvessels in control, but poorly developed ones in Atorvastatin treated mice (magnification $\times 40$). (F) Quantitative presentation of the average number of vessels density in control and treatment group of animals.

Figure 7.

The lung sections stained with H&E dye to show the degree of metastasis. (A) Huge and significant number of metastatic lesions observed in lungs of control mice (a and b) while few and poorly developed metastatic lesions are present in the Atorvastatin treated group of mice (c and d) (magnification $\times 40$). (B) Bar graph showing the metastatic lung nodules per animal. *Statistically significant ($p < 0.05$).