Simvastatin-dependent apoptosis in Hodgkin's lymphoma cells and growth impairment of human Hodgkin's tumors *in vivo*

Bastian von Tresckow, Elke Pogge von Strandmann, Stephanie Sasse, Samir Tawadros, Andreas Engert, Hinrich P. Hansen

From the Laboratory of Immunotherapy, Clinic I of Internal Medicine, University Hospital Cologne, Cologne, Germany, Center for Molecular Medicine, University Hospital Cologne, Cologne, Germany.

Acknowledgments: we would like to thank Mrs. Anne Kruessmann and Mr. Hermann Straub for their expert technical assistance.

Funding: supported by grants from the Koeln Fortune Program/Faculty of Medicine, University of Cologne (102/2005) (BvT) and the Deutsche Krebshilfe (106473) (HPH).

Manuscript received November 9, 2006.
Manuscript accepted March 8, 2007.

Correspondence:
Bastian von Tresckow, M.D., University Hospital Cologne, Laboratory of Immunotherapy, Department of Internal Medicine I, LFI-Eb.4, room 703, Kerpener Str. 62, 50924 Cologne, Germany.
E-mail: bastian.von-tresckow@uk-koeln.de

ABSTRACT

Statins are used to treat hypercholesterolemia and seem to have a preventive effect against cancer through pleiotropic effects including prenylation-inhibition. So far nothing is known about the activity of statins or more specific prenylation-inhibitors in Hodgkin's lymphoma (HL). We, therefore, evaluated the anti-HL activity of simvastatin and specific prenylation-inhibitors. Two µM Simvastatin induced caspase-related apoptosis via depletion of prenylation-substrates in several HL-cell lines. Furthermore, it effectively impaired tumor growth in a mouse model for HL. Since the prenylation-inhibitors FTI-277 and GGTI-298 were also effective against HL-cells, we conclude that statins and specific prenylation-inhibitors should be evaluated in HL patients.

Key words: lymphoma, apoptosis, growth control, statins.

Haematologica 2007; 92:682-685

Today, most patients with Hodgkin's lymphoma (HL) can be cured, but the treatment of patients with progress or relapse needs to be improved. In addition, late treatment-related toxicities are seriously affecting up to 30% of patients. Hence, new therapies should be established. There is growing evidence for a preventive effect of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA)-reductase-inhibitors, known as statins, in a wide range of cancers.[1] Recently, the EPILYMPH study indicated a reduced risk of lymphoma, including HL, in statin-treated patients.[2] Statins are normally used to treat hypercholesterolemia and prevent cardiovascular disease. They block the conversion of HMG-CoA to mevalonate and thus the synthesis of downstream mevalonate products like farnesyl-transferase (FTase) substrate farnesyl-pyrophosphate and geranylgeranyl-transferase (GGTase) substrate geranylgeranyl-pyrophosphate. By depleting these isoprenoids, statins inhibit prenylation of anti-apoptotic proteins such as ras and ras-related molecules and induce apoptosis in several malignancies. More specific prenylation-inhibitors are currently being evaluated in clinical trials.[3] So far nothing is known about the therapeutic action of statins or specific prenylation-inhibitors in HL. We report how simvastatin promotes caspase-related apoptosis in HL-cells via the isoprenoid pathway and exhibits high anti-tumor activity in a xenograft model for human HL. Both statins and prenylation-inhibitors could therefore have preventive or therapeutic potential for the treatment of HL.

Design and Methods

Cell lines, antibodies and reagents

HL-cell lines (DEV, KM-H2, L540, L540Cy, HD-LM2, L428) and the erythroleukemia cell line K562 have been previously described.[4,5] All *in vitro* experiments used an initial concentration of cells of $2x10^4$/mL. Anti-poly (ADP-ribose) polymerase (PARP) antibody (#9542) was from Cell Signalling Technology. Anti-Actin antibody (sc-1616-R) was from Santa Cruz. Annexin V-FITC Apoptosis Detection Kit I was from BD Biosciences. Suicide-Track™ DNA Ladder Isolation Kit, simvastatin (sodium salt), FTI-277 and GGTI-298 were from Calbiochem. Complete protease

inhibitor tablets were from Roche. All other reagents were from Sigma. Dimethylsulfoxid (DMSO) was used to prepare stock solutions of simvastatin (5 mg/mL), FTI-277 (4.46 mg/mL) and GGTI-298 (4.46 mg/mL). Ethanol was used to prepare stock solutions of Farnesol (50 mg/mL) and Geranylgeraniol (50 mg/mL).

XTT assay

XTT assay was performed as reported.[6] After incubation of 200 μL cell aliquots in 96-well plates, 50 μL of culture medium with tetrazolium compound and electron-coupling reagent was added. Absorbance was measured after 20 h.

Apoptosis assays

Annexin V-binding assay, DNA ladder isolation and immunoblotting of PARP were performed according to the manufacturers' instructions.

Xenograft model of human HL

The subcutaneous xenograft model of human HL has been previously described.[6] Simvastatin tablets (ZOCOR, MSD) were mixed with food and pressed into pellets by Altromin GmbH at a ratio of 1.67 mg of simvastatin per 1 g of food. Untreated animals received pellets without simvastatin. Simvastatin-pellets were mixed in different ratios with untreated food, and uptake was measured by weighing the remaining simvastatin-pellets twice weekly. The Institutional and national guides for the care and use of laboratory animals were observed.

Results and Discussion

Anti-tumor activity of simvastatin in HL-cells via caspase-related apoptosis

Cellular viability was analyzed after simvastatin treatment. Four out of six HL-cell lines were sensitive to simvastatin in the XTT assay (IC50 < 2 μM) and the Annexin V-binding assay (Figures 1A-B). Apoptosis induction was verified by DNA laddering in L540 cells (Figure 1C). Increased DNA laddering after simvastatin treatment was also detectable in L540Cy cells but very weak in DEV and KM-H2 cells (*data not shown*). Whereas L540 and L540Cy cells displayed constitutive DNA laddering dependent on cell number (Figure 1C, lane 2). These apoptotic DNA fragments were barely detectable in DEV and KM-H2 cells even when large amounts were analyzed. Therefore, DNA laddering might not be a suitable assay for detection of simvastatin-related apoptosis in the latter cell lines. Consequently, a PARP cleavage immunoblot was performed (Figure 1D). The increase in the 89 kD cleaved PARP fragment in simvastatin treated KM-H2 cells confirmed caspase-related apoptosis[7] to be the major mechanism of simvastatin-induced cell death.

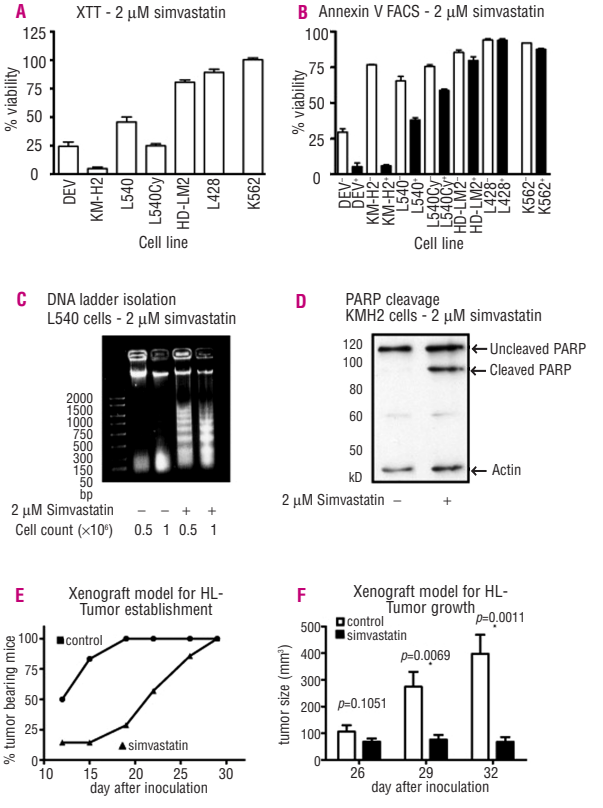

Figure 1. Apoptosis-related cytotoxicity of simvastatin in HL *in vitro* and *in vivo*. A. XTT assay: Viability (% untreated) of a panel of HL-cell lines and the control cell line K562 after 6 days exposure to 2 μM simvastatin. Mean ± standard deviation (SD) of at least three independent experiments. B. Annexin V-binding assay after 5 days exposure to 2 μM simvastatin (+) or vehicle (-) incubation: HL-cell lines and the control cell line K562 (open columns – untreated; filled columns - treated). Means and SD of two independent experiments. C. DNA ladder isolation after 7 days exposure of L540 cells to 2 μM simvastatin (+) or vehicle (-) incubation. Number of analyzed cells is indicated. First lane: base pair (bp) marker. (D) PARP immunoblot of whole KM-H2 cell lysate of 4×10^4 cells per lane in SDS-PAGE sample buffer (45 mM Tris-HCl, 10% glycerol, 1% SDS, 0,01% bromphenol blue, 50 mM DTT, pH 6,8) after 3 days exposure as indicated. Actin was simultaneously stained for control of equal protein loading. Molecular weights of standard proteins are given. E-F. Xenograft model for human HL: Tumor establishment (%) in control (L) and treated (G) mice (E) and growth of established tumors (open columns – control; black columns – treated; means and standard error of the mean) with indication of statistical significance ($p<0.05$) by asterisks (F). *p* values were estimated with the unpaired, two-tailed *t* test using GraphPad™ Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Statin treatment started one day after inoculation of cells. Control group and treatment group consisted of seven animals each. For the first 14 days animals were fed low dose simvastatin (average uptake 64 mg/kg/day ranging from 51 mg/kg/day to 103 mg/kg/day) and for the following 17 days animals were fed high dose simvastatin (average uptake 202 mg/kg/day ranging from 163 mg/kg/day to 258 mg/kg/day). One animal in the control group was excluded because of signs of disease, and one animal in the treatment group died on day 32 of the experiment without any previous sign of disease.

Simvastatin treatment also increased PARP cleavage in L540 cells. However, high constitutive PARP cleavage in untreated L540Cy and DEV cells meant this test was not suitable to use for these cells (*data not shown*).

Statins have previously been reported to have cyto-

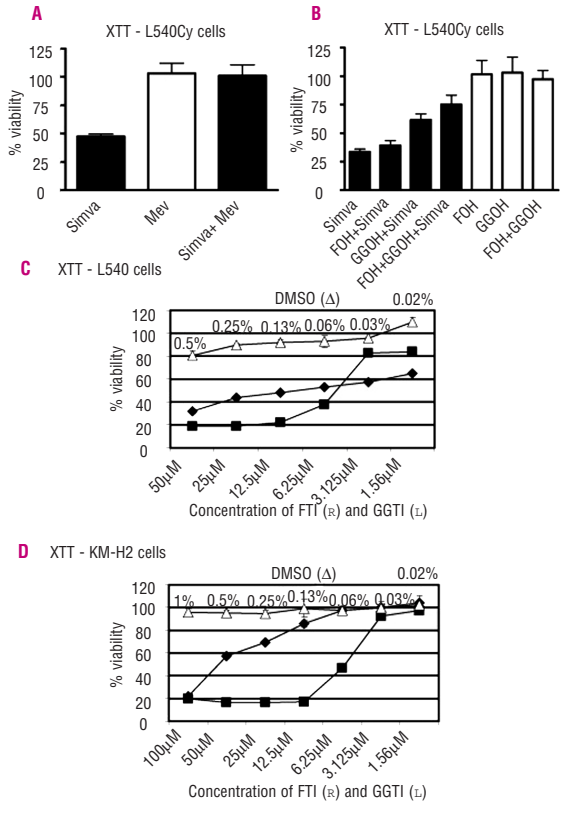

Figure 2. Prenylation-dependent cytotoxicity of simvastatin in HL-cells. (A-B) XTT assays after 5 days exposure of L540Cy cells to 2 μM simvastatin (Simva) ± 100 μM mevalonolactone (Mev) (A), 10 μM farnesol (FOH) and/or 10 μM geranylgeraniol (GGOH) (B). Mean (% untreated) ± SD of at least three independent experiments. (C-D) XTT assays after 48 hours exposure of L540 (C) or KM-H2 (D) cells to FTI-277 (R) or GGTI-298 (L) with specification of vehicle (DMSO, g) concentration as indicated. Mean (% untreated) ±SD of at least three independent experiments.

toxic effects in several malignant cell lines.[1] However, with few exceptions, these effects required higher simvastatin concentrations (5-400 μM).[8,9] High doses of statins in humans could achieve plasma levels in the 2 μM range.[10,11] This concentration induced apoptosis in our experiments. Thus, these results strongly suggest the efficacy of statins against HL *in vivo*.

Anti-tumor activity of simvastatin in a xenograft model of human HL

To assess the effect of simvastatin in HL *in vivo*, mice were given food containing simvastatin, starting one day after the inoculation of L540Cy tumor cells. Tumor establishment and growth were compared with control animals. For the first 14 days, mice received simvastatin in a preventative dose of an average of 64 mg/kg/day. For the following 17 days, animals received high therapeutic doses of simvastatin (average dose 202 mg/kg/day). This led to a significant impairment of both tumor establishment (Figure 1E) and growth (Figure 1F). On day 19 after inoculation, all mice in the control group (6/6) had tumors compared with two animals in the treatment group (2/7). Only by day 29 had all mice in the treatment group developed tumors. Measurement of established tumors beginning at day 26 revealed that the average tumor-volume of control mice increased four-fold in 6 days, while there was no increase in the tumor-volume of treated mice.

These data confirm that simvastatin is effective in a xenograft model of human HL. To date, there have been few reports of successful *in vivo* experiments with statins in xenograft models for human hematological malignancies.[12] This could be because effective statin concentrations are difficult to obtain *in vivo*. In a recent study in patients with relapsed or refractory myeloma or lymphoma, high-dose simvastatin (15 mg/kg/d) given immediately prior to chemotherapy has been found to be safe and tolerable.[13] Since mice metabolize statins more rapidly than humans, a dose of 15 mg/kg/d is comparable to 202 mg/kg/d used in our mouse model.[14] Hence, high dose simvastatin before chemotherapy is a promising combination for the treatment of patients with HL.

Given the preventive effect of statins reported in the EPILYMPH study and the results of this report, it could be interesting to evaluate simvastatin at a dosage of 80 mg/d as a preventative drug for high risk HL patients in remission. Furthermore, statins have been seen not only to increase antitumor action but also to reduce cardiotoxicity of doxorubicin in tumor models in animals.[15] Because doxorubicin is part of most regimens used in HL patients and heart failure is one of the most common late toxicities, it would also be interesting to evaluate a potential cardio-protective effect of simvastatin in HL patients.

Anti-tumor activity of prenylation-inhibitors in HL-cells

Besides inhibiting HMG-CoA conversion to mevalonate and causing subsequent inhibition of protein prenylation,[16] induction of apoptosis by statins has been attributed to pleiotropic effects such as inhibition of the proteasome, inhibition of surface molecule interactions, or displacement of signalling proteins from lipid rafts[1]. The complete reversal of simvastatin-induced cell death by 100 μM of the mevalonate precursor mevalonolactone (Figure 2A) indicates that the effect of simvastatin in HL depends on HMG-CoA reductase.[14] Since farnesol and geranylgeraniol, which are precursors of the mevalonate downstream metabolites farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate respectively, both led to a partial inhibition of simvastatin-induced cell death (Figure 2B) we hypothesized that prenylation-inhibitors also have a cytotoxic effect on HL cells. Indeed, the FTase-inhibitor FTI-277 and the GGTase-inhibitor GGTI-298 induced dose-dependent cell death in two HL-cell lines (Figures 2C-D). Only inhibitor concentrations up to 50 μM were tested in L540 cells because of the significant toxicity

encountered in the vehicle DMS at >0.5%. However, in KM-H2 cells, inhibitor concentrations up to 100 μM could be tested (Figures 2C-D). In pre-clinical tests, the FTase-inhibitor FTI-277 acted on malignancies[17,18] and the well-tolerated therapeutic FTase-inhibitor tipifarnib is especially effective in hematological neoplasia.[3] It is important to note that FTI-277 in HL-cells exhibits a relatively high cytotoxicity, whereas it has little effect on the proliferation of Non-HL-cell lines.[8] In conclusion, therapeutic FTase-inhibitors like tipifarnib in HL merit clinical evaluation. Indeed, this study provides further evidence that HL cells are sensitive not only to an FTase-inhibitor but also to a GGTase-inhibitor. Consequently, therapeutic dual FTase and GGTase-I-inhibitors[19,20] could be even more effective in HL.

Authors' Contributions

BvT designed and performed research, analyzed data and wrote the paper; EPvS, AE and HPH designed research and analyzed data; SS and ST performed research and analyzed data; and all authors checked the final version of the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Demierre MF, Higgins PD, Gruber SB, Hawk E, Lippman SM. Statins and cancer prevention. Nat Rev Cancer 2005;5:930-42.
2. Fortuny J, de Sanjose S, Becker N, Maynadie M, Cocco PL, Staines A, et al. Statin use and risk of lymphoid neoplasms: results from the European Case-Control Study EPILYMPH. Cancer Epidemiol Biomarkers Prev 2006;15:921-5.
3. Mesa RA. Tipifarnib: farnesyl transferase inhibition at a crossroads. Expert Rev Anticancer Ther 2006; 6: 313-9.
4. Klimka A, Barth S, Drillich S, Wels W, van Snick J, Renauld JC, et al. A deletion mutant of Pseudomonas exotoxin-A fused to recombinant human interleukin-9 (rhIL-9-ETA') shows specific cytotoxicity against IL-9-receptor-expressing cell lines. Cytokines Mol Ther 1996;2:139-46.
5. von Kalle C, Wolf J, Becker A, Sckaer A, Munck M, Engert A, et al. Growth of Hodgkin cell lines in severely combined immunodeficient mice. Int J Cancer 1992;52:887-91.
6. Boll B, Hansen H, Heuck F, Reiners K, Borchmann P, Rothe A, et al. The fully human anti-CD30 antibody 5F11 activates NF-{kappa}B and sensitizes lymphoma cells to bortezomib-induced apoptosis. Blood 2005; 106:1839-42.
7. Soldani C, Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: an update. Apoptosis 2002;7:321-8.
8. van de Donk NW, Schotte D, Kamphuis MM, van Marion AM, van Kessel B, Bloem AC, et al. Protein geranylgeranylation is critical for the regulation of survival and proliferation of lymphoma tumor cells. Clin Cancer Res 2003;9:5735-48.
9. Katano H, Pesnicak L, Cohen JI. Simvastatin induces apoptosis of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines and delays development of EBV lymphomas. Proc Natl Acad Sci USA 2004;101: 4960-5.
10. Thibault A, Samid D, Tompkins AC, Figg WD, Cooper MR, Hohl RJ, et al. Phase I study of lovastatin, an inhibitor of the mevalonate pathway, in patients with cancer. Clin Cancer Res 1996;2:483-91.
11. Holstein SA, Knapp HR, Clamon GH, Murry DJ, Hohl RJ. Pharmacodynamic effects of high dose lovastatin in subjects with advanced malignancies. Cancer Chemother Pharmacol 2006;57:155-64.
12. Clutterbuck RD, Millar BC, Powles RL, Newman A, Catovsky D, Jarman M, et al. Inhibitory effect of simvastatin on the proliferation of human myeloid leukaemia cells in severe combined immunodeficient (SCID) mice. Br J Haematol 1998;102:522-7.
13. van der Spek E, Bloem AC, van de Donk NW, Bogers LH, van der Griend R, Kramer MH, et al. Dose-finding study of high-dose simvastatin combined with standard chemotherapy in patients with relapsed or refractory myeloma or lymphoma. Haematologica 2006; 91: 542-5.
14. Cohen JI. HMG CoA reductase inhibitors (statins) to treat Epstein-Barr virus-driven lymphoma. Br J Cancer 2005;92:1593-8.
15. Feleszko W, Mlynarczuk I, Balkowiec-Iskra EZ, Czajka A, Switaj T, Stoklosa T, et al. Lovastatin potentiates antitumor activity and attenuates cardiotoxicity of doxorubicin in three tumor models in mice. Clin Cancer Res 2000;6:2044-52.
16. Morgan MA, Sebil T, Aydilek E, Peest D, Ganser A, Reuter CW. Combining prenylation inhibitors causes synergistic cytotoxicity, apoptosis and disruption of RAS-to-MAP kinase signalling in multiple myeloma cells. Br J Haematol 2005; 130: 912-25.
17. Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD, Penn LZ. Blocking protein geranylgeranylation is essential for lovastatin-induced apoptosis of human acute myeloid leukemia cells. Leukemia 2001;15:1398-407.
18. Bolick SC, Landowski TH, Boulware D, Oshiro MM, Ohkanda J, Hamilton AD, et al. The farnesyl transferase inhibitor, FTI-277, inhibits growth and induces apoptosis in drug-resistant myeloma tumor cells. Leukemia 2003;17:451-7.
19. Martin NE, Brunner TB, Kiel KD, DeLaney TF, Regine WF, Mohiuddin M, et al. A phase I trial of the dual farnesyltransferase and geranylgeranyltransferase inhibitor L-778,123 and radiotherapy for locally advanced pancreatic cancer. Clin Cancer Res 2004;10:5447-54.
20. McCormack P, Macpherson M, Wilson D, Lindsay A, Parry T, Holt A, et al. Pharmacokinetic characterization of the novel oral prenyl transferase inhibitor AZD3409: The first analysis in healthy male volunteers. J Clin Oncol (Meeting Abstracts) 2004;22 Suppl 14:3172-5.