Gene signature indicates different antineoplastic activities of statins and bisphosphonates

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INTRODUCTION

The aim of this study was to identify the molecular mechanisms and biological pathways associated with the anticancer effects of statins and bisphosphonates, which are known to downregulate the farnesylation and geranyl-geranylation of essential membrane-associated signal-transducers such as RAS and RHO proteins.
Transcriptomic, proteomic and methylomic analyses were done from the neoplastic cell lines MDA-MB-231 breast cancer, PC-3 prostate carcinoma, MG-63 and U2-OS osteosarcoma and HMC-1 mast cell leukemia being treated for 3 days with pharmacologic doses with a representative statin (simvastatin) and a bisphosphonate (ibandronate). Bioinformatic analyses involved the gene set enrichment analysis (GSEA) and Pathvisio software as pathway recognition algorithms.

A higher percentage of microRNAs ist upregulated with statin

The mean percentage of significantly downregulated microRNAs in a total of 1199 microRNAs which were detectable in our gene-chips was 14.8% in simvastatin-treated and 14.2 % in ibandronate-treated cell lines.
MicroRNA34a, which regulates the NAD$^+$-dependent histone deacetylase SIRT1 [1] as well as HDAC1 and HDAC7 [2] was **downregulated** with simvastatin or ibandronate in all cancer cell lines investigated in this study, but most significantly in simvastatin-treated MDA-MD-231 cells.
The mean percentage of significantly upregulated microRNAs in a total of 1199 microRNAs which were detectable in our gene-chips was 21.9% in simvastatin-treated and 14.4 % in ibandronate-treated cell lines.
The most significantly **upregulated** microRNA in simvastatin-treated MDA-MB 231 cells was **microRNA612**, which is known to reduce stemness and to promote resistance against 5-fluorouracil in cancer cells [3]. MicroRNA612 was also significantly upregulated in simvastatin-treated PC-3 cells as well as in MG-63 and HMC-cells, which had been treated with simvastatin, ibandronate or decitabine.

MIR612	Basal	Treat	Fold stim
HMC Sim	7,13	7,86	1,65
MGSim	5,9	6,6	1,65
PCSim	8,1	9	1,82
MDASim	7,9	9,4	2,88
MDAIbn	7,9	7,7	-1,15
HMC Ibn	7,13	7,61	1,4

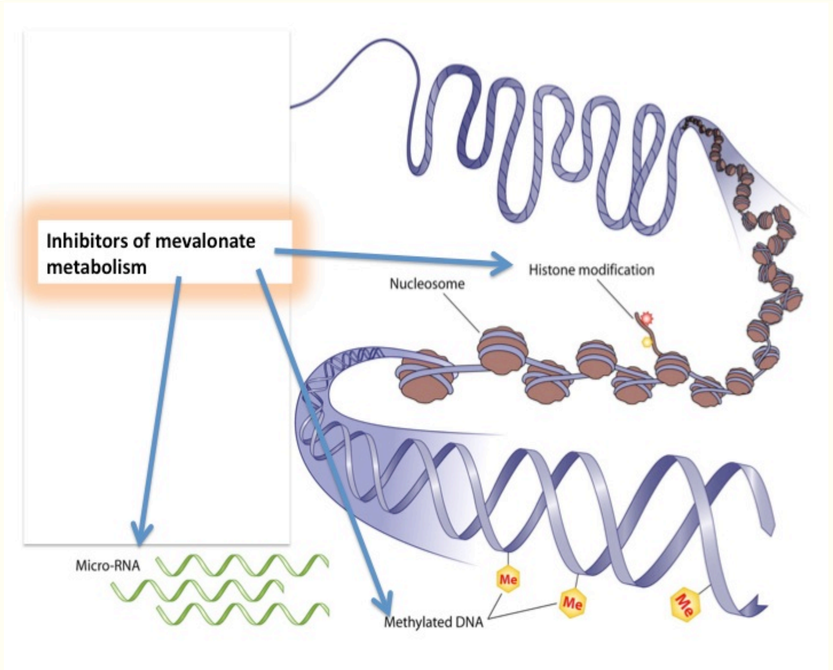
Simvastatin upregulates microRNA612, which is known to promote sensitivity against the thymidilate synthase (=TYMS)-inhibitor 5-fluorouracil.

Abbreviations: Simvastatin treated: HMC Sim = HMC1.1, MGSIm = MG63, PCSim = PC3, MDASim = MDA-MB-231; Ibandronate – treated: MDAIbn = MDA-MB-231; HMC Ibn = HMC 1.1

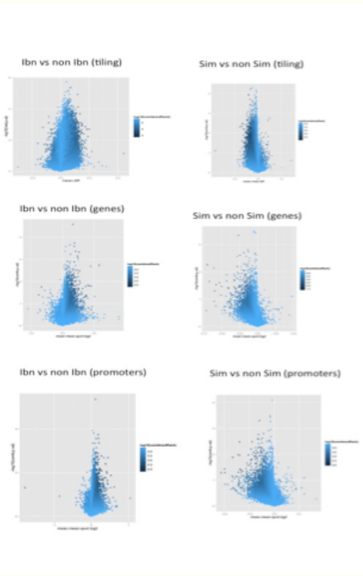
Literature

[1] Tabuchi T, Satoh M, Itoh T, Nakamura M. Micro-RNA-34a regulates the longevity-associated protein SIRT1 in coronary artery disease: effect of statins on SIRT1 and microRNA-34a expression. Clin Sci (Lond) 2012; 123:161-71.
[2] Wu MY, Fu J, Xiao X, Wu J, Wu RC. MiR-34a regulates therapy resistance by targeting HDAC1 and HDAC7 in breast cancer. Cancer letters 2014;354:311-9.
[3] Tang J, Tao ZH, Wen D, Wan JL, Liu DL, Zhang S, Cui JF, Sun HC, Wang L, Zhou J, Fan J, Wu WZ. MiR-612 suppresses the stemness of liver cancer via Wnt/beta-catenin signaling. Biochem Biophys Res Commun 2014;447:210-5.

Epigenetic modifications

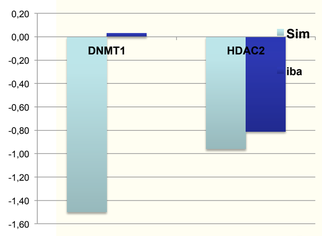


The higher rate of Simvastatin-upregulated genes could be a result from a higher rate of Simvastatin-associated promoter-demethylation & a changed NAD(P)/ NAD(P)H - relation

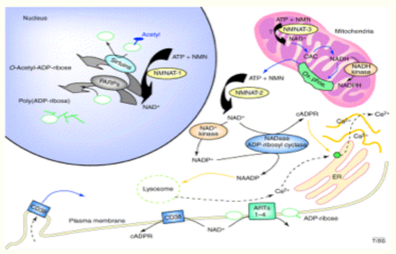


DNMT1	Sim	Iba
U2OS	-1,47	-1,21
MG63	1,06	1,02
PC3	-2,47	-1,73
MDA	-2,56	1,00
HMC	-2,06	1,09
mean FC	-1,50	0,03

HDAC2	Sim	Iba
U2OS	-1,38	-1,06
MG63	-1,30	-1,23
PC3	-1,44	-1,09
MDA	1,17	-1,65
HMC	-1,84	1,00
mean FC	-0,96	-0,81



NAD(P) biosynthesis and major NAD(P)–mediated signaling pathways in eukaryotic cells,
(modified according to Berger et al, TRENDs in Biochemical Sciences 2004 (Vol 29, p 111ff).



Simvastatin and Ibandronate induce **upregulation of the NMNAT (Nicotineamide mononucleotide acetyl-transferase)**, which synthesizes NAD from ATP and Nicotineamide mono-nucleotide)

Abbreviations: Simvastatin treated: HMC Sim = HMC1.1, MGSIm = MG63, PCSim = PC3, MDASim = MDA-MB-231; Ibandronate – treated: MDAIbn = MDA-MB-231; HMC Ibn = HMC 1.1

The drug-induced reduction of NADPH-levels appears to be associated with a downregulation of **NNT (Nicotineamide nucleotide transhydrogenase)** in the mitochondrial membrane

MNAT1 produces NAD

	basal	treated	fold stim
U2OS-Ibn	6,0	6,2	1,18
MG-63-Ibn	7,3	7,9	1,50
PC3-Ibn	6,7	6,9	1,15
MDA-Ibn	7,6	7,6	1,05
U2OS-Sim	5,6	6,1	1,37
MG-63-Sim	7,3	7,4	1,07
PC3-Sim	7,6	7,6	1,02
MDA-Sim	7,6	7,7	1,14

NNT makes NADPH from NADP

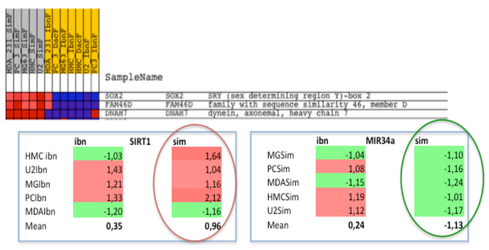
	basal	treated	fold stim
U2OS-Ibn	10,5	10,5	-1,01
MG-63-Ibn	10,7	10	-1,61
PC3-Ibn	5,8	5,6	-1,2
MDA-Ibn	10,6	10,3	-1,2
U2OS-Sim	10,4	10,4	-1,03
MG-63-Sim	10,7	10,5	-1,12
PC3-Sim	7,2	7,3	1,04
MDA-Sim	10,6	9,3	-2,53

Results from GSEA (Gene Set Enrichment) analyses

Simvastatin regulates EMT (epithelial mesenchymal transition):

1. The SOX2-SIRT1-MIR34a axis
SOX2 is crucial for the multipotency of mesenchymal stem cells

Our data confirm an association of SOX2 with **statin-mediated activation of SIRT1*** and **downregulation of MIR34a****



	ibn	SIRT1	sim
HMC Ibn	-1,03		1,64
U2Ibn	1,43		1,04
MGIbn	1,21		1,16
PCIbn	1,33		2,12
MDAIbn	-1,20		-1,16
Mean	0,35		0,96

	ibn	MIR34a	sim
MGSim	-1,04		-1,10
PCSim	1,08		-1,16
MDASim	-1,15		-1,24
HMCSim	1,19		-1,01
U2Sim	1,12		-1,17
Mean	0,24		-1,13

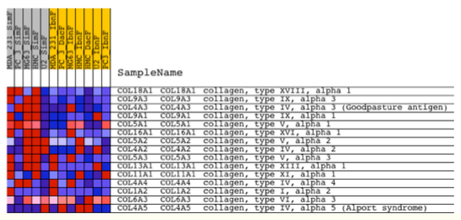
*Yoon DS et al, Stem Cells 32:3219-31, 2014; ** Tabuchi T et al, Clin Sci 123: 161-171, 2012
>>Data on statin-mediated inhibition of EMT in peritoneal dialysis are discussed by Chang TI et al, PlosOne, October 2014

Simvastatin supports EMT (epithelial mesenchymal transition):

1. The SOX2-SIRT1-MIR34a axis

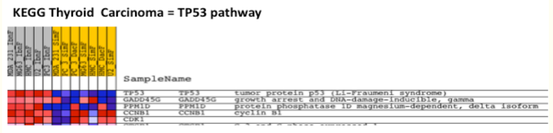
2. Simvastatin upegulates the majority of collagens

up to 11 von 15 Collagen-types (in HMC) are upregulated according to Geneontology (C5) SIM

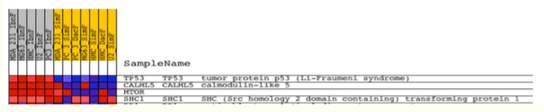


Top- regulated genes by ibandronate are associated with **DNA-repair**

KEGG Thyroid Carcinoma = TP53 pathway



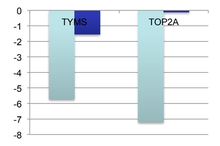
KEGG IBN Glioma= TP53 pathway



Does the co-regulation of DNMT1 with TYMS and TOP2A* play a role?
The DNA-damage – associated genes (**downstream targets of RAC, Rho-kinase and Rab-family** genes**) **TYMS** (thymidilate synthase) and **TOP2A** (topoisomerase 2A) are more efficiently downregulated with Simvastatin as compared to Ibandronate

TYMS	Sim	Iba
U2OS	-1,07	-1,05
MG63	1,00	-1,21
PC3	-7,82	-3,18
MDA	-12,40	-1,23
HMC	-8,21	-1,02
mean FC	-5,70	-1,54

TOP2A	Sim	Iba
U2OS	-1,13	1,02
MG63	-1,19	1,06
PC3	-15,08	-2,59
MDA	-14,97	-1,17
HMC	-3,58	-1,10
mean FC	-7,19	-0,12



Is the Ibandronate (and IPP induced genoprotection) mediated stimulation of TP53 responsible for this difference ???

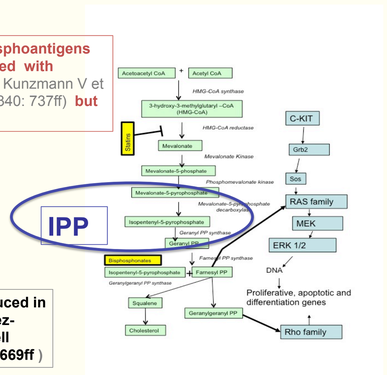
- ***Apostolou** P et al, 2014, PLOSone 9: e109741ff.
- ****Coudray** AM et al 2005, Int J Oncol 27:553ff; **Wartlick** F et al. 2013, BBA 1833: 3093ff (>TOP2A independent of TP53); **Huelsenbeck** SC 2012, JBC 287: 38590ff.; **Werner** M 2013, Naunyn-Schmiedeberg's Arch Pharmacol 386: 605ff. (simvastatin), **Okamoto** S 2014, Cell Death and Disease 5: e1517)

Anti inflammatory action of simvastatin

TP53 is upregulated with ibandronate but not with simvastatin:

Pro-inflammatory phosphoantigens like isopentenyl pyrophosphate (IPP) are downregulated with statins but not with bisphosphonates

T-cell stimulating phosphoantigens like IPP are accumulated with bisphosphonates (e.g. Kunzmann V et al, 1999, N Engl J Med 340: 737ff) **but not with statins**



Statins prevent bisphosphonate-induced , T-cell proliferation and activation in vitro (Thompson K & Rogers MJ, 2004 JBMR 19: 278ff) & is this associated with **reduced genoprotection resulting from a lack of IPP**? (Ling S et al, 2004, Mutation Research 554: 33ff.)

TP53 is known to be induced in activated T-cells (Sanchez-Jimenez C et al, 2015, Cell Death and Disease 6: e1669ff)

TP53 Expression

	Basal Control	Basal Treat	Fold Change
U2Ibn	9,3	9,7	1,30
MGIbn	10,6	11,2	1,46
PCIbn	7,1	7,3	1,09
MDAIbn	10,1	10,2	1,08
HMC Ibn	11,1	11,2	1,06
U2Sim	9,3	9,1	-1,11
MGSim	10,6	10,5	-1,06
PCSim	8,3	8,2	-1,09
MDASim	10,1	9,7	-1,28
HMC Sim	11,1	10,5	-1,56

IPP also acts as a genoprotective agent independently of TP53 (Ling, S. et al. 2004 Mutation Research 554: 33ff.)

Statin and ibandronate downregulate anti-EMT genes

Anti - EMT (epithelial mesenchymal transition)-genes from the PRC (polycomb related complex) are downregulated by both simvastatin and ibandronate

EZH2	Basal Control	Basal Treat	Fold Exp
U2Ibn	8,7	8,4	-1,25
MGIbn	10,0	10,0	-1,05
PCIbn	9,2	8,5	-1,62
MDAIbn	9,4	9,3	-1,06
U2Sim	8,7	8,4	-1,23
MGSim	10,0	9,8	-1,18
PCSim	9,7	8,8	-1,91
MDASim	9,4	8,3	-2,21

RBBP4	Basal Control	Basal Treat	Fold Exp
U2Ibn	12,0	11,9	-1,08
MGIbn	10,6	10,4	-1,12
PCIbn	11,8	11,4	-1,35
MDAIbn	10,1	10,0	-1,12
U2Sim	11,9	11,8	-1,06
MGSim	10,6	10,4	-1,12
PCSim	10,5	9,9	-1,44
MDASim	10,1	9,2	-1,90

RBBP7	Basal Control	Basal Treat	Fold Exp
U2Ibn	11,3	11,0	-1,17
MGIbn	10,4	10,3	-1,06
PCIbn	11,6	11,3	-1,30
MDAIbn	10,3	10,2	-1,09
U2Sim	11,2	11,1	-1,08
MGSim	10,4	10,4	-1,02
PCSim	10,1	9,8	-1,26
MDASim	10,3	9,1	-2,29

EZH2 = Enhancer Of Zeste Homolog 2, histone Lysine N-Methyltransferase

RBBP2 (or RBBP4) = Retinoblastoma Binding Protein, Histone Binding Protein

Conclusion

1. Inhibitors of the mevalonate pathway regulate the 3 main epigenetic levels, namely
   - DNA-(de)methylation,
   - Regulation of HDACs and
   - microRNAs.
2. However, there is a stronger impact of statin on the promotion of mediators of differentiation and downregulation of factors that are associated with DNA-repair.
3. Respective metabolites such as NADP/ NADPH which in turn regulate epigenetic enzymes and a downregulation of anti-EMT genes by both types of drugs appear to play key roles in this network.

MATERIALS AND METHODS

Cell culture and treatment : Cells were cultured in cell culture flasks at 37°C and 5% CO_2 . The culture media were as recommended by ATCC for (MDA-MB-231 breast cancer DMEM containing 10% fetal calf serum (FCS), PC-3 prostate carcinoma DMEM-F12 with 10% FCS,
MG-63 and U2-OS osteosarcoma were cultured in AlphaMEM **medium** containing 10% FBS. For the HMC1.1 cell line, we used Iscove's modified Dulbecco's medium (IMDM) . thioglycerol (260 nM) with 20% of foetal bovine serum (FBS). All culture media contained 10 µg/mL gentamycin (Sigma). To guarantee optimal growth, cells were splitted two times a week and reseeded at a density of 2 – 5x10^5 cells/ml.
One day after splitting , 32 µM Simvastatin or 150 µM ibandronate were added to the culture medium for 72 hours.

NADP/NADPH analyses were performed directly in 96-well culture plates after 24, or 48 hours according to manufacturers instructions of the NADP/NADPH Glo Assay (Promega).
Gene expression analysis: For comparative analysis of selected genes we synthesized cDNA using the First Strand cDNA Synthesis Kit as described by the supplier (Roche). The obtained cDNA was subjected to PCR amplification with a real-time cycler (Corbett Research). FAM-labeled TaqMan gene expression probes & primers-sets (all from Applied Biosystems) were used according to the conditions suggested by the suppliers. For normalization of expression we used VIC labelled GAPDH and 18S TaqMan primer & probe-sets in the same reaction vial (GAPDH 4310884E, 18S 4319413E, Applied Biosystems). Quantification of mRNA within the samples was examined using the comparative Ct method (Livak *et al.*, 2001, *Methods* **25**(4)**:** 402-408).
Transcriptomics and methylomics analysis: Analysis and data evaluation for the Affymetrix Arrays (Type Human Gene 1.0 ST Array) were commercially obtained from an internationally certified institution (Kompetenzzentrum für Biofluoreszenz, Regensburg). "Pathvisio" software (van Iersel et al., 2008, *BMC Bioinformatics* **9:** 399). was applied for specific analyses of defined pathways from Affymetrix Arrays (Type Human Gene 1.0 ST Array).
Methylomics analyses were done on Illumina 450k chips, got in a collaboration with the Austrian Institute of Technology (AIT, Working group Andreas Weinhäusel and Walter Pulverer).

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