Imatinib and spironolactone suppress hepcidin expression

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 doi:10.3324/haematol.2016.162917

Received: December 27, 2016.

Accepted: April 5, 2017.

Pre-published: April 6, 2017.

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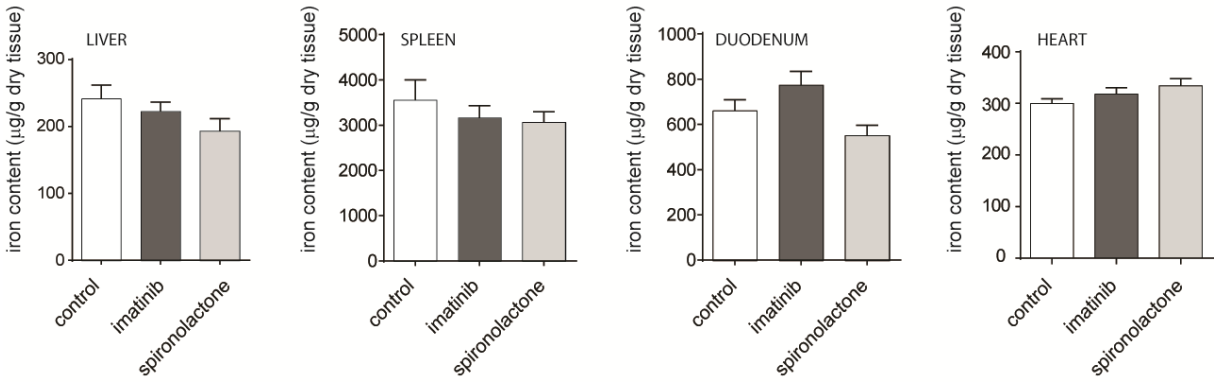
Supplementary Figure



Supplementary Figure 1. Non-heme iron content (µg iron/g dry tissue) in organs of mice administrated with drugs and respective controls. All data are presented as a mean ± SEM from 10 mice per treatment group.

Supplementary Tables

Drug	Supplier	Catalog number	Concentration in culture
Acetylsalicylic acid (Aspirin)	Sigma-Aldrich (Steinheim, Germany)	50-78-2	500 µM [1]
Amiloride hydrochloride hydrate	Sigma-Aldrich (Steinheim, Germany)	2016-88-8	50-200 µM
Captopril	Sigma-Aldrich (Steinheim, Germany)	62571-86-2	10 µM
Compound 2	Provided by prof. Olaf Witt, described in Krennhrubel et al.[2]		25 µM
Diclofenac sodium salt	Sigma-Aldrich (Steinheim, Germany)	15307-79-6	50-200 µM
Entinostat (MS-275)	Calbiochem, San Diego, CA	209783-80-2	1 µM
Imatinib Mesylate	Selleck Chemicals (Houston, TX, USA)	220127-57-1	1-10 µM
Lovastatin	Sigma-Aldrich (Steinheim, Germany)	75330-75-5	10 µM
Panobinostat (LBH 589)	Selleck Chemicals (Houston, TX, USA)	404950-80-7	100 nM [3]
Pentoxifyllin	Sigma-Aldrich (Steinheim, Germany)	64.93-05-6	100 µM [4]
Progesterone	Sigma-Aldrich (Steinheim, Germany)	57-83-0	1 µM
Ranitidine hydrochloride	Sigma-Aldrich (Steinheim, Germany)	666357-59-3	10 µM
Spironolacton	Sigma-Aldrich (Steinheim, Germany)	52-01-7	10-50 µM
Trapoxin A	Calbiochem, San Diego, CA	133155-89-2	80 nM
Vorinostat (SAHA)	Calbiochem, San Diego, CA	149647-78-9	0.5-1.5 µM [5]
Dicumarol [3,3′-Methylene-bis(4-hydroxycoumarin)]	Sigma-Aldrich (Steinheim, Germany)	66-76-2	100 µM
Eplerenone	Sigma-Aldrich (Steinheim, Germany)	E6657	0.5-20 µM
Qercetin	Sigma-Aldrich (Steinheim, Germany)	Q4951	12.5-50 µM

Table S1. List of pharmaceutical agents used in this study.

siRNA catalog number	gene symbol	gene accession numbers
MU-006147-00	CHRNE	NM_000080
MU-003109-02	CSF1R	NM_005211
MU-003129-01	FER	NM_005246
MU-009792-00	GPLD1	NM_001503
MU-005616-01	GRM1	NM_000838
MU-005623-01	GRM8	NM_000845
MU-003496-00	HDAC3	NM_003883
MU-004072-00	HDAC10	NM_032019
MU-009811-01	HMGCR	NM_000859
MU-008034-00	MPRG	NM_017705
MU-003158-01	MUSK	NM_005592
MU-019849-00	MYST3	NM_006766
MU-006334-00	NQO2	NM_000904
MU-005684-01	OPRK1	NM_000912
MU-008409-00	OSBPL10	NM_017784
MU-003163-02	PDGFRB	NM_002609
MU-008820-01	PLA2G4B	NM_005090
MU-008485-00	PLCB3	NM_000932
MU-004201-00	PLCE1	NM_016341
MU-008339-00	PLCG2	NM_002661
MU-005714-00	PTGER4	NM_000958
MU-004691-01	PTGIS	NM_000961
MU-003688-02	P2RY2	NM_002564
MU-003438-01	RARB	NM_000965
MU-007439-00	SLCO1A2	NM_021094
MU-007611-00	SLC7A10	NM_019849
MU-007459-00	SLC22A8	NM_004254
MU-007464-01	SLC24A1	NM_004727
MU-007560-00	SLC38A3	NM_006841
MU-003177-02	TEC	NM_003215
MU-006047-01	TLL2	NM_012465
MU-003181-01	TXK	NM_003328
MU-003182-01	TYK2	NM_003331
MU-008585-01	XDH	NM_000379

siRNA catalog number	gene symbol	gene accession numbers
MU-003902-01	SMAD4	NM_00535
MU-020068-01	SMAD7	NM_00590
MU-00354400	STAT3	NM_00315

Table S2. SiRNAs used in this work. SiRNAs listed on the right side served as controls for my validation assays. SiRNAs listed on the left side target the candidate genes from the genome-wide screening selected for validations.

Gene name	FW 5'-3'	RW 5'-3'
ACE2	ccagtggatgaaaaagtggtg	gtttcatcatggggcacag
ADORA1	gtcaagatccctctccggta	tcccaccacgaaggagag
ALOX15	agcctgatgggaaactcttg	aggtggtggggatcctgt
CHRNE	gcccagaccgtcttcttgt	accatgacgaaaataaggaacc
CLC	caacaatgtccctgctaccc	ttgattgtcacagtagaaccagtaga
CSF1R	tctggtcctatggcatcctc	gatgccagggtagggattc
CYSLTR1	actccagtgccagaaagagg	gcggaagtcatcaatagtgtca
FER	ggcttaaagcagattcccatt	caaagctccacacgtcactc
GABRA6	cccgtgtcagatgtggagat	ccccaaacttcaacctctcat
GPLD1	tccagccccttagcatctta	gcccatcctggttgaggt
GRIA4	gctggtggctttgatagagttc	tggcttcagaaaaggtcagc
GRM1	ctgcaaagcttgtgacttgg	aagatagcgcacaggaatgg
HMGCR	gttcggtggcctctagtgag	gcattcgaaaaagtcttgacaac
HTR2B	tgctggaggctcagaataagt	ttgcatgccagagagttcc
HTR3E	agctgcacctcttgtcatca	gggttccagctgataaatgg
KDR	gaacatttgggaaatctcttgc	cggaagaacaatgtagtctttgc
LTB4R2	gttgtttgggtcacctctgc	cccaccttctgcagtgtgta
MMP24	tctccagggcatccagaa	gagtgtagggagtggccttg
MPRG	cgggaggagtgtctggtct	cccagtttctccaatggtgt
MYST3	acaaagcccacgctgaag	tgtggtgtttgcgctttc
NQO2	ttgacatcccaggattctacg	ccgtggttacggaaaggag
OPRK1	ccttgaaggcaaagatcatca	tgcaaggagcactcaatgac
OSBPL10	acaccatggaagcccaaata	acctccaccccatcagtgt
OXTR	ggggagtcaactttaggttcg	ttcctcgggatgttcagc
P2RY11	gggaactgggtagcagacac	agttggcagggcaggact
P2RY12	tttgcctaacatgattctgacc	ggaaagagcatttcttcacattct
PDGFRB	cccttatcatcctcatcatgc	ccttccatcggatctcgtaa
PLA2G4B	ggcaacctaccagctaactgaa	aggtcctggacacctctgc
PLCB3	ccccaaaaagccaactacag	ctcctcagtggcattcacct
PTGER3	gatccttcttcgaaagttttgc	tggcagggtaaggaggtg
PTGER4	ctccctggtggtgctcat	ggctgatataactggttgacga
PTGIS	tgaaaaggccaggatgaaac	ggaggttggtatacatggcttc
RARB	tcggcacactgctcaatc	gaagcagggtttgtacactcg
SLC12A1	tccgtggaaaattacagatgc	cgaacttggcggtaactctt
SLC22A8	tctcacctttgtgcccttg	gcagctgaaggagctggata
TEC	gtgtatgaggtgatgctgagatg	cagcagatcttcgaaagaagg
TLL2	catcccctacgtcattggag	catggcctgcttaaaaatgg
TMPRSS2	cgctggcctactctggaa	ctgaggagtcgcactctatcc
TRPV5	ctgtccttcctggagcttgt	tggggtctgttccagaattt
TYK2	tggcttgcttgagttgacac	atccccaacgggcttact
XDH	aaccatctcagccctcaaga	agctcctccttccagagctt
GABRD	acggtggagaacaagctca	ggccacagtggaggtgat
GABRR1	tgcccacaagcaagtcag	ggtcatctatcctcagaagctgtt
GLRA1	aagcggagacatcacaagga	ccatcccataggcagagaag
GRM8	agctttcatccccatcttttt	gacagtaagtgttgttgtctggatg
HDAC10	tgggaagctcctgtacctctt	ggctggagtggctgctatac
HDAC3	gacctatgacaggactgatgagg	gaactcattgggtgcctctg

MUSK	cagatatgcaagcggactttc	tcccgacagcacacactc
P2RY2	gcaccctgagaggagaagc	gcatttttctgggcaggtag
PLA2G2E	cacgtgctggtgttccttt	tcatcaccccaaactgaacc
PLCE1	gtgactaagggcagcatgttt	tcaatttccagtttaagtctgatga
PLCG2	accccattaaagcagtcaaatc	atgtcctcgctgtacccatt
PSORS1C2	ctttgcctgcacaccaga	gcctcctctcggtcctct
SLC7A10	ggactacgcctacgtcacaga	ggtggggtacatgatgagga
SLCO1A2	ggggcatgcaggatatatga	tggaacaaagcttgatcctctta
HJV	cacccggaagctcaccat	taggggaagtgggtgtctc
JAK1	agtgccctgagctacttgga	cactgtcgattccctcacg
SMAD4	tggcccaggatcagtaggt	catcaacaccaattccagca
SMAD7	cgatggattttctcaaaccaa	attcgttccccctgtttca
STAT3	catatgcggccagcaaagaa	atacctgctctgaagaaact
TRAF4	ggaccagcttcctctggact	ggataggcaggcccaatact
GAPDH	catgagaagtatgacaacagcct	agtccttccacgataccaaagt
HEPCIDIN	ctctgttttcccacaacagac	taggggaagtgggtgtctc
mm hepcidin-1	ataccaatgcagaagagaagg	aacagataccacactgggaa
mm Rpl19	aggcatatgggcatagggaagag	ttgaccttcaggtacaggctgtg

Table S3. Primers used for quantitative PCR analysis.

Supplementary Methods

Cell culture

The Huh7 hepatocarcinoma cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose; Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated low-endotoxin fetal calf serum (FCS, Invitrogen), and 1% penicillin/streptomycin (PAA, Pasching, Austria).

Murine hepatocytes were isolated from hepatic lobes of male C57Bl/6N mice as described previously.[6] Briefly, liver lobes were perfused first with Liver Perfusion Medium and subsequently with Liver Digest Medium (both from Life Technologies). Perfused lobes were disrupted, the cell suspension was forced through a 100 μm Cell Strainer (Falcon-BD Biosciences), and after a washing step alive cells were separated from debris employing Percoll (GE Healthcare/Sigma) gradient centrifugation. Cells were washed, centrifuged and then plated onto collagen-coated plates (Corning - BioCoat) in William's medium E (Biochrom, Berlin, Germany) containing 100 nM dexamethasone (Sigma Aldrich), 10 μg/ml bovine insulin (Sigma-Aldrich, 1% GlutaMAX (Invitrogen), 10% heat-inactivated low-endotoxin FCS (Invitrogen) and 1% penicillin/streptomycin (PAA). After plating, cells were cultured for five to six hours to allow cell attachment. Afterwards, cells were washed vigorously with PBS and cultured in William's medium E (Biochrom, Berlin, Germany)

containing 1% L-glutamine (GlutaMAX, Invitrogen), 1% penicillin/streptomycin (PAA) and 10% heat-inactivated low-endotoxin FCS (Invitrogen).

Primary human hepatocytes were isolated from human liver tissue samples as described previously[7,8] and delivered overnight on ice, in cell suspension. Upon arrival, cells were washed twice with ice-cold PBS (centrifuged at 4°C, 5 min, 80 g) and then plated in Williams Medium E (Biochrom), containing 1% L-glutamine (GlutaMAX, Invitrogen), 1% penicillin/streptomycin (PAA), 0.8 μg/ml hydrocortisone (Sigma), 15 mM HEPES (Sigma), 1% sodium pyruvate (Invitrogen), 1.6 μg/ml human insulin (Sigma), 1% non-essential amino acids (Invitrogen) and 10% heat-inactivated low-endotoxin FCS (Invitrogen). Alive cells (as assessed by Trypan Blue staining) were plated onto collagen-coated plates (Corning - BioCoat). Seven hours after plating cells were washed with PBS and further cultured in Williams Medium E (Biochrom), containing: 1% L-glutamine (GlutaMAX, Invitrogen), 1% penicillin/streptomycin (PAA) and 10% heat-inactivated low-endotoxin FCS (Invitrogen).

All cell cultures were maintained at 37°C under 5% CO_2.

Pharmaceutical agents

Drugs used in this study are listed in Supplementary Table 1. Concentrations used in cell culture were as indicated in the table unless stated otherwise. Amiloride hydrochloride hydrate, Captopril, Diclofenac sodium salt, Imatinib Mesylate, Panobinostat, Pentoxifyllin, Ranitidine hydrochloride, Spironolacton, Trapoxin A, Vorinostat and 8-Methoxypsoralen were all prepared in DMSO. Lovastatin and Acetylsalicylic acid were prepared in 100% Ethanol. 3.3'-Methylene-bis (4-hydroxycoumarin) was dissolved in 0.13 M NaOH. The manufacturer's recommendations regarding storage and handling of drugs were followed. Stock solutions of all drugs were stored in aliquots at -20°C.

Amyloride hydrochloride hydrate was kindly provided by the laboratory of Prof. Dr. med. Marcus Mall (MMPU, Heidelberg, Germany). The panHDAC-inhibitors Vorinostat (SAHA), Trapoxin A, Panobinostat (LBH 589), Entinostat (MS-275) and Compound 2 were kindly provided by the laboratory of Prof. Dr. med. Olaf Witt (DKFZ, Heidelberg, Germany).

Drug administration in mice

SAHA

According to the studies reported in document NDA 21-991 [U.S. Food and Drug Administration (FDA) approval for the use of Vorinostat], the highest single oral dose of the

drug which does not cause significant side effects [the no-observed-adverse-effect-level (NOAEL)] in mice is 2000 mg/kg. In addition, the repeated dose toxicity studies in rats showed that within the first two weeks of treatment with a dose 50 mg/kg/day no weight loss, food consumption alterations or mortality was observed. We thus selected this dose for our experiments.

SAHA was added to the drinking water, as outlined in a study published by Hocly et al.[9] SAHA is difficult to dissolve in aqueous solutions. Thus, as reported in the study above, we increased SAHA solubility by complexing with 2-hydroxypropyl-β-cyclodextrin (HOPβ-CD), a well-known solubilizer of pharmaceuticals.[10] Applying the recommended ratio between SAHA and HOPβ-CD under the assumption that a mouse drinks approx. 3 ml of water per day, we dissolved 0.34 g SAHA and 9 g of HOβ-CD per 1 l water.

Diclofenac

According to study 840321 (4.2.3.2.1. eCTD Section), reported in the document NDA 22-122 (FDA approval for the use of Diclofenac), the highest oral dose of the drug which does not cause significant side effects (NOAEL) is 1 mg/kg/day (for 28 days). Based on these data we prepared a solution of the drug (Diclofenac sodium salt; Sigma) in drinking water, in order to obtain the desired dose (under the assumption that a mouse drinks approximately 3 ml water per day). Since solubility of diclofenac is also significantly increased by cyclodextrins,[10] we additionally added the same dose of HOβ-CD as for SAHA.

As a vehicle control for SAHA and diclofenac, mice administrated with the equal dose of HOPβ-CD were employed.

Imatinib

Imatinib mesylate is an antineoplastic agent for the treatment of chronic myeloid leukemia (CML) and other malignancies. The addition of 1.13 mM imatinib mesylate to drinking water of 14-week-old mice corresponds approximately to a dose of 75 mg/kg/day.[11] This dose induced biological effects (antiosteolytic activity)[11] and was comparable to a dose that prolonged survival in a murine CML model, without significant side effects.[12] Another study showed that this dose did not cause cardiac toxicity at the 2-week time point.[13] Therefore, we applied the dose of 75 mg/kg/day and we used acidified water (pH 5.5) to prepare solutions. Based on the fact that imatinib solubility is significantly increased by complexing with cyclodexrins,[10] we used HOPβ-CD (1.1 g/l) as a solubilizing agent.

Spironolactone

In a study by Voelkl et al, spironolactone was dissolved in drinking water at 80 mg/l and prevented pathological calcification.[14] In this study, spironolactone treatment was applied for a life-span without significant adverse effects. Here, we used a two-times higher dose of 160 mg/l water for a 2-week treatment interval. This dose corresponds to approx. 24 mg/kg/day. According to the Material Safety Data Sheet for Aldactone (spironolactone-containing medicine; Pfizer), the lowest-observed-adverse-effect level (LOAEL) dose for oral spironolactone administration in rats is 50 mg/kg/day for 78 weeks and LD50 for a single oral dose is above 1000 mg/kg. Thus, the selected dose is not expected to provoke significant side effects within the treatment period. Based on literature reports,[15] to increase solubility and bioavailability of this drug, we applied HOPβ-CD, in a molar ratio 1:3 (spironolactone: HOPβ-CD), in acidified water (pH 5.5).

As a vehicle control for imatinib and spironolactone treatment, mice were administrated an equal HOPβ-CD dose, in acidified water.

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