Suppression of RUNX1/ETO oncogenic activity by a small molecule inhibitor of tetramerization

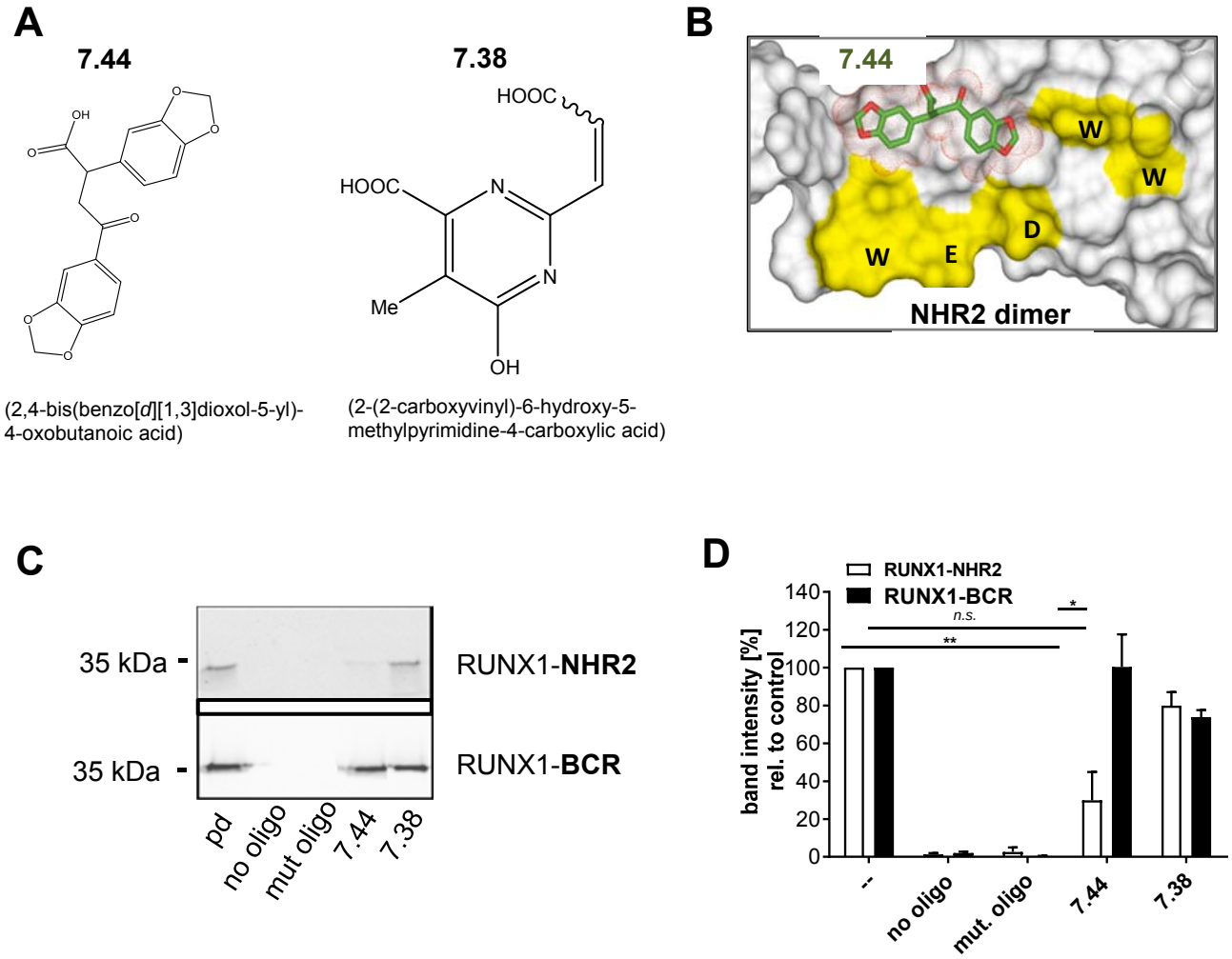
Julia Schanda,[1,§] Chun-Wei Lee,[2,§] Katharina Wohlan,[2,§] Uta Müller-Kuller,[1,3] Hana Kunkel,[1] Isabell Quagliano-Lo Coco,[1,3] Stefan Stein,[1] Alexander Metz,[4] Joachim Koch,[1] Jörn Lausen,[1] Uwe Platzbecker,[5] Hind Medyouf,[1] Holger Gohlke,[4] Michael Heuser,[2] Matthias Eder,[2] Manuel Grez,[1,§,] Michaela Scherr[2,§] and Christian Wichmann[1,6,§]*

[1]Institute for Tumor Biology and Experimental Therapy, Georg-Speyer-Haus, Frankfurt; [2]Department of Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School; [3]German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), Heidelberg; [4]Institute for Pharmaceutical and Medicinal Chemistry, Department of Mathematics and Natural Sciences, Heinrich Heine University Düsseldorf; [5]Department of Hematology, Medical Clinic and Polyclinic I, University Hospital Carl Gustav Carus, Dresden and [6]Department of Transfusion Medicine, Cell Therapeutics and Hemostaseology, Ludwig-Maximilian University Hospital, Munich, Germany

[§]JS, CWL, and KW contributed equally to this work. MG, MS and CW contributed equally and share senior authorship.

Correspondence: grez@gsh.uni-frankfurt.de
doi:10.3324/haematol.2016.161570

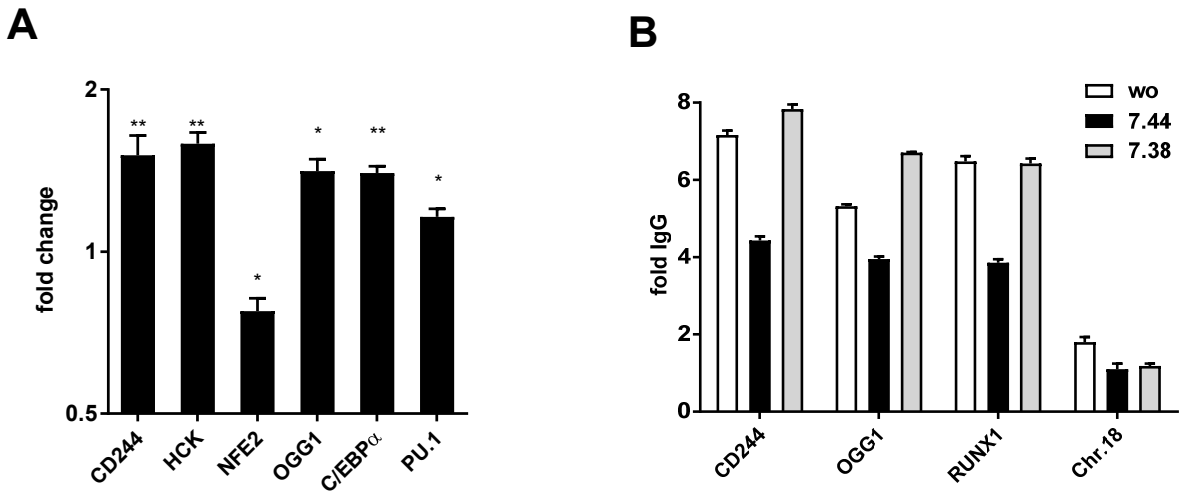
Supplementary Figure 1



Compound 7.44 blocks the binding of a RUNX1/NHR2 polypeptide to DNA.

(**A**) Chemical structure and IUPCA names of compounds 7.44 and 7.38. (**B**) Predicted binding site of compound 7.44 (green) at the NHR2 dimer interface depicted in surface representation. The tetramerization hot-spot including the 5 essential amino acids is highlighted in yellow. (**C**) Differential inhibition of RUNX1/NHR2- and RUNX1/BCR-binding to a RUNX3-oligonucleotide containing the RUNX1 binding consensus motif by compound 7.44 (*c* = 25 µM). Compound 7.38 was used as control. Representative western blot of the polypeptides bound to the RUNX3-oligonucleotide after immunoprecipitation of the polypeptide/oligonucleotide complex. Binding of the polypeptides to the RUNX3-oligonucleotide in the absence of compounds was used as control (pd). No binding was evident when no oligo or a mutated RUNX3-oligonucleotide was used (mut oligo). (**D**) Quantitative analysis of band intensity of the pulled-down proteins shown in (B) in relation to the controls.

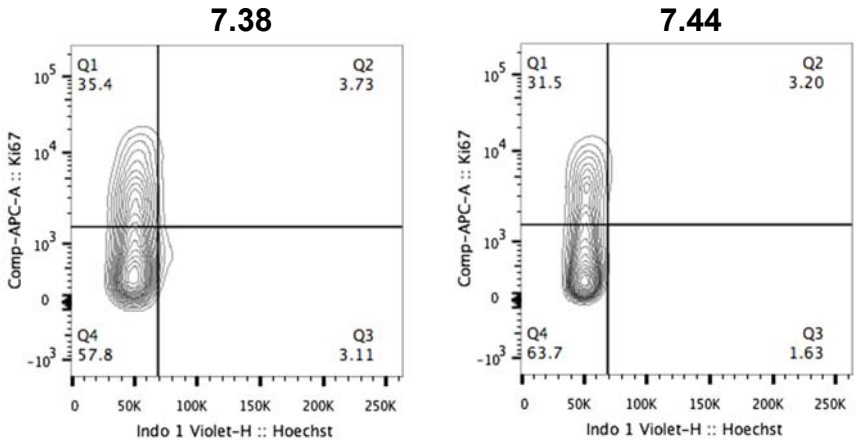
Supplementary Figure 2



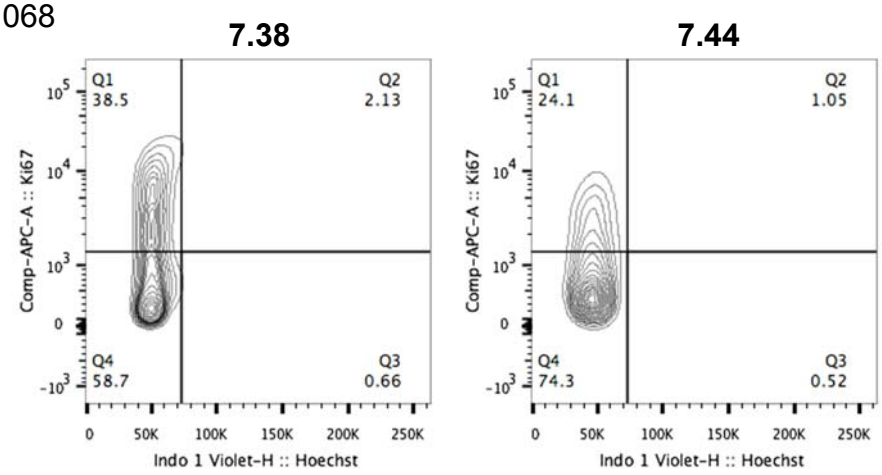
Compound 7.44 restores the expression of RUNX1/ETO-repressed genes. (**A**) Relative changes in expression levels after treatment of SKNO-1 cells with compound 7.44 or 7.38 for 3 days (*c* = 20 µM). Depicted is the ratio between the expression levels of RUNX1/ETO-target genes in 7.44- vs. 7.38-treated cells. Bars denote mean ± SEM. Statistical significance according to paired two-tailed t-test. **(B**) Reduced binding of RUNX1/ETO to RUNX1/ETO-target promoters after treatment of SKNO-1 cells as described in (A). A heterochromatic DNA region within chromosome 18 was used to monitor the specificity of the immunoprecipitation. The graphic depicts the amount of immunoprecipitated, chromatin–bound RUNX1/ETO in untreated as well as 7.44- or 7.38-treated cells as fold of the IgG control. * $p < 0.05$, ** $p < 0.01$. n=3

Supplementary Figure 3

Pat# 060



Pat# 068



Compound 7.44 impacts on the proliferation of primary CD34⁺ AML cells. Primary AML samples were collected from diagnostic bone marrow aspirations of AML patients after written informed consent. Samples were cultured in duplicates for five days in serum free media in the presence of SCF (50ng/ml), FLt3L (50ng/ml), TPO (25ng/ml), Il3 (10ng/ml), IL6 (10ng/ml) and SR1 (1 µM). Compound 7.44 or 7.38 were added daily to the cultures to a final concentration of 75 µM. Cell proliferation was assessed by Ki67/ Hoechst staining on CD34⁺ gated cells.

Supplementary Material & Methods

Cell culture and transduction

Kasumi-1 (ACC-220), SKNO-1 (ACC-690), 293T (ACC-635), HEL (ACC-11) and K562 (ACC-10) cells were obtained from the German Collection of Microorganisms and Cell Cultures (www.dsmz.de) and cultured as previously described.[1] Cells were maintained for a maximum of 4 months in culture and then replaced by fresh cells. Granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood $CD34^+$ cells were obtained from the German Red Cross (Blutspendedienst) Frankfurt, after informed consent and approval by the local ethic committee and cultured in Iscove's modified Dulbecco's medium (Life Technologies, Karlsruhe, Germany) supplemented with 20% fetal calf serum, 20 ng/ml Flt-3L, 20 ng/ml GM-CSF, 20 ng/ml stem cell factor (SCF), 20 ng/ml thrombopoietin, 20 ng/ml interleukin (IL)-6, 10 ng/ml IL-3 (all cytokines were obtained from Peprotech, Hamburg, Germany), 100 U/ml penicillin and streptomycin, and 2 mM L-glutamine.

Cell culture of primary bone marrow samples from AML patients

AML samples were collected from diagnostic bone marrow aspirations of AML patients treated in the Department of Hematology and Oncology of the University Hospital in Dresden, Germany, after written informed consent. RUNX1-ETO primary AML were culture in serum free media (STEMSPAN, Stem Cell Technologies, Vancouver BC, Canada), in the presence of SCF (50 ng/ml), FLt3L (50 ng/ml), TPO (25 ng/ml), Il3 (10 ng/ml), IL6 (10 ng/ml) and SR1 at 1μM.[2] Cultures were maintained for up to 5 days. Compound 7.44 or 7.38 were added daily at a final concentration of 75 μM. The number of viable cells was assessed by trypan blue exclusion. Cell proliferation was assessed by Ki67/Hoechst staining on a Becton-Dickinson LSR Fortessa flow cytometer.

Transduction of $CD34^+$ cells

Retroviral transduction of $CD34^+$ cells with pMIGR1-REtr-ires-eGFP (kindly provided by Dong-Er Zhang, Scripps Research Institute, La Jolla, CA, USA) and long-term cultivation of the cells were performed as previously described.[3]

Treatment of cells with small molecular weight compounds

Compounds 7.44 and the inactive control compound 7.38 were obtained from the National Cancer Institute[4] and dissolved in dH_2O/25% ammonia to a final concentration of 50 mM. For the treatment of cells, compounds were further diluted in the appropriate cell culture media as indicated in the text. Cell proliferation was measured with the cell proliferation kit II (XTT, Roche Applied Science).

Tumor formation in immunodeficient mice

Kasumi-1 cells were transduced with a lentiviral vector SLIEW encoding both enhanced green fluorescent protein, for *in vitro* analysis, and firefly luciferase, for *in vivo* bioluminescent imaging.[5] Individual clones were isolated, expanded and analysed for stable EGFP and luciferase expression. Recipient NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice received 2 Gy total body irradiation (137 Cs γ-source) prior to the inoculation of Kasumi-SLIEW cells. Kasumi-SLIEW cells ($2x10^6$) were adoptively transplanted into recipients intravenously. Eight weeks thereafter, animals were randomized into two groups and treated with compound 7.44 (n=8) or compound 7.38 (n=7). Compounds (200 – 250 µg/Kg) were injected i.p. 5 times per week into the animals in a vehicle consisting of 65% dextrose water, 30% propylene glycol and 5% Tween 80. D-Luciferin (1 mg/mouse) (AppliChem, Darmstadt, DE) was injected intraperitoneally for *in vivo* bioluminescent assay. Life imaging of tumor growth was detected weekly (IVIS Lumina II, Caliper Life Sciences, Hopkinton, MA). Survival was monitored daily. Living Image 4.0 software was used to analyze the bioluminescent intensity. The significance of survival curves was assessed using the Log-rank test. All mice used in the experiments were 8 to 10 weeks old. The animals were housed and treated in the central animal laboratory of Hannover Medical School and were approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit [No. 33.14-42502-04-12/0759].

RUNX1 peptides

RUNX1/NHR2 and RUNX1/BCR polypeptides were produced according to Metz *et a*l,.[6] Briefly, polypeptides were expressed in *Escherichia coli* (*E. coli* BL21) after induction with IPTG. After lysis of the cells by sonification, the proteins were purified on HisTrap HP columns (GE Healthcare, Feiburg, Germany) according to the manufacturer. The identity of the purified proteins was analyzed by SDS-PAGE, Western Blotting, and

size exclusion chromatography using a Superdex 200 column (GE Healthcare) according to the manufacturer.

Protein-DNA interaction assay

For protein-DNA interaction (ABCD) assays a double-stranded biotinylated oligonucleotide sequence from the RUNX3 promotor containing two binding sites for RUNX1 was used.[1] As a control we generated a mutated oligonucleotide bearing mutations in the RUNX1 binding motifs (mut oligo). For pulldown of DNA-protein complexes streptavidin-coated magnetic beads were used (Dynabeads M-280 streptavidin, Life Technologies, Darmstadt, Germany). Oligonucleotide sequences: RUNX3 A (sense) biotin-GCCTG GTCCCTC AACCACA GAACCACAA GGCCAGGCCCT, RUNX3 B (anti-sense) AGGGCCT GGCCTTGT GGTTCTGT GGTTGAG GGACCAGGC, mut oligo A (sense) biotin-GCCTGGTC CCTCACTAA CAGACTAACA AGGCCAGGCCCT, mut oligo B (anti-sense) AGGGCCT GGCCTTGT TAGTCTGT TAGTGAGG GACCAGGC.

Real time PCR

For the qPCR experiments SKNO-1 cells were treated daily with compounds 7.44 or 7.38 at a concentration of 20 µM for 3 days in three independent experiments. Total RNA was isolated at day 3 of treatment (RNeasy Kit, Qiagen) and 1 µg of each sample transcribed to cDNA (RETROscript Kit, Ambion). The qPCR was performed according to the supplier's protocol (TaqMan realtime PCR Assay, Life Technologies). Expression levels were normalized to TBP expression using the ΔΔCt method. The following assays were used: TBP (Hs00427620_m1), CD244 (Hs00175566_m1), HCK (Hs00176654_m1), ITGAM (Hs01064813_gH), NFE2 (Hs00232351_m1), OGG1 (Hs00249899_m1), C/EBPα (Hs00269972_s1), PU.1 (Hs02786711_m1), RUNX1 (Hs01021970_m1) and β2M (Hs99999907_m1). TBP and B2M were used as standards.

Flow cytometry analysis

For the analysis of cell surface markers, we used FITC (fluoresceinisothiocyanate)-, PE (phycoerythrin)-, or APC (allophycocyanin)-conjugated anti-human CD11b and CD117 antibodies as well as mouse immunoglobulin G1 isotype control antibodies (all obtained from BD Pharmingen, Heidelberg, Germany).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to the Diagenode protocol (iDeal ChIP-seq Kit for Transcription Factors) in at least two independent experiments. For this, $25x10^6$ SKNO1 cells were treated with 7.38 and 7.44 at a concentration of 20 µM for 3 days. The cells were fixed, lysed and afterwards sonicated for 20 min. The chromatin of ca. $4x10^6$ cells was used for immunoprecipitation with the following antibodies: 1 µl IgG rabbit (Diagenode, Seraing, Belgium) and 4 µl AML1-ETO (C15310197, Diagenode) as recommended by the supplier. The ChIP-DNA was eluted in 50 µl buffer and analyzed by SYBR green based quantitative PCR using 2 µl of chromatin. Primers used are listed below.

gene	sense (5`- 3`)	antisense (5`- 3`)
CD244	AGTTTGGCTTGTGGGAACTG	GACAAGGCCACTGAGAAAGC
ITGAM	GCTTCCTTGTGGTTCCTCAG	AGGAGCCAGAACCTGGAAG
OGG1	CCACCCTGATTTCTCATTGG	CAACCACCGCTCATTTCAC
RUNX1	CTGTGGGTTGGTGATGCTC	AGCCTGGCAGTGTCAGAAGT
Chr.18	ACTCCCCTTTCATGCTTCTG	AGGTCCCAGGACATATCCATT

Statistical analysis

Statistical significance was determined by unpaired two-tailed t-test unless stated otherwise. Bar diagrams show mean ± SD, except for Fig 3A. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

References

1. Wichmann C, Becker Y, Chen-Wichmann L, et al. Dimer-tetramer transition controls RUNX1/ETO leukemogenic activity. Blood. 2010;116(4):603-613.

2. Pabst C, Krosl J, Fares I, et al. Identification of small molecules that support human leukemia stem cell activity ex vivo. Nat Methods. 2014;11(4):436-442.

3. Mulloy JC, Cammenga J, Berguido FJ, et al. Maintaining the self-renewal and differentiation potential of human CD34+ hematopoietic cells using a single genetic element. Blood. 2003;102(13):4369-4376.
4. Monga M, Sausville EA. Developmental therapeutics program at the NCI: molecular target and drug discovery process. Leukemia. 2002;16(4):520-526.
5. Scherr M, Elder A, Battmer K, et al. Differential expression of miR-17~92 identifies BCL2 as a therapeutic target in BCR-ABL-positive B-lineage acute lymphoblastic leukemia. Leukemia. 2014;28(3):554-565.
6. Metz A, Schanda J, Grez M, Wichmann C, Gohlke H. From determinants of RUNX1/ETO tetramerization to small-molecule protein-protein interaction inhibitors targeting acute myeloid leukemia. J Chem Inf Model. 2013;53(9):2197-2202.