Combined targeting of STAT3 and STAT5: a novel approach to overcome drug resistance in chronic myeloid leukemia

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 doi:10.3324/haematol.2016.163436

Received: January 2, 2017.

Accepted: June 7, 2017.

Pre-published: June 8, 2017.

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Supplemental data to: Gleixner et al: Combined targeting of STAT3 and STAT5: a novel approach to overcome drug resistance in chronic myeloid leukemia

A) Supplemental Methods

Reagents

- CDDO-Me was kindly provided by Dr.E.Sausville (National Cancer Institute, Bethesda, MD) under the Rapid Access to Interventional Development program and by Dr.M.Sporn (Dartmouth Medical College, Hanover, NH) and was dissolved in dimethyl-sulfoxide (DMSO) (Merck, Darmstadt, Germany).
- The water-soluble HO-1 inhibitor styrene-maleic acid-micelle-encapsulated ZnPP (SMA-ZnPP) was produced as reported.[1,2] Stock solutions of SMA-ZnPP were prepared by dissolving in H_2O.
- The STAT5-inhibitor AC-3-019 (compound 13a) was synthesized as described[3] and was dissolved in DMSO.
- Imatinib, nilotinib, dasatinib, and ponatinib were purchased from Chemietek (Indianapolis, IN) and were dissolved in DMSO.
- RPMI 1640 medium, fetal calf serum (FCS), and antibiotics were purchased from PAA laboratories (Pasching, Austria).
- Murine interleukin-3 (IL-3) was purchased from PeproTech (Rocky Hill, NJ).
- Puromycin was purchased from Sigma Aldrich (St Louis, MO).
- Murine fibroblasts (M2-10B4) were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 medium with 10% FCS and antibiotics. Before co-incubation with primary CML cells, fibroblasts were irradiated with 60 Gy.
- ^{3}H-thymidine was purchased from Perkin Elmer (Waltham, MA).
- Propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO).
- AnnexinV-FITC was purchased from eBioscience (San Diego, CA)

^{3}H-thymidine incorporation assay

To examine anti-proliferative effects of CDDO-Me alone or in combination with other drugs, cell lines and primary cells were cultured in control medium or in various concentrations of CDDO-Me, BCR-ABL1 blockers and SMA-ZnPP (alone or in combination at a fixed ratio of drug concentrations) for 48 hours. Thereafter, ^{3}H-thymidine-uptake was measured as reported.[2,4]

Clonogenic assay

In 3 patients with CML (CP), MNC were grown in MethoCult H4435 medium containing methylcellulose, FCS and human recombinant cytokines (stem cell factor, IL-3, IL-6, erythropoietin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor) (Stemcell technologies) as described.[5] Cultures were maintained in the absence or presence of various targeted drugs (CDDO-Me: 0.25-1 μM; ponatinib: 0.25 μM; alone or in combination) for 14 days. Thereafter, the numbers of colonies were counted under an inverted microscope (Olympus, Tokyo, Japan).

Determination of apoptosis by flow cytometry

Cell lines were kept in control medium or in CDDO-Me (0.1–1.0 μM) for 48 hours. Then, apoptosis was determined by staining for AnnexinV-FITC and propidium iodide, and the numbers of apoptotic cells were assessed by flow cytometry on a FACScan (Becton Dickinson, San Diego, CA) as reported.[6] To analyse drug effects on survival (apoptosis) of primary CML-derived leukemic stem cells (LSC), mononuclear cells (MNC) were incubated in the presence or absence of irradiated murine fibroblasts (M2-10B4) in control medium (RPMI-1640 plus 10% FCS plus antibiotics without cytokines) or in medium with CDDO-Me (0.1-1 μM), ponatinib (0.1-1 μM) or a combination of both drugs at 37°C for 48 hours. Then, cells were incubated with APC-H7-conjugated mAb HI30 against CD45, PE-conjugated mAb 581 against CD34 and APC-conjugated mAb HIT2 against CD38 (15 minutes). After incubation, cells were washed with Annexin-binding buffer (ABB) (Aqua, CaCl2, NaCl, Hepes, pH 7.4) and rh Annexin-V (FITC-conjugated) was added (15 minutes). Then, cells were washed and resuspended in ABB-containing DAPI (100 ng/ml) to exclude non-viable cells. The percentage of apoptotic $CD34^+/CD38^-$ stem cells and $CD34^+/CD38^+$ progenitor cells was analyzed on a FACSCanto (Becton Dickinson, San José, CA).

shRNA-based knockdown experiments

shRNA-based knockdown of STAT3 and STAT5 was conducted as described.[7] For knockdown of STAT5, a modified pLKO.1 lentiviral vector encoding a shRNA against human and murine STAT5A and STAT5B was used. In select experiments, a modified version of the vector, where the puromycin-resistance gene is replaced by mCherry, was applied. For knockdown of STAT3, six different shRNAs targeting STAT3 were obtained from Dharmacon (GE Healthcare Europe, Vienna, Austria) in a pGIPZ backbone (encoding GFP and a puromycin-resistance gene as selection markers). Two hairpins (clone #V3LHS_376016 and clone #V3LHS_641818) produced a strong STAT3 knockdown and were used in consecutive experiments. A non-targeting shRNA served as control. Production of recombinant VSV-G pseudotyped lentiviruses and transduction of target cells was performed in K562 and KCL22 as described.[7] Cells were selected with puromycin (2 μg/ml, pGIPZ) or by FACS sorting of GFP+ cells (pGIPZ) or mCherry-positive cells (pLKO.1) on a FACSAria (Becton Dickinson). Knockdown of STAT3 or STAT5 was confirmed by Western blotting.

B) Supplemental Tables

Supplemental Table S1

Cell lines and culture conditions

Cell line (Name)	Origin / Provider	Comments
K562	Dr.M.Deininger (University of Utah, Salt Lake City, UT, USA)	-
K562-R	Kindly provided by Dr.J.D.Griffin (Harvard Medical School, Boston, MA, USA)	Imatinib-resistant; kept in the presence of 1 µM imatinib
KU812	Kindly provided by Dr.K.Kishi (Niigata University, Niigata, Japan)	Basophil-committed
KCL-22	Purchased from the German Collection of Microorganism and Cell Culture (DSMZ, Braunschweig, Germany)	Imatinib-resistant
Ba/F3p210^{T315I}	Dr.M.Deininger (University of Utah, Salt Lake City, UT, USA)	Imatinib-resistant; cell lines generated as described[8]
Ba/F3p210^{E255K}		
Ba/F3p210^{G250E}		
Ba/F3p210^{H396P}		
Ba/F3p210^{F359V}		
Ba/F3p210$^{T315I/E255V}$	Dr.T.Lion (Children's Cancer Research Institute (CCRI), Vienna, Austria)	Ponatinib-resistant; cell lines were generated as described[9]
Ba/F3p210$^{T315I/F311L}$		
Ba/F3p210$^{T315I/F359V}$		
Ba/F3p210$^{T315I/G250E}$		
Ba/F3	DMSZ, Braunschweig, Germany	Maintained in IL-3

Human and murine cell lines expressing *BCR/ABL1* were purchased or provided by cooperation partners as listed in the table. All cell lines were maintained in RPMI 1640 medium with 10% FCS and antibiotics. Untransfected Ba/F3 cells were kept in murine IL-3 (10 ng/ml). K562-R were maintained in the presence of 1 µM imatinib. However, imatinib was removed shortly before ^{3}H-thymidine uptake experiments were performed.

Supplemental Table S2A

Antibodies used for Western blotting

Protein/Epitope	Clone	Source	Dilution	Company
p-STAT5(pY694)	47	mouse	1:500	BD Biosciences
STAT5	89	mouse	1:250	BD Biosciences
p-STAT3(Tyr705)	M9C6	mouse	1:1,000	Cell Signalling
STAT3	124H6	mouse	1:1,000	Cell Signalling
p-CrkL(Tyr207)	polyclonal	rabbit	1:1,000	Cell Signalling
CrkL	32H4	mouse	1:1,000	Cell Signalling
p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	polyclonal	rabbit	1:2,000	Cell Signalling
p44/42 MAPK (Erk 1/2)	polyclonal	rabbit	1:1,000	Cell Signalling
p-S6 ribosomal protein (Ser235/236)	polyclonal	rabbit	1:2,000	Cell Signalling
S6 ribosomal protein	polyclonal	rabbit	1:1,000	Cell Signalling
p-Src (Tyr416)	polyclonal	rabbit	1:1,000	Cell Signalling
Src	polyclonal	rabbit	1:1,000	Cell Signalling
p-JAK2 (Tyr221)	polyclonal	rabbit	1:1,000	Cell Signalling
JAK2	polyclonal	rabbit	1:1,000	Cell Signalling
HO-1	polyclonal	rabbit	1:1,000	Enzo Life Sciences
β-Actin	polyclonal	rabbit	1:1,000	Sigma Aldrich
Actin	polyclonal	goat	1:1,000	Santa Cruz

p: phosphorylated; Tyr: tyrosine. Antibodies were purchased from BD Biosciences (San Jose, CA), Cell Signaling Technology (Berverly, MA), Enzo Life Sciences (Farmingdale, NY), Sigma Aldrich (St. Louis, MO) and Santa Cruz Biotechnology (Santa Cruz, CA).

Supplemental Table S2B

Antibodies used for flow cytometry and high speed sorting

CD Antigen	Clone	Source	Isotype	Fluorochrome	Company
CD34	581	mouse	IgG1	PE	BioLegend
CD38	HIT2	mouse	IgG1	APC	BD Biosciences
CD45	HI30	mouse	IgG1	APC-H7	BD Biosciences

PE: Phycoerythrin; APC: allophycocyanin. Antibodies were purchased from BioLegend (San Diego, CA) and BD Biosciences (San Jose, CA).

Supplemental Table S3

Effects of CDDO-Me and tyrosine kinase inhibitors (TKI) on CML cell lines

Cell line (Name)	IC_{50} values obtained with				
	Imatinib	**Dasatinib**	**Nilotinib**	**Ponatinib**	**CDDO-Me**
K562	100-500 nM	1-10 nM	10-50 nM	1-10 nM	250-350 nM
K562-R	>1,000 nM	10-50 nM	100-500 nM	10-50 nM	350-500 nM
KU812	100-500 nM	0.1-1 nM	1-10 nM	0.1-1 nM	250-350 nM
KCL-22	500-1,000 nM	1-10 nM	10-50 nM	1-10 nM	100-250 nM

The effects of BCR-ABL1-targting TKI and CDDO-Me on proliferation in human CML cell lines were determined in ^{3}H-thymidine uptake experiments. Results represent the ranges of IC_{50} values obtained in three independent experiments.

Supplemental Table S4

Effects of ponatinib on Ba/F3 cells expressing various mutations of *BCR-ABL1*

Cell line (Name)	Effects of Ponatinib (IC_{50})
Ba/F3p210^{WT}	1–5 nM
Ba/F3p210^{T315I}	5–10 nM
Ba/F3p210$^{T315I/E255V}$	50–100 nM
Ba/F3p210$^{T315I/F311L}$	50–100 nM
Ba/F3p210$^{T315I/F359V}$	100–150 nM
Ba/F3p210$^{T315I/G250E}$	100–150 nM

The effects of ponatinib on proliferation of Ba/F3 cells containing wild type (WT) BCR-ABL1 or various mutant forms of BCR-ABL1 were determined in ^{3}H-thymidine uptake experiments. Results are expressed as IC_{50} values obtained in 3 independent experiments.

C) Supplemental Figures

Supplemental Figure S1

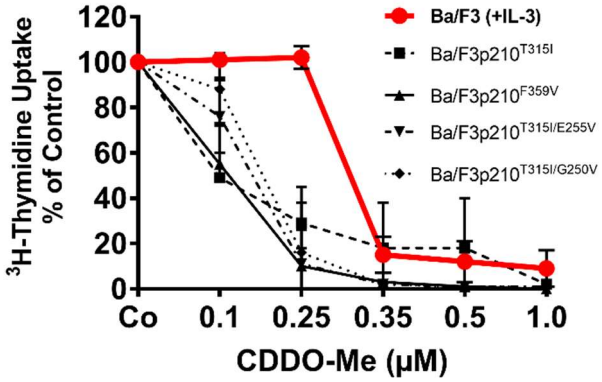


Figure S1A

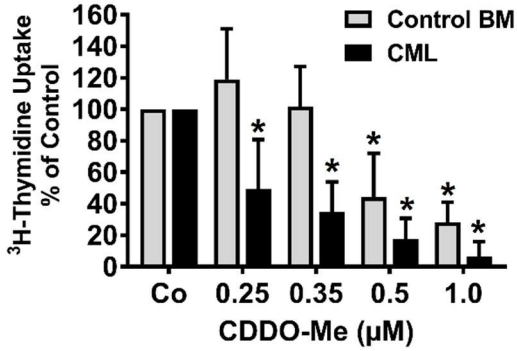


Figure S1B

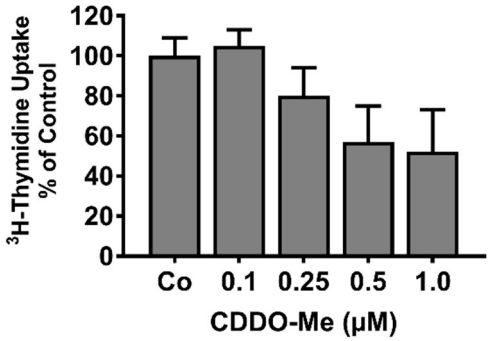
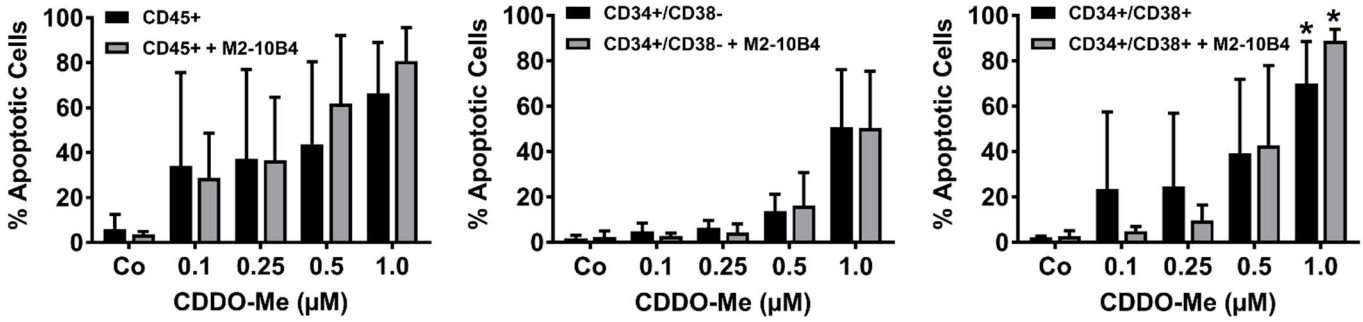


Figure S1C

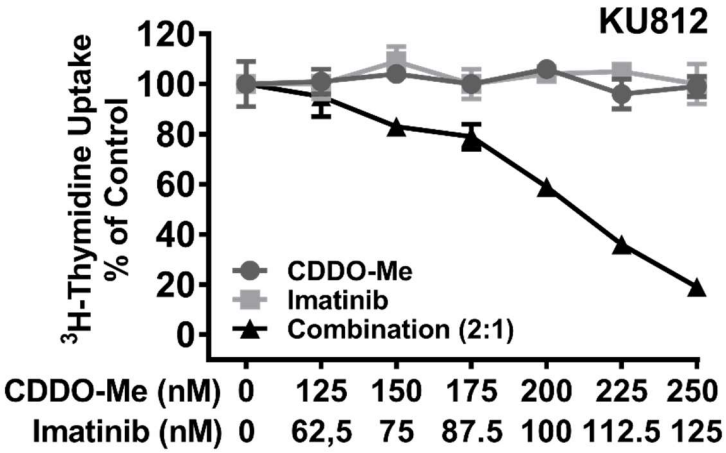
Effects of CDDO-Me on normal bone marrow mononuclear cells (BM MNC), normal CD34$^+$ hematopoietic precursor cells and untransfected Ba/F3 cells. Figure S1A: Untransfected (BCR-ABL1-negative) Ba/F3 cells (kept in 10 ng/ml IL-3) (red line, ●-●) and Ba/F3 cells containing various BCR-ABL1 mutant forms (black lines, as indicated) were exposed to control medium (Co) or various concentrations of CDDO-Me at 37°C for 48 hours. Thereafter, ^{3}H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of 3 independent experiments. Figure S1B: Normal BM MNC (Control BM, grey bars) obtained from four donors and primary CML cells (CML, black bars) obtained from the peripheral blood (PB) of 16 patients were kept in control medium (Co) or in various concentrations of CDDO-Me (as indicated) at 37°C for 48 hours. Thereafter, ^{3}H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of all 16 donors. Asterisk: $p<0.05$ compared to medium control. Figure S1C: Normal purified CD34$^+$ BM cells were exposed to control medium (Co) or to various concentrations of CDDO-Me at 37°C for 48 hours. Then, ^{3}H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of triplicates

Supplemental Figure S2

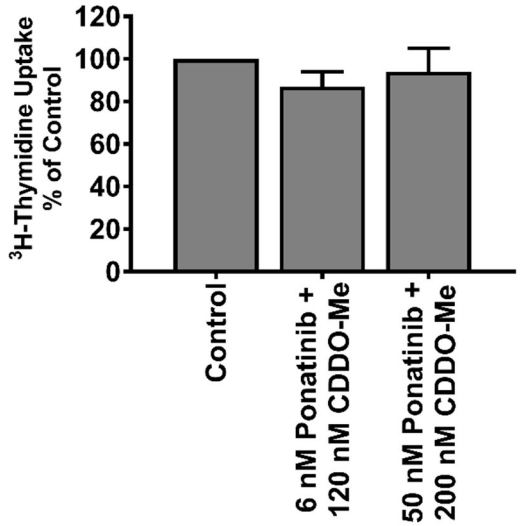


Effects of CDDO-Me on CML cells and their stem- and progenitor cells in the presence or absence of co-cultured stromal cells. MNC isolated from the PB of 3 CML patients (#9 and #11, both chronic phase, CP; and #17, blast phase, BP) were cultured in the absence (black bars) or presence of murine M2-10B4 fibroblasts (grey bars). Cells were kept in control medium (Co) or in various concentrations of CDDO-Me (as indicated) at 37°C for 48 hours. Thereafter, cells were stained with mAb against CD45, CD34 and CD38. After incubation, Annexin-V (FITC-conjugated) was added and the percentage of apoptotic (Annexin V+) cells in each fraction ($CD45^+$ cells (left panel), $CD34^+/CD38^-$ stem cells (middle panel), and $CD34^+/CD38^+$ progenitor cells (right panel) was analyzed by multi-color flow cytometry on a FACSCanto. Asterisk (*): $p<0.05$ compared to control (Co).

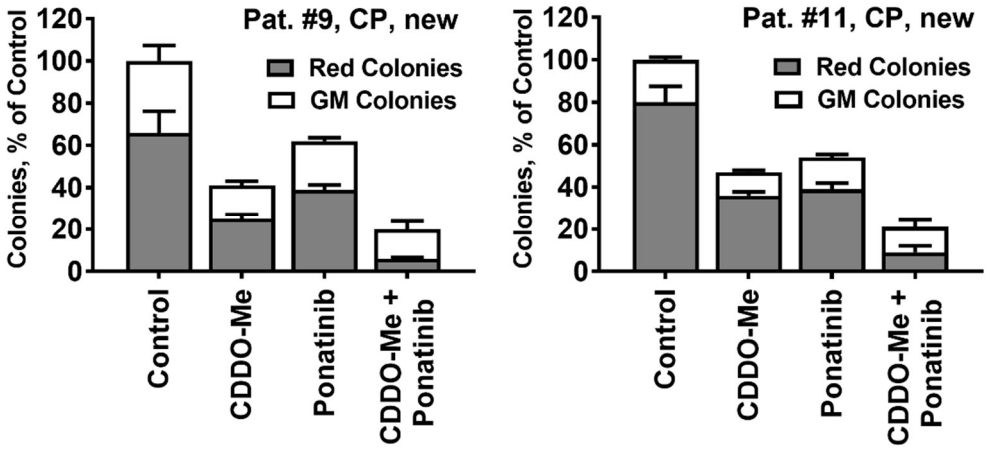
Supplemental Figure S3



Supplemental Figure S3A: CDDO-Me synergizes with imatinib in inhibiting cell proliferation. KU812-cells were incubated in control medium (0) or in various concentrations of CDDO-Me (●-●), imatinib (■-■), or a combinations of both drugs at a fixed ratio (2:1) of drug-concentrations as indicated (▲−▲) for 48 hours. Thereafter, 3H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of triplicates.



Supplemental Figure S3B: Effects of "CDDO-Me + ponatinib" on untransfected Ba/F3 cells. Untransfected (BCR-ABL1-negative) Ba/F3 cells (kept in IL-3, 10 ng/ml) were exposed to control medium (Co) or to drug combinations consisting of CDDO-Me (120 or 200 nM) and ponatinib (6 or 50 nM) at 37°C for 48 hours. Thereafter, ^{3}H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of three independent experiments. As shown in Figure 3B, the drug concentrations applied here in this control experiment were found to produce almost complete growth inhibition in BCR-ABL1+ Ba/F3 cells (Ba/F3p210^{F359V}, Ba/F3p210^{G250E}, Ba/F3p210^{T315I}, and Ba/F3p210$^{T315I/F311L}$).



Supplemental Figure S3C: Effects of the drug combination "CDDO-Me+ponatinib" on colony formation of primary CML cells. Peripheral blood mononuclear cells from 2 newly diagnosed patients with chronic phase (CP) CML (#9 – left panel and #11 – right panel) were cultured in methylcellulose with cytokines in the absence (Control) or presence of CDDO-Me (250 nM), ponatinib (250 nM) or a combination of both drugs (250 nM each) as indicated for 14 days. Then, the numbers of red cell-containing (burst-forming plus erythroid) colonies (grey bars) and granulocyte/macrophage (GM) colonies (white/open bars) were counted under an inverted microscope. Results are expressed in percent of control (100% = red colonies + GM colonies in the absence of CDDO-Me) and represent the mean±S.D. of triplicates.

Supplemental Figure S4

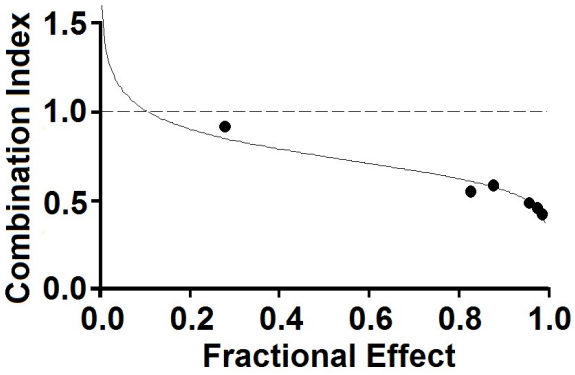


Figure S4A

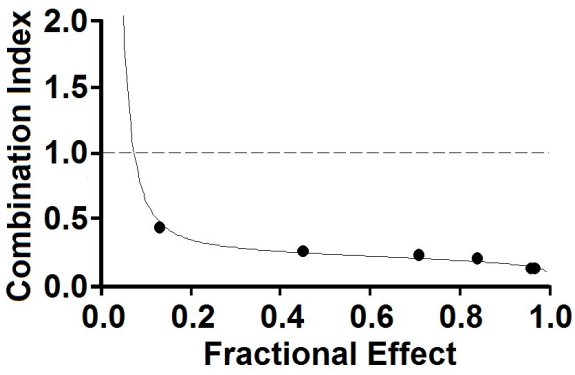


Figure S4B

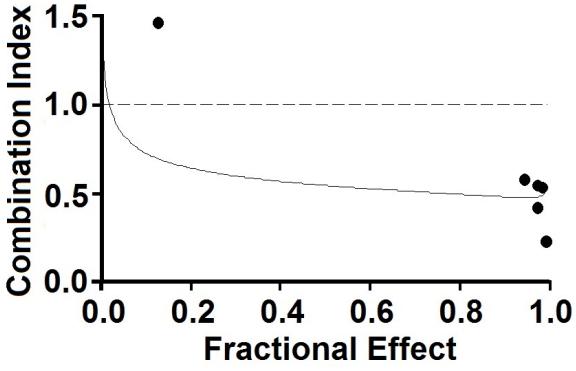


Figure S4C

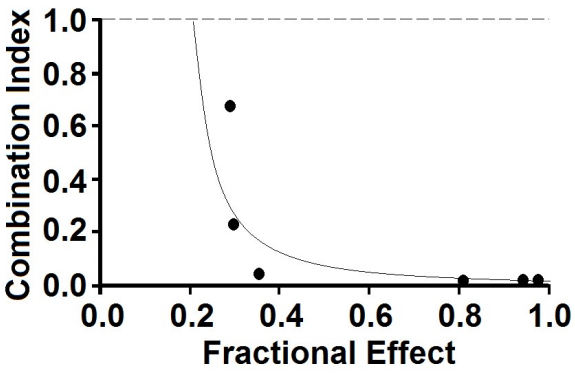


Figure S4D

Supplemental Figure S4: Synergistic drug-interactions as determined by Calcusyn software. For all drug combinations tested and shown, the nature of drug interaction (additive versus synergistic) was determined by calculating combination index (CI) values using Calcusyn software as described (ref. 46). A CI value of 1 indicates an additive effect, whereas CI values below 1 indicate synergistic drug effects. Some examples of resulting graphs are shown in Supplemental Figure 1. Figure S4A: KU812 cells were incubated in various concentrations of CDDO-Me, dasatinib or a combination of both drugs at a fixed ratio of 500:1 for 48 hours as shown in Figure 3A (lower left panel). Figure S4B: Ba/F3p210^{G250E} cells were incubated in various concentrations of CDDO-Me, ponatinib or a combination of both drugs at a fixed ratio of 20:1 for 48 hours as shown in Figure 3B (upper right panel). Figure S4C: KU812 cells were incubated in various concentrations of CDDO-Me, SMA-ZnPP, or a combination of both drugs at a fixed ratio of 1:20 for 48 hours as shown in Figure 5B (middle panel). Figure S4D: Ba/F3p210$^{T315I/F359V}$ cells were incubated in various concentrations of CDDO-Me, SMA-ZnPP, or a combination of both drugs at a fixed ratio of 1:50 for 48 hours as shown in Figure 5C (lower panel).

Supplemental Figure S5

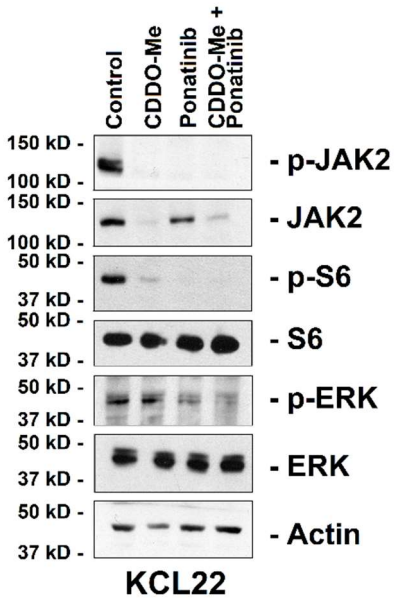


Figure S5A

Supplemental Figure S5A: Effects of CDDO-Me and ponatinib on phosphorylation of target kinases in KCL22 cells. KCL22 cells were incubated with CDDO-Me (1 µM), ponatinib (1 µM) or a combination of both drugs (1 µM each) for 4 hours. Thereafter, cells were subjected to Western blot analysis using antibodies against p-JAK2, JAK2, p-ERK, ERK, p-S6, S6 or actin (loading control) as indicated.

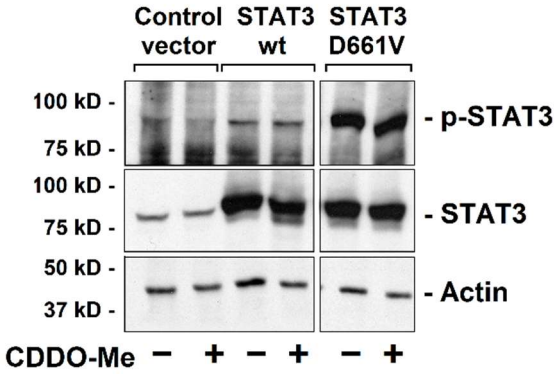


Figure S5B

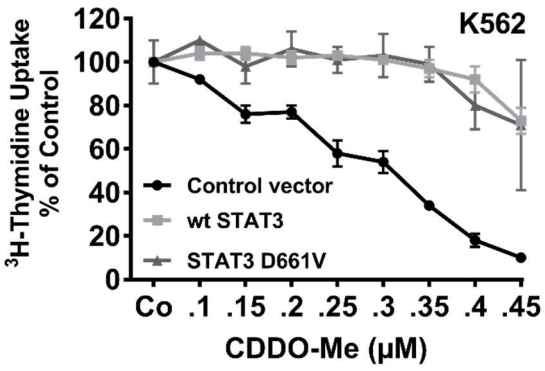
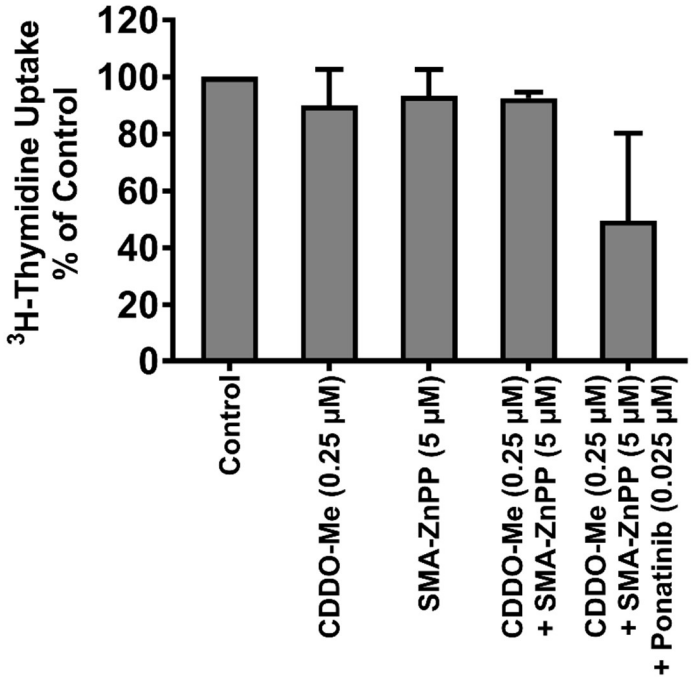


Figure S5C

Supplemental Figure S5B and S5C: STAT3 overexpression induces resistance against CDDO-Me in CML cells. K562 cells were transduced with a control vector or vectors containing the coding sequence of wild type (wt) STAT3 or oncogenic STAT3 D661V. Overexpression of STAT3 and p-STAT3 was confirmed by Western blotting (Figure S5B). Figure S5C: cells were exposed to control medium (Co) or to various concentrations of CDDO-Me at 37°C for 48 hours. Then, ^{3}H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of triplicates. Similar results were obtained in 2 other experiments.

Supplemental Figure S6



Supplemental Figure S6: Effects of the drug combinations "CDDO-Me+SMA-ZnPP" and "CDDO-Me+SMA-ZnPP+ponatinib" on normal bone marrow (BM) cells. Normal BM mononuclear cells (MNC) obtained from three donors were kept in control medium (Control), CDDO-Me (0.25 µM), SMA-ZnPP (5 µM), a combination of both drugs, and the drug combination CDDO-Me (0.25 µM) + SMA-ZnPP (5 µM) + ponatinib (25 nM) at 37°C for 48 hours. Then, ^{3}H-thymidine incorporation was measured. Results are expressed in percent of control and represent the mean±S.D. of three independent experiments. As shown in Figure 5, the same two-drug concentrations that were applied here in this control experiment, were found to show significant cooperative anti-leukemic effects in primary CML cells (Figure 5D).

D) References

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