5-lipoxygenase is a candidate target for therapeutic management of stem cell-like cells in acute myeloid leukemia

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Conflict of interest
The authors declare that they have no conflict of interest.

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Abstract
Non-steroidal anti-inflammatory drugs such as sulindac inhibit Wnt-signaling, which is critical to maintain cancer stem-cell like cells (CSC), but they also suppress the activity of 5-lipoxygenase (5-LO) at clinically feasible concentrations. Recently, 5-LO was shown to be critical to maintain CSC in a model of chronic myeloid leukemia. For these reasons, we hypothesized that 5-LO may offer a therapeutic target to improve the management of acute myeloid leukemia (AML), an aggressive disease driven by CSC. Pharmacological and genetic approaches were used to evaluate the effects of 5-LO blockade in a PML/RARα-positive model of AML. As CSC models we used Sca-1+/lin- murine hematopoietic stem and progenitor cells (HSPC), which were retrovirally transduced with PML/RARα. We found that pharmacological inhibition of 5-LO interfered strongly with the aberrant stem cell capacity of PML/RARα-expressing HSPC. Through small molecule inhibitor studies and genetic disruption of 5-LO, we also found that Wnt and CSC inhibition is mediated by the enzymatically inactive form of 5-LO which hinders nuclear translocation of β-catenin. Overall, our findings revealed that 5-LO inhibitors also inhibit Wnt signaling, not due to the interruption of 5-LO-mediated lipid signaling but rather to the generation of a catalytically inactive form of 5-LO which assumes a new function. Given the evidence that CSC mediate AML relapse after remission, eradication of CSC in this setting by 5-LO inhibition may offer a new clinical approach for immediate evaluation in AML patients.
Introduction

Actually, one of the major clinical challenges in oncology is to target the cancer stem-cell like cell (CSC). Current therapeutic strategies, regardless of whether conventional cytotoxic or novel molecular approaches, often fail to eradicate cancer completely, because they are unable to efficiently target CSC.

In this respect, a key signaling pathway for the maintenance of CSC in many cancers, including colorectal, breast and pancreatic cancer, is the Wnt-signaling pathway (1-4).

In acute and chronic myeloid leukemias (AML and CML) the role of the Wnt-signaling activation for the pathogenesis and the maintenance of the CSC is well understood (5-7). The fact that in leukemia, inhibition of the Wnt-signaling directly targets the CSC (leukemic stem cell - LSC), means that compounds and related signaling pathways which are able to inhibit Wnt-signaling offer considerable potential within the setting of maintenance therapy of leukemia. Deregulated activation of the Wnt-signaling leads to aberrant self-renewal of the LSC in AML and CML and is fundamental to its maintenance (7-9). The activation of Wnt-signaling in leukemia is mainly due to either a direct deregulation of key effectors such as APC, Axin, β-catenin, γ-catenin or TCF/Lef by i.e. mutations, or to functional inhibition of their key regulators by leukemia associated fusion proteins (LAFPs), including PML/RARα, AML-1/ETO, MLL/AF9 or BCR/ABL. These LAFPs are generated by chromosomal translocations such as t(15;17)(PML/RARα), t(8;21)(AML-1/ETO), t(9;11)(MLL/AF9) or t(9;22)(BCR/ABL) (6, 7, 10-12). The aberrant self-renewal of the LSC depends on the aberrant activation of Wnt-signaling by the LAFPs (6, 7, 10). The overexpression of γ-catenin, one of the key players in Wnt-signaling in HSPCs, leads to the induction of leukemia in vivo (12).

Non-steroidal anti-inflammatory drugs (NSAIDs) developed as COX-1/2 inhibitors are efficient Wnt-signaling inhibitors (13-15). Therefore, compounds such as sulindac, indomethacin, or ibuprofen have been employed in therapeutic approaches to stem cell therapy in models of AML and CML (6, 16). In fact, sulindac inhibits aberrant stem cell
capacity induced by PML/RARα or PLZF/RARα (X-RARα) in human as well as in primary murine HSPCs. Sulindac targets both β-catenin and γ-catenin in X-RAR expressing progenitor cells (16), whereas indomethacin is effective in the model of MLL/AF9 induced AML (6).

However, for targeting Wnt-signaling and stem cell activity in AML or CML models, NSAID concentrations were required that highly exceeding those sufficient for COX-1/2 inhibition (5, 6, 16). This calls into question whether NSAIDs target COX-1/2 in order to inhibit Wnt-signaling in AML and CML or whether another player(s) is/are involved.

Recently, it has been shown that NSAIDs, such as sulindac or celecoxib, which are capable of inhibiting Wnt-signaling, also suppress 5-lipoxygenase (5-LO) activity. Interestingly, 5-LO-inhibitory concentrations were similar to those for suppression of Wnt-signaling and the aberrant self-renewal of LSCs (16, 17).

Together with COX-1/2, 5-LO is a key enzyme in arachidonic acid metabolism. 5-LO is crucial for the synthesis of pro-inflammatory leukotrienes. Initial evidence for a role of 5-LO in the regulation of LSC activity was provided by the finding that 5-LO activity is indispensable for the induction of a CML-like disease by BCR/ABL in vivo. Both genetic targeting of 5-LO in Alox−/− mice and pharmacological targeting by a selective 5-LO inhibitor abolished the leukemogenic potential of BCR/ABL. In this model, 5-LO has been identified as a critical regulator of LSC in CML-like disease (18). In the present work, we investigated i) whether effects of 5-LO targeting of LSC in CML are extendible to the LSC in AML, ii) whether 5-LO is involved in the regulation of Wnt-signaling, in order to better understand how NSAID inhibit both Wnt-signaling and the aberrant self-renewal of LSCs in AML and CML; and iii) whether and how 5-LO represents a novel target for stem cell therapy approach in AML.
Materials and Methods

Plasmids

The retroviral vectors, PINCO- PML/RARα and PINCO-HA-β-cateninS33A, as well as the expression vector, pCDNA3.1-5LO, have been described elsewhere (16). The TopFlash/FopFlash system and the Renilla luciferase pRL-TK construct (Promega, Mannheim, Germany) have also been described previously (19). The pGL3-γ-catenin construct and the pGL3basic and pRL-cytomegalovirus (pRL-CMV) vectors were already described (12).

Cell Lines and Chemicals

293T NB4, and THP-1 cells were obtained from German Resource Centre for Biological Material (Braunschweig, Germany). 293T cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco, Darmstadt, Germany), supplemented with 10% FCS (Gibco). NB4, THP-1 and U937-PR9 cells were maintained in RPMI medium supplemented with 10% FCS (Gibco). The identity of these cells are routinely controlled by immunodetection (immunofluorescence or western blotting) of PML/RARα (NB4 and U937-PR9) or the induction of 5-LO activity (THP-1), and the response to all-trans retinoic acid induced granulocytic differentiation (NB4), respectively. CJ-13,610 was synthesized in-house (see supplementary material) and calcium ionophore A23187, arachidonic acid, zileuton, indomethacin and diclofenac were purchased from Sigma–Aldrich (München, Germany). The sEH inhibitor trans-4-[4-(3-adamantan-1-ylureido) cyclohexyloxy]-benzoic acid (t-AUCB) 1 was kindly provided by Bruce Hammock (UC Davis).

Isolation of Sca1+/lin- hematopoietic stem and progenitor cells (HSPCs)

Sca1+/lin- HSPCs were isolated from either 8 to 12-week-old female C57BL/6N mice (Janvier, Saint Berthevin, France) or male and female B6.129S2-Alox<sup>fl/fl</sup>Fum<sup>-js1Fun</sup>/J (Jackson Lab, Bar Harbor, Maine, USA) and B6.129X-Ephx<sup>fl/fl</sup>Gonz/J mice (provided by Frank Gonzalez, May 20, 2017. © 2014 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2014 American Association for Cancer Research.
NIH, Bethesda, MD) after killing by CO₂-asphyxiation. The isolation of Sca1⁺/lin⁻ cells was performed as described previously (20). Two days after the isolation cells were retrovirally transfected.

**Retroviral infection**

Ecotropic Phoenix packaging cells were transfected with PINCO-PML/RARα or empty vector by calcium-phosphate precipitation according to widely established protocols. Retroviral supernatant was collected at days 2 and 3 after transfection. Infection of the target cells was performed as described previously (12). The minimal accepted infection efficiency was 70%, as assessed by the detection of GFP positive cells by Fluorescence-Activated Cell Sorting (FACS). Differences in the infection efficiency between samples did not exceed 10%.

**Determination of 5-LO product formation in THP-1 and Sca1⁺/lin⁻ cells**

To induce 5-LO expression, differentiation of THP-1 or transduced Sca1⁺/lin⁻ cells were either induced by the addition of transforming growth factor-β₁ (TGFβ₁, 1 ng/mL) and 1,25-dihydroxyvitamin D₃ (50 nM) or by addition of GM-/G-CSF (20 ng/mL, 60 ng/mL)(Cell Concepts) for 4 days. Determination of 5-LO product formation was performed as previously described (21). For Sca1⁺/lin⁻ cells leukotriene formation in the supernatant was measured with a LTB₄-ELISA, according to the manufacturer's instructions (Enzo Life Science, Lörrach, Germany). 5-LO product formation of CJ,13,610, Zileuton and Sulindac treated THP-1 cells were analyzed by HPLC as described previously (22). For indomethacin leukotriene levels in the supernatant were analyzed by LC–MS/MS (liquid chromatography coupled with tandem mass spectrometry) as described previously (23). 5-LO product formation included 5-HETE for CJ-13,610 and Sulindac and LTB₄ for Indomethacin.

**Immunohistochemistry**

The spleens were embedded in paraffin blocks according to a conventional tissue processing
procedure. Immunohistochemistry (IHC) was performed on 5 μm sections of each paraffin block. The sections were deparaffinized with xylol (Sigma) and rehydrated through graded alcohol series (100%, 95%, 80%, 50%, H2O). Antigen unmasking was achieved through heating (95°C) with 0.25 mM EDTA buffer for 50 min. The endogenous peroxidase activity was blocked using 3% hydrogen peroxide (5 min, room temperature), followed by preincubation with antibody (Ab) mix (TBS, 2% BSA, 2% normal goat serum, 0.02% Tween20) for 20 min at room temperature. The primary antibody (mouse monoclonal anti-β-catenin; BD, Heidelberg, Germany) and biotinylated isolectin B4 (dilution 1/25; Sigma) were diluted in the Ab mix. This Ab mixture was applied, and samples were then incubated for 2h at RT. After washing with TBS, the secondary Ab (DAKO Envision Dual-Link System HRP; DAKO, Glostrup, Denmark) was added, and samples were further incubated for 30 min at RT. DAKO AEC-high sensitivity substrate chromogen was applied for 15 min and after intense washing with H2O, slides were counterstained with hematoxylin (Sigma) for 15 sec and washed under running H2O. Immunohistomount (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used for mounting.

**Competitive Re-Population Assay (CRA)**

The CRA was based on the CD45.1 - CD45.2 chimerism of C57BL/6N mice, as described previously (24). CD45.2+/Sca1+/lin- were retrovirally transduced and cultivated in DMEM supplemented with 10% FCS (Gibco), mIL-6, mSCF and mIL-3 upon exposure of either 0.001% DMSO, 0.3 μM and 3 μM CJ,13610 for 7 days. 1 x 10^4 CD45.2+/Sca1+/lin- cells were injected into lethally (11 Gy) irradiated CD45.1 and 2 positive female recipients (B6DJLF1/JRj; Janvier) together with 5 x 10^5 normal Ly5.1+ BM cells. The proportion of CD45.2+ donor cell-derived hematopoietic cells was determined by FACS analysis of cells from the peripheral blood or spleen. The cells were stained with conjugated monoclonal antibodies specific for CD45.1 and CD45.2 or unstained control (Miltenyi).
Transactivation Assays

pCDNA3 or pC3-5LO expression plasmids were either co-transfected with pRT-LK, pGL3-OT, or pGL3-OF or pGL3-γ-catenin, pGL3-basic and pRT-CMV into U937-PR9 cells by nucleofection according to the manufacturer’s instructions (Nucleofector Kit C, Lonza, Basel, Switzerland). Two hours later, transgene expression was induced by the exposure of the cells to 100 μM ZnSO₄ (Sigma) as described (25). Twenty-four hours after Zn²⁺ treatment, luciferase activity was determined using the Dual-luciferase Reporter Assay System (Promega) according and all assays were normalized to co-transfected Renilla activity.

Immunofluorescence

Cells were applied on positively charged coverslips (Menzel-Gläser, Braunschweig, Germany), washed with TBS (10 mM Tris-HCl pH 8, 150 mM NaCl) and fixed in 4% paraformaldehyde (Applichem, Darmstadt, Germany) for 30 min followed by blocking and permeabilisation with 5% (w/v) non-fat dry milk (Roth, Karlsruhe, Germany) and 0.2 % Triton X-100 (Roth) for 30-45 min. Cells were incubated with monoclonal anti-5-LO (BD), polyclonal anti-β-catenin (clone H102; Santa Cruz Biotechnology Inc.) and polyclonal anti-RARα (Santa Cruz Biotechnology Inc.) antibodies, respectively. After extensive washing in TBS, cells were stained with Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 594-conjugated goat anti-rabbit Ig antibodies (Invitrogen). Nucleus staining was obtained using TO-PRO 3 (Invitrogen). The coverslips were mounted with Moviol (Sigma). Images were acquired by a Leica TCS-SP5 confocal microscope (Leica, Wetzlar, Germany) under identical conditions for pinhole opening, laser power, photomultiplier tension and layer number. During data elaboration by Fiji software (www.fiji.sc), identical parameters were applied for all samples.

Statistics

Statistical significance was determined using one-way ANOVA with Bonferroni posttest using GraphPad Prism 5.0 (Graph Pad, San Diego, CA, USA).
Results

5-LO is inhibited by NSAIDs and is expressed in normal and malignant hematopoietic stem cell compartments

Some NSAIDs, such as Sulindac, Indomethacin or Celecoxib, are able to inhibit Wnt-signaling, but only at high concentrations, exceeding those for the suppression of COX-1/-2 (Table 1) (6, 7, 13-16, 26-29). It has recently been shown that Sulindac sulfide (SSi, active metabolite of sulindac) is able to inhibit 5-LO at clinically relevant concentrations (17). Therefore, we investigated whether other NSAIDs such as Indomethacin are able to inhibit 5-LO. Monocytic THP-1 cells, known to express 5-LO and to have inducible 5-LO activity (30), were pre-incubated with inhibitors. 5-LO product formation was induced by exposure to Ca²⁺-ionophore A23187 (21). CJ-13,610, a selective 5-LO inhibitor, was used as a positive control. We found that Indomethacin was able to suppress 5-LO product formation in a concentration-dependent manner (Figure 1A). Indomethacin suppressed 5-LO activity at higher concentration as needed for COX inhibition. (Figure 1A). Noteworthy for both inhibitors, Indomethacin and Sulindac Sulfd the 5-LO-inhibitory concentrations were similar to those required for suppression of Wnt-signaling and the aberrant self-renewal of LSCs (16, 17). Therefore, we investigated in the subpopulation of cells which should be targeted in a “stem cell therapy” approach. We studied 5-LO product formation in a highly enriched Sca1⁺/lin⁻ population in order to disclose whether 5-LO is expressed in HSPCs. Differentiation was induced by GM/G-CSF. 5-LO activity was assessed by the formation of LTB₄, by ELISA. Here we show for the first time, that the HSPC compartment not only expressed 5-LO, but also exhibited 5-LO enzyme activity, which was only slightly induced by the GM-CSF induced differentiation process (Figure 1B). These findings were confirmed by western blot analysis of Sca1⁺/lin⁻ HSPCs (data not shown).
Targeting 5-LO by selective inhibitors reduces the stem cell capacity of PML/RARα-positive HSPC

Because NSAID concentrations able to inhibit aberrant LSC capacity lie within the range needed to suppress 5-LO, we speculated whether the effects of the NSAIDs on LSCs are mediated by inhibition of 5-LO. Therefore, we studied the effects of CJ-13,610, a potent and selective inhibitor of 5-LO, on LSC capacity.

The effects of 5-LO inhibition on aberrant LSC capacity were addressed in serial replating efficiency assays in PML/RARα-positive Sca1+/lin− HSPCs. Serial replating revealed the combined effect of PML/RARα on proliferation, differentiation and self-renewal of HSPCs (12, 31). Therefore, we retrovirally expressed PML/RARα in Sca1+/lin− murine HSPCs and plated them in semi-solid medium supplemented with mIL-3, mIL-6 and mSCF in the presence/absence of 0.3 and 3 µM of CJ-13,610 (Figure 2A). Empty vector-transduced cells were used as controls. Serial replatings were performed as graphically described in Figure 2A.

CJ-13,610 abolished the aberrant serial replating capacity of PML/RARα-positive HSPCs at a concentration of 0.3 µM, starting from the second plating (Figure 2B). An effect of the treatment was already seen at the first plating, as revealed by the modifications in the morphology of the colonies in the presence of 3 µM CJ-13,610 (Figure 2C). The rare colonies in the controls also disappeared upon treatment (Figure 2A).

Additionally to the 5-LO and COX-pathway there is also a third arachidonic acid pathway - CYP (cytochrome P450)/sEH (soluble epoxide hydrolase) pathway - known to be involved in Wnt-signaling, proliferation and mobilization of HSC (32). In order to investigate the role of CYP/sEH in the effects of stem cell suppression and to confirm that only 5-LO inhibition is responsible for the observed effects, we extended the treatment of PML/RARα-positive Sca1+/lin− cells, to SSi, in COX-inhibitory concentrations, as well as to tAUCB, an sEH inhibitor(33) (Supplementary Figure 1). In fact neither treatment with SSi or tAUCB alone nor in combination was able to suppress the replating efficiency of PML/RARα. Only the...
presence of CJ-13,610 led to a complete inhibition of the aberrant replating efficiency of PML/RARα (Supplementary Figure 1). Also the genetic inhibition of sEH, using murine Sca1+/lin- cells with a sEH-/- background, showed no effect on the replating efficiency of PML/RARα-expressing HSPCs (Supplementary Figure 2).

In summary, these data indicate that the pharmacological inhibition of 5-LO is sufficient to interfere with the aberrant replating capacity of PML/RARα-positive HSPCs.

The abolition of the stem cell capacity of PML/RARα-positive HSPCs by CJ-13,610 is accompanied by the inhibition of Wnt-signaling

CJ-13,610 not only inhibited serial replating capacity of PML/RARα-positive but also inhibited colony formation of empty vector-transduced HSPCs. Thus we investigated whether the inhibition of 5-LO generally suppresses stem cells or only LSCs. First, we studied the effects of CJ-13,610 on short term hematopoietic stem cells (ST-HSC) and early immature progenitors in a colony forming unit spleen day 12 (CFU-S12) assay (34). Therefore, Sca1+/lin- HSPCs were retrovirally transduced with PML/RARα or with empty vector and exposed in liquid culture to 0.3 or 3 µM CJ-13,610. After 7 days, cells were counted, harvested and inoculated into lethally irradiated mice (Figure 3A). On day 12, the mice were sacrificed, spleens were fixed and the spleen colonies were counted. As reported in Figure 3B, the exposure to CJ-13,610 led to a concentration-dependent and significant reduction of the number of spleen colonies expressing PML/RARα. The exposure to 3 µM CJ-13,610 almost completely abolished these colonies (Figure 3B and C). Interestingly, 3 µM CJ-13,610 allowed the formation of a few colonies in the controls, indicating that CJ-13,610 did not exert stem cell toxicity (Figure 3B).

In order to determine which stem cell compartment is targeted by CJ-13,610, we studied its effects on the frequency of short-term (ST) and long-term (LT) HSC in a competitive repopulation assay (CRA). In this assay the ST-HSC population was detected at 12 weeks (Figure 3F, left panel) and the LT-HSC population after 6 months (Figure 3F, right panel).
Therefore, Sca1+/lin- HSPCs were retrovirally transduced with PML/RARα or with empty vector and exposed in liquid culture to 0.3 or 3 µM CJ-13,610. After 7 days, cells were counted, harvested and inoculated into lethally irradiated mice (Figure 3E). As shown in Figure 3F, exposure to CJ-13,610 led to a reduction of both ST- and LT-HSC, as revealed by the percentage of CD45.2 positive cells, with respect to untreated controls at 12 weeks and 6 months, respectively (Figure 3F). Treatment of control cells led to a higher frequency of ST- and LT-HSCs, confirming that targeting of 5-LO is not stem cell toxic. Very similar results were obtained using another selective 5-LO inhibitor, Zileuton (supplementary Figure 3).

Interestingly, the effects of CJ-13,610 were not related to the induction of apoptosis, excluding a cytotoxic effect of the 5-LO inhibitor (data not shown).

The effects of NSAIDs on cancer stem cells are mainly attributed to an inhibitory activity on β-catenin and the Wnt-signaling. Therefore, we investigated whether suppression of the PML/RARα-induced aberrant stem cell capacity by CJ-13,610 was related to an inhibitory effect on β-catenin and Wnt-signaling. Thus, we performed immunohistochemical staining of the spleen colonies induced by PML/RARα, using an antibody raised against human β-catenin (Figure 3D). To confirm the specificity of the β-catenin staining, we used an unstained control, only incubated with the secondary antibody. PML/RARα activation of Wnt-signaling was reversed by CJ-13,610. As shown in Figure 3D, spleen colonies derived from PML/RARα-positive HSPCs exposed to solvent exhibited a strong β-catenin signal (red staining) in comparison to controls and colonies derived from PML/RARα-positive HSPCs treated with CJ-13,610. To confirm these data we performed qRT-PCR analysis of the expression of the direct Wnt-target genes Axin 2 and Cyclin D1 in the PML/RARα- positive cell line NB4 after treatment with 3µM CJ-13,610. As a control for Wnt-signaling inhibition we used Indomethacin. We could observe that treatment with CJ-13,610 led clearly to a reduction of mRNA expression levels of Axin 2 as well as Cyclin D1 (Supplementary Figure 4).
Taken together, these data demonstrate a selective effect of CJ-13,610 on the LSC compartment, accompanied by the inhibition of Wnt-signaling without toxicity to the normal hematopoietic stem cell compartment.

**Loss of 5-LO expression does not recapitulate the inhibitory effects of the inhibitors on the leukemic phenotype by PML/RARα**

To further investigate the role of 5-LO in the maintenance of normal and leukemic stem cells, we investigated the effect of targeted genetic inhibition of 5-LO on leukemogenic potential and the aberrant stem cell capacity induced by PML/RARα in Alox5−/− mice (35). PML/RARα was retrovirally expressed in Sca1+/lin−, Alox5−/− and Alox5+/+ HSPCs, respectively. First, we performed serial replatings assays. As shown in Figure 4A, the loss of 5-LO expression not only failed to inhibit the replating efficiency of PML/RARα-expressing HSPCs, but also led to increased proliferation of colony forming cells, as revealed by an increased number of colonies in comparison to Alox5+/+ controls expressing PML/RARα (Figure 4A and B).

Next, we tested the LT-HSC capacity of Alox5−/− and Alox5+/+ HSPCs expressing PML/RARα in a CRA. Alox5−/− and Alox5+/+ HSPCs did not show significant differences in the frequency of the PML/RARα-positive LT-stem cell population (CD45.1-positive), as revealed 9 months after transplantation into lethally irradiated recipient mice (CD45.1 and 2 population) (Figure 4C).

Taken together, these data show that loss of 5-LO expression does not interfere with the replating efficiency and LT-stem cell capacity of PML/RARα-positive HSPCs. The data strongly suggest that stem cell inhibition may be mediated by the inhibited/inactive form of 5-LO, indicating a pathophysiologically relevant difference between enzymatically active and inactive 5-LO.
Co-Expression of catalytically inactive 5-LO inhibits the PML/RARα-induced activation of Wnt-signaling in human leukemic U937 cells

Assuming that the catalytically inactive form of 5-LO is responsible for stem cell inactivation, it should, like the NSAIDs, also inhibit Wnt-signaling. Therefore we investigated activation of Wnt-signaling in a cellular model in the absence and presence of 5-LO. We took advantage of the fact that PR9 cells, promonocytic U937 cells expressing PML/RARα under the control of the Zn²⁺-inducible metallothionein1 (MT-1) promoter do not express endogenous 5-LO due to the methylation of the 5-LO promoter (36). PML/RARα activates Wnt-signaling in these cells, through the activation of β-catenin and γ-catenin(10, 12). The lack of 5-LO expression in both control and P/R9 cells was confirmed by Western blotting and by PCR (data not shown). Noteworthy, transfected 5-LO is unable to synthesize leukotrienes in undifferentiated U937 cells (37). This may relate to cell type specific features governing 5-LO activity, such as the redox state, the phosphorylation status of 5-LO, the activity of intracellular tyrosine kinases, structural characteristics in the cytoskeleton, and the individual expression of further enzymes involved in leukotriene biosynthesis (38). Notably, in myeloid cells, the activity of 5-LO was already reported by us to depend on the differentiation status and the activity of cellular peroxidases which may also be true in 5-LO-transfected U937 cells (22). We expressed 5-LO in these cells under the control of a CMV promoter. Activation of Wnt-signaling by PML/RARα was addressed by the transactivation of the “Topflash/Fopflash” reporter system, in which the activation of the reporter is under the control of a TCF/LEF responsive element (19). Here we show that the expression of 5-LO prevented the activation of the Wnt-target promoter by PML/RARα (Figure 5A).

In order to further understand the mechanism by which 5-LO suppresses Wnt-signaling, we investigated the effect of 5-LO on γ-catenin dependent transactivation. β- and γ-catenin are closely related and are both key mediators of Wnt-signaling. In contrast to β-catenin, which exhibits transforming potential only upon constitutive stabilization by mutations, the over-expression of γ-catenin alone is able to transform cells (39). We used a construct in which
the luciferase reporter was controlled by the γ-catenin promoter, which is activated by γ-catenin itself (12). As reported in Figure 5B, only a slight reduction of the transactivation of γ-catenin promoter by PML/RARα was seen in the presence of 5-LO (Figure 5B). In summary, these results suggest that 5-LO interferes with the PML/RARα-induced activation of Wnt-signaling by inhibiting β-catenin- and not γ-catenin-dependent transactivation. This ability is mediated by inactive 5-LO.

5-LO and β-catenin show a direct interaction

To test the possibility of a direct influence of 5-LO on Wnt-signaling, we investigated the interaction between 5-LO and β-catenin. Therefore, we co-expressed 5-LO in 293T cells together with a HA-tagged constitutive active β-catenin mutant, S33A, which lacks the phosphorylation sites for proteasomal degradation (Supplementary Figure 5, right panel). Co-immunoprecipitation using anti-5-LO or high affinity anti-HA antibodies coupled to magnetic beads, followed by western blotting, revealed a direct interaction between 5-LO and active β-catenin (Supplementary Figure 5, left panel: IP 5-LO and IP HA). The slight bands of 5-LO in β-catenin in the respective anti-β-catenin or anti-5-LO precipitates were most likely due to specific binding of endogenous proteins. 293T cells expressed both endogenous β-catenin and 5-LO (data not shown). Nevertheless, upon over-expression of both proteins the amount of precipitated 5-LO or β-catenin, respectively, was clearly higher than that seen in the controls showing a direct interaction between the two proteins.

In summary, our data show a direct interaction between 5-LO and β-catenin, the key regulator of the Wnt-signaling pathway, which may account for the inhibitory effect of inactive 5-LO on Wnt-signaling.

5-LO co-localizes with β-catenin and hinders β-catenin from entering the nucleus.

To confirm the biological relevance of the interaction between 5-LO and β-catenin revealed in vitro, we sought to disclose the mechanism by which this interaction interferes with Wnt-
signaling. Therefore, we studied the influence of the expression of 5-LO on the localization of β-catenin in PR9 cells by indirect immunofluorescence. Thus, we expressed 5-LO in PR9 cells and induced them to express PML/RARα by Zn²⁺ treatment. To investigate the co-localization of 5-LO and β-catenin, we performed double anti-5-LO /anti-β-catenin staining. As controls, we used empty-vector transfected P/R9 cells.

The expression of PML/RARα upon Zn²⁺ induction was confirmed by anti-RARα staining which revealed the typical microspeckled pattern of PML/RARα (40, 41) (Supplementary Figure 6). The β-catenin expression in U937 cells showed a more scattered pattern than in other cell lines, most often fibroblasts, where the distribution of β-catenin is more diffuse (42-44). As shown in Figure 6A, upon Zn²⁺-induction the proportion of nuclear β-catenin (red fluorochrome) increased, confirming activation of Wnt-signaling upon PML/RARα induction.

In contrast, in the absence of PML/RARα, anti-β-catenin staining was seen exclusively in the cytoplasm. The expression of 5-LO in the PR9 cells led to perinuclear anti-5-LO staining (green fluorochrome), whereas empty vector transfected cells did not reveal any specific anti-5-LO staining, further confirming that these cells do not express 5-LO. The anti-5-LO staining was not influenced by the presence of PML/RARα. In contrast, in the presence of 5-LO, no nuclear anti-β-catenin staining was seen. The superimposition of anti 5-LO (green fluorochrome) and anti-β-catenin (red fluorochrome) staining showed partial co-localization of 5-LO and β-catenin (yellow) (Figure 6A). Four confocal sections (a-d) of the same cell taken on the horizontal axis of the microscope (0.25-0.33 micron interval) are shown in Figure 6B. To view the co-localization in greater detail, a further electronic 4-fold magnification of the indicated area of each layer (a’-d’) is shown (Figure 6B). A 3D reconstruction of all the layers of the cell (data not shown) illustrated the same co-localization seen in Figure 6B. The apparently small amount of co-localizing 5-LO and β-catenin accords with the data obtained by co-immunoprecipitation.

To investigate whether CJ-13610 is capable of inducing co-localization of 5-LO and β-catenin, human NB4 acute promyelocytic leukemia cells exhibiting well-defined cytosolic 5-
LO expression were exposed to 3 µM CJ-13,610. As can be seen treatment with CJ-13,610 led to partial co-localization of 5-LO and β-catenin (supplementary figure 7) strengthening the hypothesis of a 5-LO-mediated suppression of Wnt-signaling induced by CJ-13,610. Taken together, immunofluorescence studies could confirm the direct interaction of 5-LO with β-catenin, triggered by CJ-13,610, suggesting also a novel mechanism for Wnt-signaling inhibition by which β-catenin is prevented by 5-LO from entering the nucleus.

Discussion

The aim of our study was to determine whether the inhibition of 5-LO may account for the effects of NSAIDs on the Wnt-signaling pathway and to assess their capacity to suppress CSC in leukemia. We could show that the pharmacological inhibition of 5-LO at clinically feasible concentrations of selective inhibitors, CJ-13,610 or Zileuton (45-47) suppresses the aberrant stem cell capacity of PML/RARα-positive HSPCs via inhibition of Wnt-signaling. Additionally, inhibitors of the other arachidonic acid metabolizing enzymes, COX and sEH, failed to suppress PML/RARα-positive HSPCs. Finally, a number of subsequent experiments substantiated the CJ-13610-independent suppressive action of ectopically expressed 5-LO on Wnt signaling and leukemic cells. Thus, suppression of aberrant stem cell capacity should mainly result from the drug’s ability to specifically target 5-LO. However, pleiotropic off target effects of CJ-13610 cannot ultimately be excluded. Nevertheless, even the existence of such possible off-target effects, contributing to the anti-leukemic effect of the drug, would not detract from the documented key finding of a pivotal role of 5-LO in AML.

Based on our findings that the 5-LO directly interacts with β-catenin, we conclude that the Wnt-inhibitory effect of NSAIDs in our leukemia model is mediated not by inhibition of the COX-1/2 and prostaglandin E$_2$ synthesis as observed in models of colorectal cancer (48). This is supported by the finding that the activation of Wnt-signaling in LSC is not related to up-regulation of COX, but is related to the presence of aberrant chimeric transcription factors...
resulting from chromosomal translocations such as t(15;17)-PML/RARα or t(8;21) AML-1/ETO fusion proteins (10, 12). The inhibitory effect on tumor stem cells of the inhibition of 5-LO is not limited to our leukemia model but has also been described in glioblastoma. Here, the selective 5-LO inhibitor, Nordy, was able to reduce sphere formation and the frequency of stem cells in xenograft tumors (49).

Both the genetic disruption and pharmacological inhibition of 5-LO suppress the tumor initiating cells of BCR/ABL-driven CML-like disease in mice (18). This is, at least partially, in contrast to our findings on the PML/RARα-driven AML-model, in which the genetic targeting of 5-LO did not replicate the effects of pharmacological inhibition on the stem cell capacity. This is in accordance to recent findings showing full leukemogenic potential of AML-1/ETOex9 expressing HSPCs in an Alox5-/- background although an impairment of the in vitro self renewal of HSPCs expressing either AML-1/ETOex9, PML/RARα or MLL/AF9 was seen (50). CML is a myeloproliferative disease characterized by proliferation of hematopoietic progenitors, which are still able to fully differentiate. PML/RARα induces an acute leukemia characterized by aberrant self-renewal of the HSCs and differentiation block. The stem cell compartment of these two diseases differ, being more mature for the CML-like disease and very primitive HSPCs for PML/RARα (Oancea et al, submitted for publication and 31). Furthermore, the induction and the maintenance of AML-LSC depend on the activation of Wnt-signaling, whereas for the induction of CML-like disease, Wnt-signaling seems to be non-essential (5). Therefore, we hypothesize that the presence of the enzymatically inactive form of 5-LO is needed in order to inhibit Wnt-signaling in the PML/RARα-positive HSPCs by blocking the transition of β-catenin to the nucleus. That 5-LO is able to alter nuclear trafficking of a protein, was shown for p53, since 5-LO antagonized genotoxic stress-induced apoptosis by inhibiting binding of p53 to the promyelocytic leukemia protein (PML) and its relocalization into PML-nuclear bodies (51).

The exact molecular mechanism by which inhibitor-bound and catalytically inactive 5-LO interacts with β-catenin and possibly other Wnt components remains unclear. Possibly, 5-LO
inhibitors, such as CJ-13,610 and Zileuton, induce certain conformational changes in the 5-LO enzyme, thereby allowing suppressive interactions of the enzyme with other proteins of the Wnt-signaling pathway. Similarly, AA and competitive 5-LO inhibitors mimicking AA were observed to trigger changes in subcellular localization of 5-LO which may relate to the effects observed in this study with CJ-13,610 (52). As consequence, cytosolic and perinuclear β-catenin bound to 5-LO may be trapped and hindered from entering the nucleus suggesting that the protein cannot drive anymore the pro-leukemic transcription of Wnt target genes, as demonstrated in our study. Notably, in PR9 cells ectopically transfected 5-LO is capable of suppressing Wnt signaling, whereas in other cell types including the PML/RARα-positive HSPC, the presence of CJ-13610 is required to convert 5-LO to an active Wnt suppressor. We conclude that the environment of PR9 cells into which the ectopically transfected 5-LO is imbedded renders 5-LO catalytically inactive by still unknown mechanisms thereby allowing inhibition of Wnt signaling. Further biophysical studies on protein structure are required addressing in detail the possible conformational changes in 5-LO after binding to 5-LO inhibitors allowing interaction with β-catenin.

Our study demonstrated for a crucial role of 5-LO in maintenance of LSCs in AML confirming recent data in murine models of AML (50). The fact that in a subset of tumors the inhibition of 5-LO is able to suppress the tumor stem cell, renders feasible a stem cell therapy, as a maintenance regimen after induction and consolidation therapy protocols. The use of 5-LO inhibitors, instead of COX inhibitors, would further avoid the adverse effects of COX inhibitors, such as gastrointestinal bleeding and ulcerations or cardiovascular side-effects, including hypertension, myocardial infarction and stroke. Taken together, our data establish pharmacological inhibition of 5-LO as a novel approach of stem cell therapy in leukemia, by targeting Wnt-signaling.
References


22. Werz O, Steinhilber D. Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells. The presence of peroxidase-


Table 1. COX-1/2 IC$_{50}$ values of NSAIDs used as Wnt-signaling inhibitors.

<table>
<thead>
<tr>
<th>NSAIDS</th>
<th>COX-1 IC$_{50}$ values a (µM)</th>
<th>COX-2 IC$_{50}$ values a (µM)</th>
<th>Concentrations used for Wnt-signaling inhibition (µM) b</th>
<th>Literature</th>
</tr>
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<tbody>
<tr>
<td>Sulindac sulfide c</td>
<td>1,02</td>
<td>10,43</td>
<td>50-200</td>
<td>(13, 15, 16, 26)</td>
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<tr>
<td>Indomethacin</td>
<td>0,16</td>
<td>0,46</td>
<td>40-600</td>
<td>(5, 6, 14, 15, 26, 28)</td>
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<tr>
<td>Diclofenac</td>
<td>0,14</td>
<td>0,05</td>
<td>&gt;100</td>
<td>(15, 26)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>4,75</td>
<td>&gt;30</td>
<td>1000</td>
<td>(26, 28)</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>21,9</td>
<td>0,2</td>
<td>60-100</td>
<td>(27, 29)</td>
</tr>
</tbody>
</table>

a in human whole blood assay (endotoxin-induced PGE$_2$ synthesis)

b in different cell systems (see literature)

c active metabolite of sulindac

Figure Legends

Figure 1. Effects of NSAIDs on 5-LO product formation and expression of 5-LO in PML/RARα expressing murine HSCs.

A) Effect of NSAIDs on 5-LO product formation in THP-1 leucocytes. Cells were preincubated with CJ-13,610, sulindac sulfide (pharmacological active form of sulindac) or indomethacin for 15 min. 5-LO product formation was triggered by addition of 5 µM A23187 (Ca^{2+} -ionophore) and 10µM arachidonic acid (AA). Downstream 5-LO products (LTB4, 5-HETE (5(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid)) were analyzed with reversed HPLC (CJ-13,610, sulindac sulfide) and LC–MS/MS (indomethacin). Data are given as means ± SEM vs vehicle control (100%) of four (CJ-13,610, sulindac sulfide) and two (indomethacin) independent experiments. (B) Sca1+ /lin- HSCs were transduced with PML/RAR. Empty vector-transduced cells were used as controls. Cells were treated with +/-GM/G-CSF to induce granulocytic/monocytic differentiation. After 4 days in culture, cells were stimulated with 2.5 µM A23187 and 20 µM AA for 5-LO activation. Leukotriene formation was measured with LTB4-ELISA. Data are shown from a single representative experiment of two independent experiments performed, which yielded similar results.

Figure 2. Effect of CJ-13,610 on replating efficiency of PML/RARα expressing murine HSC.

A) Experimental strategy for studying the influence of CJ-13,610 on the biology of PML/RARα expressing murine HSCs. B) Serial replating (I-V) of empty vector control and PML/RARα-positive cells treated with 0.3 µM, 3 µM CJ-13,610 or vehicle control (DMSO). C) Colony morphology of platings I-V. Data are shown from a single representative experiment of three independent experiments performed, which yielded similar results.

Figure 3. Effect of CJ-13,610 on stem cell capacity in PML/RARα positive HSPCs.

(A) Experimental strategy for studying the influence of CJ-13,610 on short-term stem cell
capacity of PML/RARα expressing murine HSPCs (CFU-S12). (B) CFU-S12 colony numbers and (C) spleen morphology from cells treated with 0.3 µM and 3 µM CJ-13,610 (3 mice per group). Statistical analysis was performed by one-way ANOVA with Bonferroni posttest (***, p< 0.001). Data are shown from a single representative experiment of three independent experiments performed, which yielded similar results. (D) Immunohistochemical staining of β-catenin in CFU-S12 spleen slices. Empty vector control and PML/RARα expressing spleens of a CFU-S12 experiment in which the Sca1+/lin− HSPCs were retrovirally transduced and treated with 3 µM CJ-13,610. Slides were stained with either an anti β-catenin and/or only secondary antibody (unstained). (E) Experimental strategy for studying the influence of CJ-13,610 on ST- and LT-stem cell capacity of PML/RARα expressing murine HSCs (competitive repopulating assay). (F) Competitive repopulating assay (CRA). Sca1+/lin− BM cells from CD45.2 mice were retrovirally infected with empty vector control or PML/RARα. Cells were treated with 3 µM CJ-13,610. After 7 days in liquid culture, cells were co-transplanted with CD45.1 BM cells into lethally (11Gy) irradiated CD45.1+2 recipient mice (4-5 mice per group). An analysis of donor chimerism was performed at 12 weeks and 6 months after transplantation. The plots show a representative donor-derived chimerism in (left panel) peripheral blood (PB) after 12 weeks and (right panel) spleen after 6 months from an individual mouse.

**Figure 4.** Effect of 5-LO−/− (Alox5−/−) on replating efficiency and long-term stem cell capacity of PML/RARα expressing HSCs.

(A) Sca1+/lin− BM cells (wild-type and Alox5−/−) were retrovirally infected with empty vector control and PML/RARα and plated in methyl-cellulose medium supplemented with mIL3, mIL6 and mSCF to assess primary colony formation. The colony numbers were counted on day 10. After determining the colony number, the cells were harvested and serially replated every 10 days. (B) Colony morphology of platings I-VI. Data are shown from a single
representative experiment of three which yielded similar results. (C) CRA. Sca1+/lin- BM cells from wild-type and Alox5-/- CD45.2+ mice were retrovirally infected with empty vector control and PML/RARα. After 7 days in liquid culture, cells were co-transplanted with CD45.1+ BM cells into lethally (11Gy) irradiated CD45.1 and 2+ recipient mice (7-8 mice per group). An analysis of donor chimerism was performed at 9 months after transplantation to determine the LT-stem-cell capacity. The graph shows a donor-derived chimerism at 9 months in peripheral blood (PB). Statistical significance was tested using students t-test (**p< 0.001).

Figure 5. Effect of 5-LO on PML/RARα-mediated Wnt pathway activation.
Transactivation assay for Wnt-signaling-related transcription (A) β-catenin (TCF/LEF) or (B) γ-catenin dependent transcription. The expression vectors pCDNA3.1 or pCDNA3.1-5-LO were co-transfected by nucleofection with either (A) Topflash (OT) or Fopflash (OF) reporter constructs or (B) pGL3-γ-catenin or pGL3 basic constructs into 5-LO negative U937 cells expressing PML/RARα, under the control of a Zn²⁺ inducible metallothionein1 (MT-1) promoter. The transgene was induced by exposure to 100µM ZnSO₄. Luciferase activity was measured 24 hours later and normalized with Renilla activity. The data are expressed as the means of two (A) and three (B) independent experiments with SD. Statistical analysis was performed by students t-test (*p<0.05).

Figure 6. Co-localization between 5-LO and β-catenin in PR9 cells.
Cells were transfected by nucleofection with either empty vector control or 5-LO. Two hours after nucleofection, transgene expression was induced by 100 μM ZnSO₄. Twenty-four hours later, (A) cells were imaged in phase contrast (grey, a), stained with TO-PRO-3 (blue, b) and stained with anti-5-LO and anti β-catenin antibody, respectively. Alexa Fluor 594 conjugated anti rabbit-Ig (red, c) and Alexa Fluor 488, anti-mouse-Ig (green, d) were used for immunostaining. Merged images (e) were obtained by electronic overlapping of images recorded in panels a-d. (B) Cells, treated and stained as in (A), were scanned in 30 layers.
(0.25 – 0.33 micron interval) on Z-axis. Electronic overlapped merged images of phase contrast, TO-PRO-3 (nuclei), red fluorescence (β-catenin) and green fluorescence (5-LO) are shown in (B). Four confocal sections (a-d) are shown on the left part. Co-localizations are indicated with arrows. A 4x magnification (panels a’-d’) is reported on the right side of (B).
Figure 1

(A) Graph showing the 5-LO product formation in THP-1 cells as a percentage of the control against drug concentration (µM). The drugs include CJ-13,610, Sulindac sulfide, and Indomethacin.

(B) Bar graph depicting LTB4 levels (pg/ml) in Sca1+lin- cells with and without GM/G-CSF stimulation and PML/RARa expression.
Figure 4

(A) and (B) show colony number and cell morphology comparisons among different genotypes and treatments. (C) illustrates the percentage of CD45.2 positive cells under various conditions. The results indicate significant differences (***p < 0.001) in CD45.2 positive cells between control and PML/RARα treated groups.

Figure 4
5-lipoxygenase is a candidate target for therapeutic management of stem cell-like cells in acute myeloid leukemia

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