University of Nevada, Reno

Effect of CDDO-me on Myelopoiesis in Naïve Mice and in Mice Undergoing Bone Marrow Transplantation (BMT)

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of Science in Cell and Molecular Biology

Major Immunology

By

Salif, Harouna (Salif Harouna Sow)

Dr. William J. Murphy/ Thesis Advisor

December, 2009

THE GRADUATE SCHOOL

We recommend that the thesis
prepared under our supervision by

HAROUNA SALIF (SALIF HAROUNA SOW)

entitled

Effect Of CDDO-Me On Myelopoiesis In Naïve Mice And Mice Undergoing Bone Marrow Transplantation (BMT)

be accepted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

William J. Murphy, Ph.D, Advisor

Kenneth Hunte, Sci.D, Committee Member

Doug Redelman, Ph.D, Committee Member

Graca Almeida-Porada, Ph.D, Graduate School Representative

Marsha H. Read, Ph. D., Associate Dean, Graduate School

December, 2009

ABSTRACT

To treat cancer and other hematological diseases, large numbers of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) are needed. The objectives of this study were to examine the toxicity effect of the synthetic triterpenoid CDDO-me *in vivo*, to investigate the effect of CDDO-me on myeloid expansion and mobilization, to determine the hematopoietic growth factor production in the serum of mice that received CDDO-me, and to look at the effect of CDDO-me on immune system recovery after bone marrow transplantation (BMT). Our findings show that CDDO-me at 240 µg/dose BID is a lethal dose for C57BL/6 female mice. In contrast, CDDO-me at 120 µg/dose BID was well tolerated in pre-BMT, but lethal in pre- and post-BMT and in post-BMT. CDDO-me at 60 µg/dose BID was well tolerated in pre- and/ or post-BMT in a murine model. We also found that treatment with CDDO-me (120 µg/ dose, BID) significantly increased myeloid, erythroid, and CFU-HPPs in the spleen and the bone marrow. In addition to inducing CFU-GM expansion, treatment with CDDO-me caused a significant increase in myeloid and erythroid progenitors in the spleen and blood. The level of
G-CSF in the serum was significantly increased by CDDO-me treatment. Moreover, a significant increase in spleen CFU-GM in the mice that received CDDO-me (60 µg/dose BID) at day 14 post-BMT was observed. From these results we concluded that: treatment with CDDO-me (120 µg/dose BID) enhanced myelopoiesis and caused mobilization of myeloid and myeloid progenitor cells into the spleen and blood.

CDDO-me (60 μg/dose BID) had a positive effect on myeloid and myeloid progenitor cell recovery at day 14 post-BMT. Finally, the level of G-CSF in the serum was significantly increased after CDDO-me treatment.

Acknowledgements

First I would like to thank Dr William J. Murphy for being such a great mentor. I thank him for giving me the opportunity to pursue my studies in his laboratory, as well as for his patience, support and suggestions. I would like to thank my committee members: Doug Redelman, Ph.D, Kenneth Hunter Sci.D, and Dr Graca Almeida-Porada for their time, support, and encouragement throughout this project. I would like to thank Dr. Lisbeth Welniak, Ph.D, who for many years has been my mentor and helped me in Science. Her advice, critical evaluation of results and her encouragement during this project were always more than welcome. I would like to thank Dr Ruth Gault for all that she did for me. I would like to thank all of the members of Dr. Murphy's laboratory: Danice, Ming, Kory, Will, Mariem, Erik, Maite, Debrorah, Kai, Isabel, Weighong, Megan, Maria and others who been in Dr Murphy's laboratory.

I wish to acknowledge and express my appreciation to the many people who contributed directly or indirectly in this project: Dr Willian E. Courchesne, Dr Mark Hall, Dr Varough, Mr. Abou Yobi ,Mr. Aw Tidjane, Mr. Nathan Hiatt and all my co-workers from Charles River Laboratories for their help, encouragement and suggestions. I would like to thank the microbiology department personnel, especially Michelle Whitaker, Carla and the CMB program.

I would like to thank my dearest friends and fellow Mauritanians in Reno, NV. I would like to reserve special thanks for my family, particularly to my lovely wife Binta Yaya and my children Farough Sow, Oumar Sow and Aissata Sow. I would like also to thank my parents, my brothers and sisters. I would like to thank my friends: Sow Oumar Alassane, Ndiaye Ali Alassane, Dia Moctar Malal, Sene Soh, Oumar Deh,

Tijanel Aw, Dr. Sow Amadou Demba, Sow Aboubakry Moussa (Wolis), Mr. Cissoko Adama and last but not least Pr. Lo Baidy for his advice and encouragement. Thank you all for your help and support.

Table of contents

Introduction--- 2

Reference List -- 17

Chapter 1 -- 28

Abstract--- 29

Introduction-- -----31

Materials and Methods--- 33

Results and Discussion--40

Conclusion -- 49

Figures and Legends---50

Reference List --61

Chapter 2 -- 64

Abstract--- 65

Introduction-- 66

Materials and Methods---68

Results and Discussion--72

Conclusion -- 77

Figures and Legends-- 79

Reference List -- 88

Effect of CDDO-me on myelopoiesis in naïve mice and in mice undergoing bone marrow transplantation (BMT).

INTRODUCTION

Haematopoiesis

Hematopoietic stem cells (HSC): Pluripotent stem cell

Hematopoietic stem cells (HSCs) are pluripotent cells that are mostly found in umbilical cord blood or in the bone marrow (BM). HSC can give rise to all adult hematopoietic lineages. HSCs and hematopoietic progenitors cells (HPCs) are commonly used in bone marrow transplantation (BMT) to replace the hematopoietic system in patients with hematological disorders, such as asplastic anemia, or in cancer patients who are undergoing high dose chemotherapy [1-3]. Bone marrow studies have shown that there are two types of HSC: long term repopulating stem cells and short term repopulating stem cells [4]. The long term repopulating stem cells are characterized by their capacity to rescue irradiated animal through the restoration of the hematopoietic system over some months [5-7]. These cells are capable of self-renewal. Short-term repopulating progenitor or precursor cells are relatively immature cells that are precursors to a fully differentiated cell of the same tissue type. These cells can immediately regenerate all the different types of blood cells but cannot renew themselves over the long term [8].

Myeloid cells

Origin and differentiation

The pluripotent stem cell is the progenitor of the two multipotential stem cell lineages, myeloid and lymphoid stem cells [5, 7, 9-11]. The lymphoid stem cells are precursor of lymphocytes (Figure 1*A*), while the myeloid cells are precursors of granulocytes, monocytes, macrophages, red blood cells (RBCs), and platelets (Figure 1*B*). Both lymphoid and myeloid cells derivate from hematopoietic stem cells in the bone marrow (BM) (Figure 1) [12]. The myeloid stem cell becomes the colony forming unit, granulocyte-erythrocytes-monocyte-megakaryocyte (CFU-GEMM). The CFU-GEMM leads to the formation of burst forming unit erythroid (BFU), megakaryocyte (CFU-Meg) and granulocyte, monocyte/macrophage (CFU-GM) which gives rise to CFU-G (granulocyte), CFU-M (macrophage), CFU-Eo (eosinophil) and CFU-Bas (basophil) as presented in figure 1*B* [13].

The myeloid cells represent an important population in the peripheral blood; they are the first line of defense against many microorganisms (e.g. bacteria, fungi, and viruses) in what is known as innate immunity. Among myeloid cells, the dendritic cells (mDCs) are important in both the initiation of immune response and the maintenance of peripheral tolerance; they are the main producer of IL-12 that activates T cells [14-16]. During the early stages of infection, DCs migrate into secondary lymphoid tissues to initiate a specific immune response by presenting the antigen to activate T cells [14-18]. Monocytes become macrophages in various tissues after maturation: Kupffer cells in the liver, osteoclasts in the bone, alveolar macrophages in the lungs and mesangial macrophages in the kidneys. Monocytes give rise to Langerhans cells in the skin and dendritic cells in the lymphoid organs [7, 12, 13, 19].

cytokines, such as GM-CSF, TNF, and TGF-β [27-29]. Interleukin-6 (IL-6) in collaboration with Notch signaling promotes proliferation of HSCs [6]. GM-CSF, IL-3; IL-6, granulocyte colony stimulatory factor (G-CSF) and steel factor (SF) are identified as early acting lineage factors [30]. IL-3, GM-CSF and interleukin (IL-4) appear to support the proliferation of multipotential progenitors but only after they exit from G_0 [31]. These factors can act as intermediate-acting lineage nonspecific factors. It has been shown that GM-CSF alone or in combination with IL-3 and SF increases production of granulocyte progenitors [5, 9, 32, 33]. Erythropoietin (Epo), macrophage colony stimulator factor (M-CSF), G-CSF and interleukin-5 (IL-5) are identified as late-acting lineage-specific factors. These factors control the proliferation and maturation of committed progenitors. For example G-CSF is known to regulate granulocyte production, GM-CSF promotes neutrophil, monocyte, eosinophil, megakaryote and DC production [5, 32] (Figure1).

Other cytokines are involved in myeloid expansion, such as interleukin-17 (IL-17). This cytokine is primarily produced by activated T cells and invariant natural killer T (iNKT) cells [34, 35]. Schwarzenberger et al. [35] has shown that IL-17 can induce granulopoiesis [35]. Moreover, it has been shown that one of the mechanisms by which IL-17 expands myeloid progenitors is by inducing G-CSF and SCF in the BM stromal cells [35].

Chemokines are another class of compounds that are important regulators of hematopoiesis. They are composed of a large family of proteins that mediate a variety of processes such as inflammation, cell migration, development and angiogenesis [36]. In hematopoiesis, chemokines can inhibit progenitor growth and regulate migration of hematopoietic progenitor cells (HPCs). The peptidase CD26 (DPPI peptidylpeptidase) is expressed by T cells and other cells. The chemokine SDF-1 (which binds the receptor CXCR4)

The results of several years of investigation from Bell [20] and Wada [21] indicate the existence of progenitors possessing T and myeloid potential within the thymus; this is incompatible with the current dominant model of hematopoiesis in which there is a standard model of a binary split between lymphoid and myelo-erythroid lineages [20, 21]. These results suggest that the model for lineage commitment during hematopoiesis should be redrawn.

Haematopoiesis regulation

Cytokines are a broad family of proteins that can have positive or negative effects on cellular quiescence, apoptosis, proliferation, and differentiation. Cytokines generally function by engaging a specific receptor and activating a variety of signaling pathways (i.e.: protein kinase C (PKC), Jun kinase (JNK)) [22]. The most characterized environmental regulators of hematopoiesis are cytokines. Some cytokines such as interleukin-3 (IL-3) and granulocyte-macrophage colony stimulatory factor (GM-CSF) can induce cell proliferation, while other cytokines can protect cells from apoptosis and sensitize them to growth-promoting cytokines [23]. Other cytokines may facilitate the interactions between stem cells and elements in the microenvironment such as the extracellular matrix (ECM). Transforming growth factor-beta (TGF-β) and tumor necrosis factor-alpha (TNF-α) can modulate cell cycle activity and engraftment of HSC by suppressing the proliferation of primitive Lin-Thy-1+ progenitors [23, 24]. Flt-3 and stem cell factor (SCF) are involved in HSC development and in early cell survival and self-renewal [25, 26]. FL is a receptor tyrosine kinase (RTK); it stimulates the proliferation, differentiation and the mobilization of stem and progenitor hematopoietic cells. FL has been shown to stimulate the generation of dendritic cells (DCs) in mouse models [25]. FL has also been shown to stimulate the *in vitro* generation of DC and NK cells in combination with other

and the peptidase CD26 has been shown to mediate homing of HSCs and progenitors to the bone marrow after transplantation and in stem cell mobilization [37, 38]. CD26 inhibition may enhance HSC engraftment and homing to the BM [38, 39]. These results suggest that chemokines regulate blood cell trafficking and homing to sites of need and may also be negative and positive growth regulators. Some receptors such as Notch signaling have been shown to be involved in the early events of embryonic hematopoietic determination [40-44]. Notch is implicated in HSC expansion, self-renewal and survival [6]

Mobilization of HSC

Mobilization of HSCs and HPCs is a process consisting of the release of stem cells and progenitors from the bone marrow reservoir (niche) into the blood as a response to stress signals during injury, inflammation or following treatment with cytokines (i.e. G-CSF) [45]. HSCs and HPCs mobilization is important in enhanced transplantation, where an increased number of these cells is needed. It has been shown that mobilized HSCs engraft better than HSCs taken directly from the bone marrow [46-48]. The chemokine SDF-1 (CXCL12) and its receptor CXCR4 are involved in the chemotaxis [37, 46, 49, 50], homing and survival of HSCs and HPCs [47, 48, 51-53]. SDF-1-CXCR4 is implicated in the retention of HSCs and HPCs in the BM. HSC homing is the reverse of mobilization, these two processes are dependent on the level of SDF-1 and its receptor CXCR4 in the BM. During certain conditions such as cellular stress, SDF-1 and vascular endothelial growth factor (VEGF) activate matrix metallopeptidase-9 (MMP-9) which converts membrane-associated kit ligand into soluble kit ligand (skitL) that causes HSCs entry in cell cycle, mobilization and differentiation [54-56]. Many stem cell mobilizing agents are now

of donors, a high risk of graft rejection by the host, a period of immune deficiency which leaves the patient highly susceptible to opportunistic infections and graft-versus-host disease (GVHD) that is the most important cause of morbidity following allogeneic HSCT [63-66]. GVHD occurs when immunocompetent donor cells attack the genetically disparate immunocompromised host. In general, solid tissue organs such as the gut, liver, skin and lungs are the main targets.

Immune reconstitution following BMT also remains a significant issue limiting its efficacy; hematopoietic growth factors have been used during HSCT in an effort to hasten engraftment, thereby reducing complications associated with various infections by enhancing the innate immune system. The wide use of growth factors such as G-CSF and GM-CSF after HSCT diminish the cost and the length of hospitalization, shorten the period of neutropenia, and enhance the host defense that consist largely of professional phagocytes of the granulocyte and monocyte lineages, including polymorphonuclear leukocytes (PMNL). G-CSF and GM-CSF, among other growth factors, have demonstrated clinical usefulness in accelerating recovery from neutropenia [67, 68]. The treatment of mice post-BMT with rmGM-CSF delayed the recovery of platelets and erythrocytes [69, 70]. Growth factors alone or in combination should be used with caution, particularly in patients who have limited numbers of progenitor cells.

Triterpenoids

CDDO and CDDO-me

Triterpenoids are a large family that is biosynthesized in plants through the cyclization of squalene [71, 72]. More than 20,000 triterpenoids have been described in nature, most of them have weak activities [73]. Natural triterpenoids are synthesized by plants for regulation of physiological processes as part of their defense mechanism. They have been used for centuries in

used to mobilize HSCs to the peripheral blood [57]**.** Some of these agents including G-CSF, GM-CSF, AMD3100 (a symmetric bicyclam, antagonist of the CXCR4 receptor) and SCF are approved by the Food and Drugs Administration (FDA), others have been clinically tested but are waiting for FDA approval. They include: haematopoietic growth factor Flt3 ligand (Flt-3L), Progenopoietin, Gro-b truncated (SB-251353) (a novel truncated form of human CXC chemokine growth related gene product beta), CXCR2, recombinant human growth hormone (rhGH) and VLA-4 small molecule antagonist.

Hematopoietic Stem Cell Transplantation (HSCT)

Hematopoietic stem cell transplantation (HSCT) or bone marrow transplantation (BMT) is a medical procedure that consists of taking HSCs from a donor and transplanting them into a recipient. HSCT was first developed out of research to treat and understand radiation injuries, but has subsequently being used for the treatment of a variety of diseases ranging from aplastic anemia to cancer. There are two major types of HSCT [58-60]: the first is autologous bone marrow transplantation, which consists of the isolation of HSCs from a patient, and the infusion of these cells back into the patient after medical treatment [61, 62]. Autologous transplantations limit infections and have the advantage of having almost no risk of graft rejection and graft-versus-host disease (GVHD). However the limitation of autologous transplantation is the higher rate of cancer relapse. The second type of HSCT is allogeneic bone marrow transplantation, which involves two people, the HSC donor and the recipient (patient). An allogeneic HSC donor should have the same human leukocyte antigens (HLA) as the recipient (patient). This is known as an HLA-match. In addition, the recipient requires immunosuppressive medications to reduce rejection rates. Allogeneic bone marrow transplantations have some major limitations: shortages

Asian countries as traditional medicines (anti-carcinogens) [74, 75]. Synthetic oleanane triterpenoids are a new class of non-cytotoxic and are highly multifunctional drugs. They are used for the prevention and treatment of not only cancer, but also many other inflammatory diseases. The synthetic triterpenoids have been developed in an effort to increase the potency of oleanolic acid (OA) and ursolic acid (UA) for their use as chemopreventive and chemotherapeutic agents [76]. The synthetic oleanane tritrepenoid, CDDO and its derivates, such as C-28 methyl ester (CDDO-me) imidazoldes (CDDO-Im), amides (CDDO-MA, and CDDO-EA), and dinitrite (Di-CDDO), have been reported to be multifunctional molecules that can have numerous biological effects, such as anti-inflammatory, anti-proliferative and apoptosis-inducing activities [77-81]. CDDO-me is a novel synthetic tritrepenoid more potent than its parent compound, 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oic acid (CDDO) and its derivates both *in vitro* and *in vivo* [79]. CDDO and CDDO-me (Figure 2) are currently in phase I clinical trials for treatment of leukemia and solid tumors.

The synthetic triterpenoids have been shown to be highly effective in many *in vivo* models for the prevention and treatment of cancer. They suppress the growth of tumor cells, induce apoptosis in cancer cells that are highly resistant to conventional chemotherapeutics agents, and prevent a primary lung cancer induced by chemical carcinogens [71].

NF-κB, or nuclear factor kappa B, is a nuclear transcriptional factor found in all cell types and is involved in cellular responses to stimuli (cytokines, stress, free radicals, ultraviolet irradiation and bacterial or viral antigens). It plays an important role in regulating the immune response to infection. NF-κB regulation has been linked to cancer, immune response, cell survival, apoptosis, autoimmune and inflammatory diseases [82, 83].

CDDO-me is a derivative of CDDO, which is synthesized from oleanolic acids [76]. CDDO-me was found to be much more potent than CDDO-Im and CDDO in suppressing NF-κB activation [76, 80] and in decreasing cell survival [71]. CDDO-me was shown to be a novel mitochondriotoxic agent that inhibits mitochondrial electron transport and induces apoptosis [77]. It is a potent inhibitor of both constitutive and inducible NF-κB activation by tumor necrosis factor (TNF), interleukin-1 beta (IL-1B), and cigarette smoke. Shishodia et al. [79] suggested that CDDO-me suppresses NF-kB by inhibiting the activation of IkBα kinase [84-90]. CDDO-me was shown to inhibit TNF-dependent IkBα phosphorylation and degradation. It represses TNF-induced NF-kB-dependent reporter gene expression, and augments the cytotoxic effect of TNF [90, 91]. Vannini et al. [92] showed that CDDO-me is an effective agent for suppressing angiogenesis, both *in vitro* and *in vivo* [92, 93]. In human cancer cells CDDO-me was shown to induce apoptosis via the c-Jun NH_2-terminal kinase (JNK)-mediated death receptor-5 (DR-5) [94-97]. CDDO-me was reported to enhance apoptosis caused by death ligands, such as TNF and TRAIL [81, 97]. Kim et al. [71] showed that CDDO-me induces apoptosis in human non-small cell lung carcinoma cells (NSCLC) via cytochrome c-triggered caspase activation pathways without altering the level of Bcl-2 and Bcl-x_L proteins. They showed that over-expression of Bcl-2 did not protect cells from CDDO-me-induced apoptosis [80, 98-100]. CDDO was identified as ligand for a key transcription factor, peroxisome prolifertor-activated receptor-γ (PPAR γ). Wang et al have identified CDDO-me as a PPARγ antagonist, while CDDO was identified as a PPARγ agonist [101]. CDDO-me and other CDDO derivates are potent inducers of transcriptional factor nuclear erythroid 2 p45-related factor 2 (Nrf2)-regulated genes [102]. The synthetic oleanane triterpenoids such as CDDO-me, CDDO and CDDO-Im have been shown to suppress the induction of both nitric oxide synthase (iNOS)

and cyclooxygenase (COX_2) in primary macrophages stimulated with pro-inflammatory molecules, both *in vitro* and *in vivo* [75, 76].

Synthetic oleanane triterpenoids are proven to be non-cytotoxic and multifunctional drugs. They can be used not only for cancer prevention and treatment, but also for other inflammatory diseases. CDDO and its derivates CDDO-Im and CDDO-me are synthetic triterpenoids confirmed to be effective agents in controlling cancer cell growth in preclinical models of leukemia, breast cancer (4T1 cells), pancreatic cancer (COLO357 and PANC1), colon cancer (SW-480) , and lung cancer (human non-small cell lung carcinoma (NSCLC cell lines) [71, 103-106]. CDDO-me inhibits cell proliferation and induces apoptosis in numerous tumor types (lymphoid, myeloid, carcinomas). It was shown that CDDDO-me can activate intrinsic apoptosis pathway by blocking Bcl-2 phosphorylation [103]. Ling et al showed that CDDO-me inhibits 4T1 breast cancer cells growth via inactivation of STAT3 signaling [84]. CDDO-me can eliminate tumor development and metastasis in immunocompetent mice. CDDO-me pretreatment shows an increase in levels of antioxidant genes and suppression of interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin1β (IL-1β), interleukin 6 (IL-6) and lipopolysaccharide (LPS)-induced cytokines expression [107, 108]. In addition to the cancer prevention and treatment, CDDO-me significantly affects normal hematopoiesis cells including precursor populations in vivo and can promote granulopoiesis in non irradiated mice. CDDO-me did not significantly affect hematopoietic recovery when given to mice following sublethal total body irradiation.

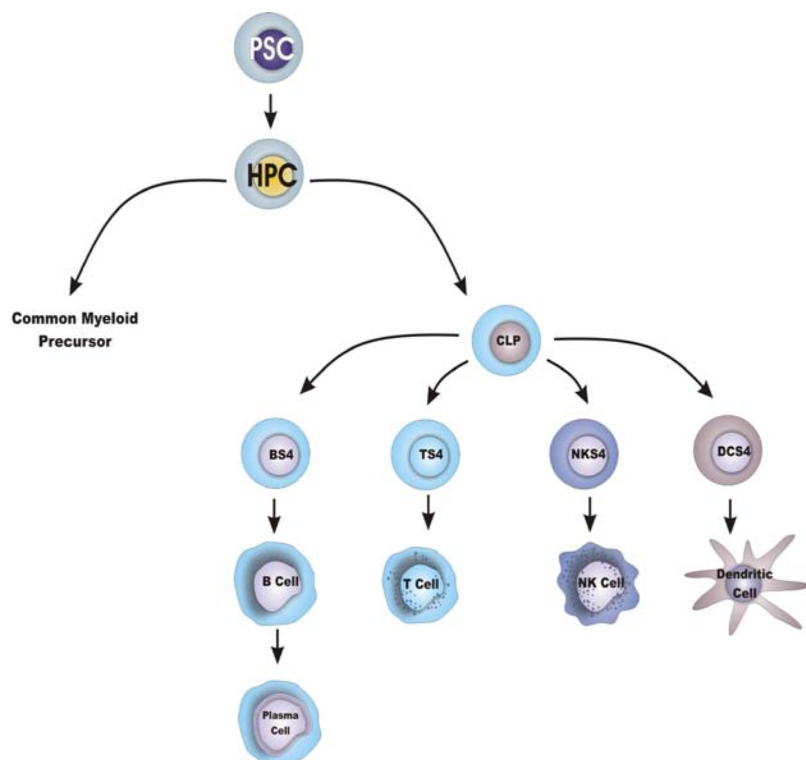
Other NF-κB blockers are used (alone or in combination) in cancer and inflammatory diseases: proteasome inhibitors and histone deacetylase (HDAC) inhibitors. Proteasome inhibitor Velcade (bortezomib, PS-341) is a small molecule inhibitor that selectively blocks

activity of the catalytic site of the 26S proteosome complex [109, 110]. PS-341 is a potential inhibitor for NF-κB activation as it enhances apoptosis in various tumor cells [111]. Velcade has shown antitumor activity in preclinical and clinical studies (e.g. refractory multiple myeloma (MM), in some solid tumor types, head and neck squamous cell carcinomas (HNSCC) and cervical cancer), it was also shown to decrease NF-κB-dependent chemo-and radio-resistance in various tumor models such as breast cancer and multiple myeloma (MM) [112, 113]. Velcade was proposed as a potential therapeutic agent for the treatment of cancer in NF-κB- dependent tumors; however, continuous exposure to bortezomib is associated with increased toxicity and development of denovo bortezomib resistance. Therefore, at minimally toxic concentrations the combination of synthetic triterpenoids (CDDO-Im) and bortezomib were used in multiple myeloma and other cancer cell lines [114]. The results indicate that the low-dose combination of CDDO-Im and bortezomib have selective anti-MM activity. The use of bortezomib and CDDO-Im in conjunction can overcome bortezomib resistance in some types of cancer [114]. Histone acetylation regulates transcription by remodeling chromatin structure; acetylated histone is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDAC). These two enzymes have opposite activities as HATs allow transcription and HDACs repress transcription [115, 116]. HDAC inhibitors induce cancer cells by modulating the function of transcription factors, like p53 and NF-kB, as opposed to normal cells [117]. HDAC inhibitors have high tolerance in cancer treatment and induce differentiation, apoptosis, and growth arrest with low toxicity [118]. HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) exhibit minimal toxicity, induce differentiation, growth arrest and apoptosis in some types of cancer [119-121]. HDAC inhibitors were used in combination with bortezomib in pancreatic cancer [122]. Given the low toxicity of CDDO derivates (e.g. CDDO-me) and HDAC inhibitors (e.g.

SAHA), the finding with proteasome inhibitors (e.g.bortezomib), may reveal the usefulness of CDDO-me and related compounds in various combination-treatment strategies toward the end of overcoming resistance to cancer treatments.

FIGURES AND LEGENDS

A



B

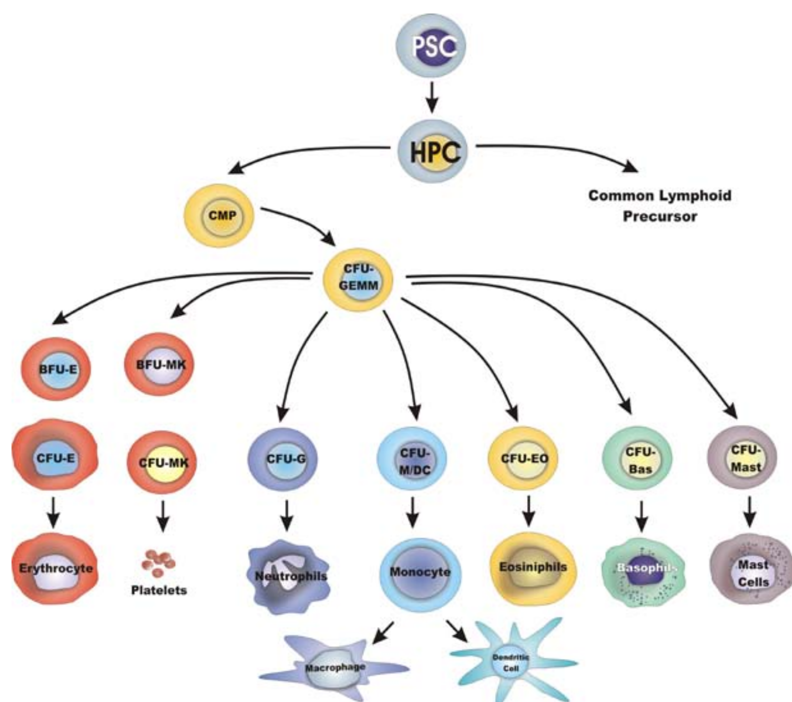


Figure 1. Hematopoiesis: Classic schema of a haematopoietic lineage tree with an early division into myeloid and lymphoid branches. *A*- Common Myeloid Precursor branch, *B*- Common Lymphoid Precursor branch, Pluripotent Stem Cell (PSC), Haematopoietic Precursor Cells (HPC), Common Myeloid Precursor (CMP), Common Lymphoid Precursor (CLP), colony forming unit granulocytes-erythrocytes-Monocytes and Megakaryotes (CFU-GEMM), Burst forming unit erythrocytes (BFU-E), Burst forming unit Megakaryotes (BFU-MK), colony forming unit Megakaryotes (CFU-MK), colony forming unit granulocytes (CFU-G), colony forming unit erythrocytes (CFU-E), colony forming unit eosinophil (CFU-EO), colony forming unit Monocytes/Dendritic cells (CFU-M/DC), colony forming unit basophiles (CFU-Bas), colony forming unit Mast cells (CFU-Mast). BS4, TS4, NKS4, DCS4, B cells, T cells, NK cells and dendritic cells respectively at stage 4.

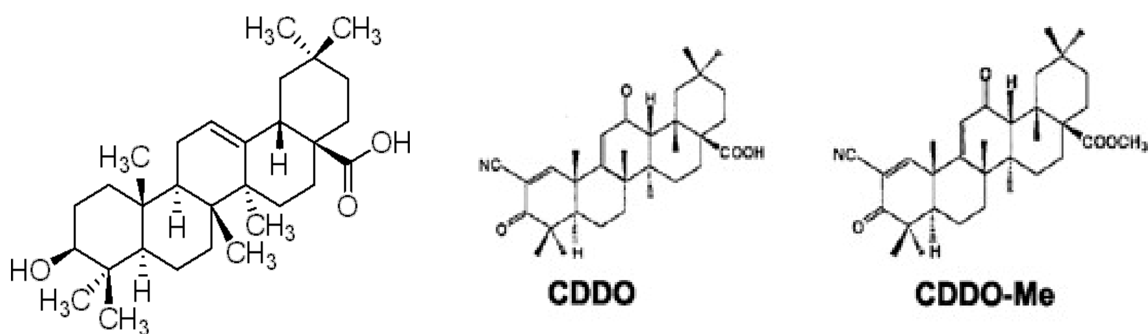


Figure 2. Oleanolic Acid and the Synthetic Triterpenoids CDDO and its Derivate C-28 Methyl ester (CDDO-me) [71, 72].

REFERENCE LIST

1. Verfaillie, C.M., *Adult stem cells: assessing the case for pluripotency.* Trends Cell Biol, 2002. **12**(11): p. 502-8.

2. Verfaillie, C.M., *Optimizing hematopoietic stem cell engraftment: a novel role for thrombopoietin.* J Clin Invest, 2002. **110**(3): p. 303-4.

3. Glimm, H., et al., *Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID-beta2 microglobulin-null mice.* J Clin Invest, 2001. **107**(2): p. 199-206.

4. Harrison, D.E. and C.M. Astle, *Short- and long-term multilineage repopulating hematopoietic stem cells in late fetal and newborn mice: models for human umbilical cord blood.* Blood, 1997. **90**(1): p. 174-81.

5. Metcalf, D., *Lineage commitment in the progeny of murine hematopoietic preprogenitor cells: influence of thrombopoietin and interleukin 5.* Proc Natl Acad Sci U S A, 1998. **95**(11): p. 6408-12.

6. Radtke, F., et al., *Notch regulation of lymphocyte development and function.* Nat Immunol, 2004. **5**(3): p. 247-53.

7. Kawamoto, H. and Y. Katsura, *[The process of lineage commitment in hematopoiesis].* Rinsho Ketsueki, 2002. **43**(4): p. 274-7.

8. Armstrong, L., et al., *Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity.* Stem Cells, 2004. **22**(7): p. 1142-51.

9. Metcalf, D., *Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: influence of colony-stimulating factors.* Proc Natl Acad Sci U S A, 1991. **88**(24): p. 11310-4.

10. Kawamoto, H. and N. Minato, *Myeloid cells.* Int J Biochem Cell Biol, 2004. **36**(8): p. 1374-9.

11. Serafini, M. and C.M. Verfaillie, *Pluripotency in adult stem cells: state of the art.* Semin Reprod Med, 2006. **24**(5): p. 379-88.

12. Paquette, R. and K. Dorshkind, *Optimizing hematopoietic recovery following bone marrow transplantation.* J Clin Invest, 2002. **109**(12): p. 1527-8.

13. Katsura, Y., *Redefinition of lymphoid progenitors.* Nat Rev Immunol, 2002. **2**(2): p. 127-32.

14. Urban, B.C., et al., *The frequency of BDCA3-positive dendritic cells is increased in the peripheral circulation of Kenyan children with severe malaria.* Infect Immun, 2006. **74**(12): p. 6700-6.

15. Urban, B.C., et al., *Frequencies of peripheral blood myeloid cells in healthy Kenyan children with alpha+ thalassemia and the sickle cell trait.* Am J Trop Med Hyg, 2006. **74**(4): p. 578-84.

16. Urban, B.C. and S. Todryk, *Malaria pigment paralyzes dendritic cells.* J Biol, 2006. **5**(2): p. 4.

17. Jenkins, N.E., et al., *The effect of Plasmodium falciparum infection on expression of monocyte surface molecules.* Trans R Soc Trop Med Hyg, 2006. **100**(11): p. 1007-12.

18. Stevenson, M.M. and B.C. Urban, *Antigen presentation and dendritic cell biology in malaria.* Parasite Immunol, 2006. **28**(1-2): p. 5-14.

19. Lu, M., et al., *The common myelolymphoid progenitor: a key intermediate stage in hemopoiesis generating T and B cells.* J Immunol, 2002. **169**(7): p. 3519-25.

20. Bell, J.J. and A. Bhandoola, *The earliest thymic progenitors for T cells possess myeloid lineage potential.* Nature, 2008. **452**(7188): p. 764-7.

21. Wada, H., et al., *Adult T-cell progenitors retain myeloid potential.* Nature, 2008. **452**(7188): p. 768-72.

22. Kolesnick, R. and D.W. Golde, *The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling.* Cell, 1994. **77**(3): p. 325-8.

23. Broxmeyer, H.E., *Regulation of hematopoiesis by chemokine family members.* Int J Hematol, 2001. **74**(1): p. 9-17.

24. Jacobsen, S.E., et al., *The growth response of Lin-Thy-1+ hematopoietic progenitors to cytokines is determined by the balance between synergy of multiple stimulators and negative cooperation of multiple inhibitors.* Exp Hematol, 1994. **22**(10): p. 985-9.

25. Feng, B., et al., *Development of mouse dendritic cells from lineage-negative c-kit(low) pluripotent hemopoietic stem cells in vitro.* Stem Cells, 2000. **18**(1): p. 53-60.

26. Sitnicka, E., et al., *Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells.* Blood, 2003. **102**(3): p. 881-6.

27. Matera, L., et al., *Individual and combined effect of granulocyte-macrophage colony-stimulating factor and prolactin on maturation of dendritic cells from blood monocytes under serum-free conditions.* Immunology, 2000. **100**(1): p. 29-36.

28. Solanilla, A., et al., *CD40-ligand stimulates myelopoiesis by regulating flt3-ligand and thrombopoietin production in bone marrow stromal cells.* Blood, 2000. **95**(12): p. 3758-64.

29. Solanilla, A., et al., *Expression of Flt3-ligand by the endothelial cell.* Leukemia, 2000. **14**(1): p. 153-62.

30. Caceres-Cortes, J.R., et al., *Steel factor sustains SCL expression and the survival of purified CD34+ bone marrow cells in the absence of detectable cell differentiation.* Stem Cells, 2001. **19**(1): p. 59-70.

31. Cooper, S.H. and H.E. Broxmeyer, *Measurement of interleukin 3 and other hematopoietic cytokines, such as GM-CSF, G-CSF, M-CSF, erythropoietin, steel factor, and Flt-3 ligand.* Curr Protoc Immunol, 2001. **Chapter 6**: p. Unit 6 4.

32. Smith, A., D. Metcalf, and N.A. Nicola, *Cytoplasmic domains of the common beta-chain of the GM-CSF/IL-3/IL-5 receptors that are required for inducing differentiation or clonal suppression in myeloid leukaemic cell lines.* Embo J, 1997. **16**(3): p. 451-64.

33. Metcalf, D., *Hematopoietic cytokines.* Blood, 2008. **111**(2): p. 485-91.

34. Michel, M.L., et al., *Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia.* J Exp Med, 2007. **204**(5): p. 995-1001.

35. Schwarzenberger, P., et al., *Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis.* J Immunol, 2000. **164**(9): p. 4783-9.

36. Baggiolini, M., *Introduction to chemokines and chemokine antagonists.* Ernst Schering Res Found Workshop, 2004(45): p. 1-9.

37. Broxmeyer, H.E., et al., *Stromal cell-derived factor-1/CXCL12 selectively counteracts inhibitory effects of myelosuppressive chemokines on hematopoietic progenitor cell proliferation in vitro.* Stem Cells Dev, 2005. **14**(2): p. 199-203.

38. Christopherson, K.W., 2nd, et al., *Modulation of hematopoietic stem cell homing and engraftment by CD26.* Science, 2004. **305**(5686): p. 1000-3.

39. Tian, C., et al., *Inhibition of CD26 peptidase activity significantly improves engraftment of retrovirally transduced hematopoietic progenitors.* Gene Ther, 2006. **13**(7): p. 652-8.

40. de Pooter, R.F., et al., *Notch signaling requires GATA-2 to inhibit myelopoiesis from embryonic stem cells and primary hemopoietic progenitors.* J Immunol, 2006. **176**(9): p. 5267-75.

41. Deftos, M.L., et al., *Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes.* Immunity, 2000. **13**(1): p. 73-84.

42. Deftos, M.L. and M.J. Bevan, *Notch signaling in T cell development.* Curr Opin Immunol, 2000. **12**(2): p. 166-72.

43. Schroeter, E.H., J.A. Kisslinger, and R. Kopan, *Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain.* Nature, 1998. **393**(6683): p. 382-6.

44. Lai, E.C., *Notch signaling: control of cell communication and cell fate.* Development, 2004. **131**(5): p. 965-73.

45. Pulliam, A.C., et al., *AMD3100 synergizes with G-CSF to mobilize repopulating stem cells in Fanconi anemia knockout mice.* Exp Hematol, 2008. **36**(9): p. 1084-90.

46. Christopherson, K.W., 2nd, et al., *CD26 inhibition on CD34+ or lineage- human umbilical cord blood donor hematopoietic stem cells/hematopoietic progenitor cells improves long-term engraftment into NOD/SCID/Beta2null immunodeficient mice.* Stem Cells Dev, 2007. **16**(3): p. 355-60.

47. Broxmeyer, H.E., et al., *AMD3100 and CD26 modulate mobilization, engraftment, and survival of hematopoietic stem and progenitor cells mediated by the SDF-1/CXCL12-CXCR4 axis.* Ann N Y Acad Sci, 2007. **1106**: p. 1-19.

48. Broxmeyer, H.E., et al., *Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist.* J Exp Med, 2005. **201**(8): p. 1307-18.

49. Levesque, J.P., et al., *Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide.* J Clin Invest, 2003. **111**(2): p. 187-96.

50. Christopherson, K.W., 2nd, G. Hangoc, and H.E. Broxmeyer, *Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells.* J Immunol, 2002. **169**(12): p. 7000-8.

51. Christopherson, K.W., 2nd, S. Cooper, and H.E. Broxmeyer, *Cell surface peptidase CD26/DPPIV mediates G-CSF mobilization of mouse progenitor cells.* Blood, 2003. **101**(12): p. 4680-6.

52. Christopherson, K.W., et al., *CD26 is essential for normal G-CSF-induced progenitor cell mobilization as determined by CD26-/- mice.* Exp Hematol, 2003. **31**(11): p. 1126-34.

53. Christopherson, K.W., 2nd and R.A. Hromas, *Endothelial chemokines in autoimmune disease.* Curr Pharm Des, 2004. **10**(2): p. 145-54.

54. Pelus, L.M., et al., *Neutrophil-derived MMP-9 mediates synergistic mobilization of hematopoietic stem and progenitor cells by the combination of G-CSF and the chemokines GRObeta/CXCL2 and GRObetaT/CXCL2delta4.* Blood, 2004. **103**(1): p. 110-9.

55. Jin, D.K., et al., *Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes.* Nat Med, 2006. **12**(5): p. 557-67.

56. Fukuda, S., C.R. Mantel, and L.M. Pelus, *Survivin regulates hematopoietic progenitor cell proliferation through p21WAF1/Cip1-dependent and -independent pathways.* Blood, 2004. **103**(1): p. 120-7.

57. Devine, S.M., et al., *Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma.* J Clin Oncol, 2004. **22**(6): p. 1095-102.

58. Maris, M.B., et al., *HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies.* Blood, 2003. **102**(6): p. 2021-30.

59. Maris, M. and R. Storb, *The transplantation of hematopoietic stem cells after non-myeloablative conditioning: a cellular therapeutic approach to hematologic and genetic diseases.* Immunol Res, 2003. **28**(1): p. 13-24.

60. Maris, M., et al., *Immunologic recovery after hematopoietic cell transplantation with nonmyeloablative conditioning.* Exp Hematol, 2003. **31**(10): p. 941-52.

61. Krause, D.S., et al., *Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell.* Cell, 2001. **105**(3): p. 369-77.

62. Perez, L.E., et al., *Xenotransplantation of immunodeficient mice with mobilized human blood CD34+ cells provides an in vivo model for human megakaryocytopoiesis and platelet production.* Blood, 2001. **97**(6): p. 1635-43.

63. Stelljes, M., et al., *Graft-versus-host disease after allogeneic hematopoietic stem cell transplantation induces a CD8+ T cell-mediated graft-versus-tumor effect that is independent of the recognition of alloantigenic tumor targets.* Blood, 2004. **104**(4): p. 1210-6.

64. Scheffold, C., et al., *Cytokines and cytotoxic pathways in engraftment resistance to purified allogeneic hematopoietic stem cells.* Biol Blood Marrow Transplant, 2005. **11**(1): p. 1-12.

65. Scheffold, C., et al., *Prediction of relapse of acute myeloid leukemia in allogeneic transplant recipients by marrow CD34+ donor cell chimerism analysis.* Leukemia, 2004. **18**(12): p. 2048-50.

66. Nolte, A., et al., *Generation of melanoma-specific cytotoxic T lymphocytes for allogeneic immunotherapy.* J Immunother, 2003. **26**(3): p. 257-69.

67. Klumpp, T.R., S.L. Goldberg, and K.F. Mangan, *Effect of granulocyte colony-stimulating factor on the rate of neutrophil engraftment following peripheral-blood stem-cell transplantation.* J Clin Oncol, 1995. **13**(8): p. 2144.

68. Klumpp, T.R., et al., *Granulocyte colony-stimulating factor accelerates neutrophil engraftment following peripheral-blood stem-cell transplantation: a prospective, randomized trial.* J Clin Oncol, 1995. **13**(6): p. 1323-7.

69. Molineux, G., et al., *An analysis of the effects of combined treatment with rmGM-CSF and PEG-rHuMGDF in murine bone marrow transplant recipients.* Stem Cells, 1997. **15**(1): p. 43-9.

70. Molineux, G., et al., *Flt-3 ligand synergizes with granulocyte colony-stimulating factor to increase neutrophil numbers and to mobilize peripheral blood stem cells with long-term repopulating potential.* Blood, 1997. **89**(11): p. 3998-4004.

71. Kim, K.B., et al., *Identification of a novel synthetic triterpenoid, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells.* Mol Cancer Ther, 2002. **1**(3): p. 177-84.

72. Suh, N., et al., *A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity.* Cancer Res, 1999. **59**(2): p. 336-41.

73. Nishino, H., et al., *Inhibition of the tumor-promoting action of 12-O-tetradecanoylphorbol-13-acetate by some oleanane-type triterpenoid compounds.* Cancer Res, 1988. **48**(18): p. 5210-5.

74. Suh, N., et al., *Synthetic triterpenoids enhance transforming growth factor beta/Smad signaling.* Cancer Res, 2003. **63**(6): p. 1371-6.

75. Place, A.E., et al., *The novel synthetic triterpenoid, CDDO-imidazolide, inhibits inflammatory response and tumor growth in vivo.* Clin Cancer Res, 2003. **9**(7): p. 2798-806.

76. Honda, T., et al., *Design, synthesis, and biological evaluation of biotin conjugates of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid for the isolation of the protein targets.* J Med Chem, 2004. **47**(20): p. 4923-32.

77. Samudio, I., et al., *A novel mechanism of action of methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate: direct permeabilization of the inner mitochondrial membrane to inhibit electron transport and induce apoptosis.* Mol Pharmacol, 2006. **69**(4): p. 1182-93.

78. Samudio, I., et al., *Inhibition of mitochondrial metabolism by methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate induces apoptotic or autophagic cell death in chronic myeloid leukemia cells.* Mol Cancer Ther, 2008. **7**(5): p. 1130-9.

79. Shishodia, S., et al., *A synthetic triterpenoid, CDDO-Me, inhibits IkappaBalpha kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor kappaB-regulated gene products in human leukemic cells.* Clin Cancer Res, 2006. **12**(6): p. 1828-38.

80. Suh, W.S., et al., *Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL.* Leukemia, 2003. **17**(11): p. 2122-9.

81. Zou, W., et al., *Coupling of endoplasmic reticulum stress to CDDO-Me-induced up-regulation of death receptor 5 via a CHOP-dependent mechanism involving JNK activation.* Cancer Res, 2008. **68**(18): p. 7484-92.

82. Gilmore, T.D., *Introduction to NF-kappaB: players, pathways, perspectives.* Oncogene, 2006. **25**(51): p. 6680-4.

83. Gilmore, T.D. and M. Herscovitch, *Inhibitors of NF-kappaB signaling: 785 and counting.* Oncogene, 2006. **25**(51): p. 6887-99.

84. Bharti, A.C., et al., *Nuclear factor-kappaB and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis.* Blood, 2004. **103**(8): p. 3175-84.

85. Bharti, A.C., et al., *Evidence that receptor activator of nuclear factor (NF)-kappaB ligand can suppress cell proliferation and induce apoptosis through activation of a NF-kappaB-independent and TRAF6-dependent mechanism.* J Biol Chem, 2004. **279**(7): p. 6065-76.

86. Shishodia, S. and B.B. Aggarwal, *Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis.* J Biol Chem, 2004. **279**(45): p. 47148-58.

87. Shishodia, S. and B.B. Aggarwal, *Nuclear factor-kappaB: a friend or a foe in cancer?* Biochem Pharmacol, 2004. **68**(6): p. 1071-80.

88. Aggarwal, B.B. and S. Shishodia, *Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning.* Ann N Y Acad Sci, 2004. **1030**: p. 434-41.

89. Shishodia, S. and B.B. Aggarwal, *Nuclear factor-kappaB activation mediates cellular transformation, proliferation, invasion angiogenesis and metastasis of cancer.* Cancer Treat Res, 2004. **119**: p. 139-73.

90. Shishodia, S., D. Koul, and B.B. Aggarwal, *Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates TNF-induced NF-kappa B activation through inhibition of activation of I kappa B alpha kinase and Akt in human non-small cell lung carcinoma: correlation with suppression of COX-2 synthesis.* J Immunol, 2004. **173**(3): p. 2011-22.

91. Shishodia, S. and B.B. Aggarwal, *Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates activation of cigarette smoke-induced nuclear factor (NF)-kappaB by suppressing activation of IkappaBalpha kinase in human non-small cell lung carcinoma: correlation with suppression of cyclin D1, COX-2, and matrix metalloproteinase-9.* Cancer Res, 2004. **64**(14): p. 5004-12.

92. Vannini, N., et al., *The synthetic oleanane triterpenoid, CDDO-methyl ester, is a potent antiangiogenic agent.* Mol Cancer Ther, 2007. **6**(12 Pt 1): p. 3139-46.

93. Dell'Eva, R., et al., *AKT/NF-kappaB inhibitor xanthohumol targets cell growth and angiogenesis in hematologic malignancies.* Cancer, 2007. **110**(9): p. 2007-11.

94. Carter, B.Z., et al., *Small-molecule XIAP inhibitors derepress downstream effector caspases and induce apoptosis of acute myeloid leukemia cells.* Blood, 2005. **105**(10): p. 4043-50.

95. Kater, A.P., et al., *Inhibitors of XIAP sensitize CD40-activated chronic lymphocytic leukemia cells to CD95-mediated apoptosis.* Blood, 2005. **106**(5): p. 1742-8.

96. Reed, J.C. and M. Pellecchia, *Apoptosis-based therapies for hematologic malignancies.* Blood, 2005. **106**(2): p. 408-18.

97. Zou, W., et al., *c-Jun NH2-terminal kinase-mediated up-regulation of death receptor 5 contributes to induction of apoptosis by the novel synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1, 9-dien-28-oate in human lung cancer cells.* Cancer Res, 2004. **64**(20): p. 7570-8.

98. Cheng, E.H., et al., *Bax-independent inhibition of apoptosis by Bcl-XL.* Nature, 1996. **379**(6565): p. 554-6.

99. Cheng, E.H., et al., *A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak.* Proc Natl Acad Sci U S A, 1997. **94**(2): p. 690-4.

100. Cheng, E.H., et al., *BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis.* Mol Cell, 2001. **8**(3): p. 705-11.

101. Wang, Y., et al., *A synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), is a ligand for the peroxisome proliferator-activated receptor gamma.* Mol Endocrinol, 2000. **14**(10): p. 1550-6.

102. Thimmulappa, R.K., et al., *Preclinical evaluation of targeting the Nrf2 pathway by triterpenoids (CDDO-Im and CDDO-Me) for protection from LPS-induced inflammatory response and reactive oxygen species in human peripheral blood mononuclear cells and neutrophils.* Antioxid Redox Signal, 2007. **9**(11): p. 1963-70.

103. Konopleva, M., et al., *Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia.* Blood, 2002. **99**(1): p. 326-35.

104. Konopleva, M., et al., *Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia.* Cancer Cell, 2006. **10**(5): p. 375-88.

105. Ikeda, T., et al., *The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance.* Cancer Res, 2003. **63**(17): p. 5551-8.

106. Hyer, M.L., et al., *Apoptotic activity and mechanism of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid and related synthetic triterpenoids in prostate cancer.* Cancer Res, 2008. **68**(8): p. 2927-33.

107. Samudio, I., et al., *Guggulsterones induce apoptosis and differentiation in acute myeloid leukemia: identification of isomer-specific antileukemic activities of the pregnadienedione structure.* Mol Cancer Ther, 2005. **4**(12): p. 1982-92.

108. Tran, T.A., et al., *The synthetic triterpenoid CDDO-methyl ester modulates microglial activities, inhibits TNF production, and provides dopaminergic neuroprotection.* J Neuroinflammation, 2008. **5**: p. 14.

109. Lun, M., et al., *Nuclear factor-kappaB pathway as a therapeutic target in head and neck squamous cell carcinoma: pharmaceutical and molecular validation in human cell lines using Velcade and siRNA/NF-kappaB.* Ann Clin Lab Sci, 2005. **35**(3): p. 251-8.

110. Lun, M., et al., *Intracellular inhibitory effects of Velcade correlate with morphoproteomic expression of phosphorylated-nuclear factor-kappaB and p53 in breast cancer cell lines.* Ann Clin Lab Sci, 2005. **35**(1): p. 15-24.

111. Adams, J., et al., *Proteasome inhibitors: a novel class of potent and effective antitumor agents.* Cancer Res, 1999. **59**(11): p. 2615-22.

112. Cardoso, F., et al., *Bortezomib (PS-341, Velcade) increases the efficacy of trastuzumab (Herceptin) in HER-2-positive breast cancer cells in a synergistic manner.* Mol Cancer Ther, 2006. **5**(12): p. 3042-51.

113. Goel, A., et al., *Synergistic activity of the proteasome inhibitor PS-341 with non-myeloablative 153-Sm-EDTMP skeletally targeted radiotherapy in an orthotopic model of multiple myeloma.* Blood, 2006. **107**(10): p. 4063-70.

114. Chauhan, D., et al., *The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance.* Blood, 2004. **103**(8): p. 3158-66.

115. Narlikar, G.J., H.Y. Fan, and R.E. Kingston, *Cooperation between complexes that regulate chromatin structure and transcription.* Cell, 2002. **108**(4): p. 475-87.

116. Papeleu, P., et al., *Differential effects of histone deacetylase inhibitors in tumor and normal cells-what is the toxicological relevance?* Crit Rev Toxicol, 2005. **35**(4): p. 363-78.

117. Glozak, M.A., et al., *Acetylation and deacetylation of non-histone proteins.* Gene, 2005. **363**: p. 15-23.

118. Vigushin, D.M. and R.C. Coombes, *Histone deacetylase inhibitors in cancer treatment.* Anticancer Drugs, 2002. **13**(1): p. 1-13.

119. Butler, L.M., et al., *Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo.* Cancer Res, 2000. **60**(18): p. 5165-70.

120. Marks, P.A., V.M. Richon, and R.A. Rifkind, *Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells.* J Natl Cancer Inst, 2000. **92**(15): p. 1210-6.

121. Ungerstedt, J.S., et al., *Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 673-8.

122. Nawrocki, S.T., et al., *Aggresome disruption: a novel strategy to enhance bortezomib-induced apoptosis in pancreatic cancer cells.* Cancer Res, 2006. **66**(7): p. 3773-81.

CHAPTER 1

Effect of CDDO-me administration on the myelopoiesis in naïve mice

Abstract:

For the treatment of cancer and other hematological diseases, large numbers of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) are needed. The objectives of this study were first, to investigate the effect of CDDO-me on myeloid expansion and mobilization and, second, to determine the hematopoietic growth factor production in the serum of mice that received CDDO-me or vehicle control. This was done by first determining the optimal timing and dosing of CDDO-me to expand CFU-GM. Then the effect of CDDO-me on myeloid expansion and mobilization was determined, and finally the production hematopoietic growth factor in the serum of mice that received CDDO-me or vehicle control was investigated. CDDO-me (120 µg /dose BID) or VC was administered to naïve mice during 7 days; the spleen, BM and blood were collected. Colony assay and flow cytometric analysis were done on the spleen, BM and blood but no flow cytometric analysis was performed on the blood. To determine the hematopoietic growth factor production, colony assay was done by adding 10% of the mice serum that were treated with CDDO-me or VC, and the remaining serum was used to determine the hematopoietic growth factor production via Luminex technology. The result showed a dosing and timing dependent CFU-GM expansion in the spleen and bone marrow after CDDO-me administration. Administration of CDDO-me (120 µg/ dose, BID) significantly increased myeloid, erythroid and CFU-HPPs in the spleen and bone marrow. In addition to inducing CFU-GM expansion, administration of CDDO-me (120 µg/ dose, BID) caused a significant mobilization of myeloid and erythroid progenitors in the spleen and blood. The results from colony assay (with 10% mice serum) and Luminex

technology assay showed an increase of G-CSF level in the serum of mice that were treated with CDDO-me. From these results it was concluded that: CDDO-me (120 µg/ dose, BID) administration enhanced myelopoiesis and caused mobilization of myeloid and myeloid progenitor cells into the spleen and blood. The level of G-CSF on the serum was significantly affected by CDDO-me administration.

Introduction

CDDO-me is a synthetic oleanane triterpenoid, which is proven to be non-cytotoxic and multifunctional drug. It can be used not only for cancer prevention and treatment, but also for other inflammatory diseases. We hypothesized that administration of CDDO-me will significantly expand myeloid, erythroid and CFU-HPPs in the spleen and bone marrow, and this expansion will be followed by a mobilization of these cells into the spleen and blood.

In the treatment of cancer and other hematological diseases, large numbers of hematopoietic stem cells (HSC) and hematopoietic progenitor cells (HPC) are needed. The current methods used for HSC expansion and mobilization include the administration of cytokines (e.g. G-CSF) and/or antagonists of chemokine receptors (e.g. AMD3100) alone or in combination with myelosuppressive chemotherapy [1, 2]. These methods fail to expand and mobilize HSC in a percentage of patients and may pose high health risks in some patients [3, 4]. New expanding and mobilizing agents with low side effects and high efficacy are needed. The C-28 methyl ester of 2-cyano-3, 12-dioxoolean-1, 9-dien28-oic (CDDO-me) is a synthetic triterpenoid based on naturally occurring ursolic oleanolic acids [5]. The synthetic triterpenoids have been shown to be highly effective in many *in vivo* models for the prevention and treatment of cancer by suppressing the growth of tumor cells, inducing apoptosis in cancer cells that are highly resistant to conventional chemotherapeutics agents and by preventing a primary lung cancer induced by chemical carcinogens [6, 7]. CDDO-me is a novel antioxidant inflammation modulator that has been shown to inhibit inflammatory cytokine production, such as IFN-

γ, Interleukin-1 and TNF-α through the suppression of NF-kB and STAT3. It can induce apoptosis and differentiation in some forms of cancer such as human leukemic and solid tumor cells [8-12]. In clinical trials, CDDO-me has shown benefits in some cancers, such as pancreatic cancer, and is well tolerated by patients [8, 12, 13]. In this chapter, our first goal was to investigate the role of CDDO-me on CFU-GM expansion, and the optimal dose of CDDO-me to use to maximize CFU-GM and CFU-HPP expansion, and to optimize the timing for CDDO-me to expand CFU-GM in naïve mice. In the second part of this chapter we analyzed the effect of CDDO-me on myeloid expansion and mobilization. Lastly, we studied the effect of CDDO-me on myeloid and myeloid progenitor expansion by determining the hematopoietic growth factor production in the serum of mice that were treated with CDDO-me or vehicle control.

MATERIALS AND METHODS

Animals

Female C57BL/6, Ly5.2 mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were kept in specific pathogen-free conditions. All animal protocols were approved and in vivo studies were performed at the University of Nevada, Reno (UNR). At the start of the experiments, mice were between eight and thirteen weeks of age.

Reagents:

CDDO-me was kindly provided by Reata Pharmaceutics, Inc and stored at 4ºC. CDDO-me was prepared in a solution of 10% dimethyl sulphoxide (DMSO) (Sigma, St Louis, MO), 10% Cremophor EL (Sigma), and 80% of a 0.9% sodium chloride solution, USP (Baxter Healthcare Corporation). CDDO-me was weighed and dissolved into DMSO, followed by the addition of Cremophor EL. Lastly, 0.9% sodium chloride solution was added and mixed for one minute. The vehicle control (VC) was prepared under the same conditions as above without the addition of CDDO-me. The solutions were filter-sterilized and stored at 4ºC for up to 7 days prior to use. CDDO-me was tested for myeloid expansion at concentrations of 0 mg/ml, 0.075 mg/ml, 0.15 mg/ml, 0.3 mg/ml and 0.6 mg/ml.

Cell preparation:

Spleens were harvested and teased apart into single cell suspension under sterile conditions. Bone marrow cells were harvested and crushed in processing buffer consisting of 1% Fetal Bovine Serum (FBS) in a PBS solution using mortor and pestle. The processed cells were run through a nylon mesh strainer to remove debris and obtain a single cell suspension. Cell suspensions were centrifuged at 250 x gravity (g) for 10 minutes at 4ºC and the supernatant was discarded. The samples were resuspended in processing buffer and cell counts were performed on a Coulter Z1 cell counter (Coulter Electronic, Hialeah, FL). Cell suspensions were kept on ice or at 4ºC prior to use for colony assay and flow cytometry.

Animal treatment

Female C57BL/6 mice (B6) received CDDO-me or vehicle control (VC) intraperitoneally (i.p) with the dose range (0 µg to 120 µg /dose) given twice a day (BID). CDDO-me was given at lengths of time with a maximum duration of seven days and a minimum duration of one day. Mice were euthanized and the spleen, bone marrow and blood were collected. All experiments were performed two to three times with 3 mice per group. All the steps were performed under sterile conditions.

Complete blood count (CBC)

Blood samples were collected by cardiac puncture in microvette capillarity blood collection tubes with EDTA (Sarstedt, Germany). The CBC analysis was performed on Hemavet 950 (Drew Scientific, Oxford, CT).

Blood collection and Separation:

Using cardiac puncture blood was collected into a tube containing 3 ml of PBS with heparin (10U/ml); the blood volume was noted before transfer into the tube and 3 ml of Lymphoprep (Cedarlane) was carefully underlayered. Tubes were centrifuged at 800 x g for 20 minutes at room temperature (RT) (approximately 20°C). The cells at the interface between the PBS and Lymphoprep were collected using a pipette. Cells were washed twice with processing buffer (1% FBS in PBS) and cell counts were performed on a Coulter Z1 cell counter (Coulter Electronic, Hialeah, FL).

Preparation of serum samples:

For the preparation of serum samples, peripheral blood was drawn into a Vacutainer Gel and a Clot activator tube (Beckton-Dickinson). It was then centrifuged in order to separate out serum. The serum was aliquoted into NUN-cryovial tubes and placed in a -70°C freezer.

Colony assay

For colony forming unit-granulocyte/monocyte (CFU-GM) and burst forming unit-erythrocyte (BFU-E) assays, bone marrow cells and spleen cells were plated in 35-mm Petri dishes at concentrations of $5x10^4$ or $5x10^5$ nucleated cells per plate, respectively. Cells were added to the media which consist of FBS (15%), penicillin/streptomycin (50 IU/mL), 2-Mercapto-ethanol ($5x10^{-3}$M), Iscove's Modified Dulbeco's Media (IMDM) and methylcellulose (2.75%). The solution was mixed, and 1mL was plated into each of three 35-mm Petri dishes. Colony formation was stimulated by 10ng/ml recombinant murine IL-3, 10ng/ml recombinant murine GM-CSF (all from PeproTech) and 5 U/ml recombinant human erythropoietin (Amgen). Plates were incubated for 7 days at 37º C in humidified atmosphere with 5% CO_2. Colonies were defined as an aggregate of more than 50 cells. Colonies that contained only red cells in single or multiple bursts were scored as BFU-E. Colonies that contained only white cells consisting of granulocytes and/or macrophages were scored as CFU-GM; colonies that were a mix (white and red) were scored as colony-forming units generating granulocytes, erythroblasts, macrophages and megakaryocytes (CFU-GEMM).

For colony forming unit-high proliferative potential (CFU-HPP), bone marrow cells and spleen cells were plated in 60-mm Petri dishes at concentrations of 5 $x10^4$ or 5 $x10^5$ nucleated cells per plate, respectively. Cells were added to the media and colony formation was stimulated with 20 ng/mL recombinant murine IL-6, 20 ng/mL recombinant murine IL-3 and 50 ng/mL stem cell factor (SCF) (all from PeproTech). Plates were incubated for 11 days at 37° C in a humidified atmosphere with 5% CO_2.

Colonies (CFU-HPP) were defined as colonies with a diameter of >2 mm and an estimated cell number of >10,000 cells.

Colony stimulating activity determination by clonogeneic assay: to determine the effect of CDDO-me serum treated mice in CFU-GM, serum from mice that were treated with CDDO-me or vehicle control (VC) was added to the colony assay media. The media for CFU-GM colony assay was the same as the previous colony assay media. The only differences were that the colony formation was stimulated by a low dose of IL-3 (0.31 ng/mL) or GM-CSF (0.31 ng/mL), and 10% of mice serum treated with CDDO-me or VC. Recombinant human G-CSF (1.5 ng/mL) (Amgen) was used as a positive control for CFU-GM.

Serum cytokine analysis by Luminex technology:

In this experiment we used Luminex 100 system (Corp., Austin, TX) to evaluate the sera of naïve mice that received CDDO-me or VC. The level of 3 cytokines consisting of G-CSF, GM-CSF and interleukin 6 (IL-6) were assessed. A single antibody bead kit of G-CSF, GM-CSF and IL-6, (Invitrogen) was used. The standard and sample wells were pre-wet with 200 µl Working Wash Solution, which was aspirated from the wells after 30 seconds (sec) using the vacuum manifold. The diluted captured bead solution was mixed (30 sec) and sonicated (30 sec), followed by the immediate addition of 25 µl to each well, and then 200 µl of 1x Working Wash Solution was added. After 30 seconds the liquid was aspirated and the wash with 200 µl of 1x Working Wash Solution was repeated. Then 50 µl of incubation Buffer was added to all wells and 100 µl standard dilution was added into designed wells. For wells designed for samples, 50 µl of diluents

was added followed by 50 µl of serum sample. The plate was incubated and agitated on an orbital plate shaker (500-600rpm) for 2 hours at room temperature (RT) in the dark. The Working Wash Solution from the wells was aspirated using the vacuum manifold and 200 µl of 1x Working Wash Solution was added after 30 seconds. This was aspirated; the wash with 200 µl of 1x Working Wash Solution was repeated. Then 100 µl of prepared 1x Biotinylated Detector Antibody was added to each well and the plate was incubated on an orbital plate shaker (500-600 rpm) for 1 hour at RT in the dark. The liquid from wells was removed by aspiration with the vacuum manifold, 200 µl of 1x Working Wash Solution was added, after 30 sec and aspirated with the vacuum manifold. This washing step was repeated, and then 100 µl of prepared 1x Streptavidin-Phycoerythrin (RPE) was added to each well and incubated for 30 min on an orbital plate shaker (500-600 rpm) at RT in the dark. The liquid from the wells was removed by aspiration with the vacuum manifold. The beads were washed by adding 200 µl of 1x Working Wash Solution to the wells allowing the beads to soak for 10 seconds, then they were aspirated with the vacuum manifold. This washing step was repeated two additional times for a total of three washes. The bottom of the filter plate was blotted on clean paper towels to remove residual liquid. Beads were re-suspended in the plate with 100 µl 1x Working Wash Solution to each well. The plate was agitated on an orbital shaker (500-600 rpm) for 2-5 min to resuspend the beads; and samples were analyzed with in an hour using the Luminex 100 system. The readout for the concentration of each cytokine was detected as median fluorescence intensity (MFI) by the instrument. These values were subsequently converted to pg/mL of cytokine based upon the MFI values from a set of standard curve that we ran simultaneously in the assay.

Flow Cytometric Analysis

One million (1 x 10^6) bone marrow cells or splenocytes were centrifuged into a pellet and resuspended in 50 µl of blocking buffer, incubated for 15-20 minutes, then incubated with antibodies for 20-30 minutes at 4ºC and washed twice with staining buffer. The following antibodies were used for cell staining: FITC-conjugated anti-mouse CD11b (clone M1/70, Pharmingen), PE-conjugated anti-mouse Gr-1 (clone 8C5, eBioSciences) and isotype control antibodies. The stained cells were fixed with 1% paraformaldehyde in PBS and then analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest software. The flow cytometric analysis was performed using Flowjo software (Tree Star Inc).

Statistical Analysis

Statistical analysis was performed by a log-rank test, one way ANOVA analysis of the mean or Student's t test and the data were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Dosing: CFU-GM expansion is CDDO-me dose dependent

The first goal of this study was to determine the role of CDDO-me on CFU-GM expansion *in vivo;* and the optimal timing and dosing of CDDO-me that would cause the maximum CFU-GM expansion. Naïve female C57BL/6 mice between eight and thirteen weeks of age were treated with CDDO-me ranging from 120 µg to 1.75 µg. The result showed that 120 µg/dose BID expanded CFU-GM to greater degree than 30 µg, 7.5 µg and 1.75 µg doses but this difference was not statistically significant (Figure 1). Thus, this treatment schedule was maintained in subsequent experiments.

Timing: CFU-GM expansion by CDDO-me administration is time dependent

After determining that 120 µg/dose BID of CDDO-me expanded CFU-GM better than other tested doses (30 µg/dose, 7.5 µg/dose , and 1.75 µg/dose, BID) , we next determined the optimal time for CDDO-me to expand myeloid cells, this was done by giving CDDO-me or vehicle control at over varying durations, ranging from 1 to 7 days. Bone marrow (femur) and spleen cells from mice that received CDDO-me or vehicle control were plated in colony assays as described in the Material and Methods section. CDDO-me (120 µg/dose BID) was administered daily and mice were assessed the day following the last treatment. The result showed a time dependent expansion of CFU-GM in the spleen and bone marrow. Administration of CDDO-me over 7 days showed

greater expansion of CFU-GM and Gr-1+/CD11b+ (result not shown) compared to 5 days (figure *2*).

Effect of CDDO-me on myeloid expansion

In this part of the study, our goal was to investigate the effect of CDDO-me on myeloid expansion and mobilization. Our previous data showed that administration of CDDO-me (120 µg/ dose, BID) over 7 days significantly expanded CFU-GM in the spleen and bone marrow. We investigated whether CDDO-me administration resulted only in CFU-GM expansion or both expansion and mobilization of CFU-GM into the blood and spleen. Resting female C57BL/6 mice were treated with CDDO-me or vehicle control intraperitoneally (i.p) using 120 µg/ dose BID during 7 days. Recombinant human granulocyte-colony stimulating factor (rhG-CSF at 2.5 µg/dose BID) was used as a control for myeloid expansion and mobilization. Spleen, femur and blood samples were collected and processed as described in the Material and Methods section. The schedule and doses for CDDO-me, VC and rhG-CSF are represented in figure 3.

Effect of CDDO-me administration on spleen and BM cellularity in naive mice

CDDO-me administration in naive mice did not significantly impact cellularity in the spleen and BM. However, a slight increase on the cellularity of the mice that received CDDO-me was detected compared to mice that received vehicle control (VC), but this difference was not statistically significant ($p<0.05$) (as shown in Figure 4).

For the complete blood count (CBC): No significant change was detected between mice that received CDDO-me and mice that received vehicle control on the complete blood count after CDDO-me administration (data not shown).

CDDO-me administration caused a significant increase of CD11b+/Gr-1+ cells

Mice were treated with vehicle control or CDDO-me (120 µg /dose) twice a day intraperitoneally from day 0 to day 6, and mice were euthanized on day 7. BM and spleen cells were processed and stained with the following antibodies: FITC-conjugated anti-mouse CD11b, and PE-conjugated anti-mouse Gr-1. The immature myeloid cells (Gr-1+CD11b+) represent about 20-30% of normal bone marrow cells and 2-4 % of all nucleated normal splenocytes. Myeloid lineage differentiation antigen Gr-1 is expressed on myeloid precursor, granulocytes, and transiently on monocytes [14]. CD11b (Mac-1) is mostly expressed on the surface of monocytes/ macrophages, DC, granulocytes and activated lymphocytes B and T cells [14, 15]. The flow cytometry findings showed that CDDO-me (120 µg/dose BID) administration caused a marked increase in the number of Gr-1+/CD11b+ cells in the spleen and bone marrow after 7 days of treatment. Gr-1+/CD11b+ cells were increased by 3-fold and 2-fold in the spleen and BM, respectively, after CDDO-me administration. The immature myeloid (Gr-1+/CD11b+) made up a high percentage of cells in the spleens (6.6 vs 3.13) and bone marrow (78.7 vs 24.8) for the mice that received CDDO-me versus VC (Figure 5*A* and 5*B*). The cytokine rhG-CSF (2.5 µg/dose BID) known to promote expansion and mobilization of hematopoietic precursors

from bone marrow was used as a control (Figure 5*B*). Our results showed that CDDO-me (120 µg/dose BID) had no significant deleterious effect on normal B and T level in the spleen and Thymus (data not shown). Result from Kress CL et al. [16] showed that CDDO-Im has less toxicity for normal B or T cells than leukemic B cells. The results from our laboratory showed that CDDO-me (120 µg/dose BID) treatment *in vivo* decreased B and T cells level in the spleen and Thymus. The decrease on B and T cells level is not significant compared mice that were treated with CDDO-me and mice that were treated with VC.

Colony Assay

CDDO-me administration caused a significant increase of CFU-GM, BFU-e and CFU-HPP in the spleen and BM

Knowing that CDDO-me administration could cause a significant expansion of myeloid cell, and we wanted to determine whether CDDO-me acted primarily on terminal stages of differentiation or at early phases in the development of hematopoiesis (committed and/or primitive progenitors). We performed a clonogeneic assay for myeloid (CFU-GM), erythroid (BFU-e) and high proliferative potential (CFU-HPP) precursors to determine at what levels CDDO-me acts to expand myeloid cells and/or myeloid progenitors. Naïve C56BL/6 mice were treated with CDDO-me (120 µg/dose) or vehicle control twice a day for 7 days. Spleen and bone marrow cells were collected at day 7 and processed in the clonogeneic assay. The results showed a significant increase of CFU-GM in the spleen and BM from mice that received CDDO-me compared to the mice that received vehicle control. The CFU-GM content of spleen and BM significantly

increased 19.5-fold and 2-fold, respectively, at day 7 after CDDO-me versus VC injection. The BFU-e content of the spleen increased by 5-fold, but the femoral (BM) BFU-e increase (1.6-fold) was not as significant as in the spleen BFU-e (Figure 6).

CDDO-me administrations resulted in significant expansions of primitive, high proliferative potential (CFU-HPP) cells in the spleen and bone marrow (Figure 6). The CFU-HPP content of the mice that received CDDO-me was significantly increased (5.5-fold) compared to the mice that received VC. Administration of CDDO-me significantly increased myeloid, erythroid and CFU-HPPs in the spleen and bone marrow. CDDO-me administration resulted in significant expansion of myeloid and erythroid progenitors in the spleen but not to the same extent as in the mice that received rhG-CSF (2.5 µg/dose BID) as a positive control (Figure 6).

CDDO-me administration resulted in a significant mobilization of CFU-GM into the blood

Our previous results showed that administration of CDDO-me significantly expanded myeloid, erythroid and CFU-HPPs in the spleen and bone marrow. We wanted to determine whether CDDO-me administration resulted only in myeloid, erythroid and CFU-HPP expansion or both expansion and mobilization of these cells. To achieve this goal, blood and spleen from naïve C56BL/6 mice which were given CDDO-me (120 µg/dose BID) or vehicle control for 7 days were collected and processed for clonogeneic assays. The cytokine rhG-CSF (2.5 µg/dose BID) known to promote expansion and mobilization of hematopoietic precursors from bone marrow was used as a positive control. The results showed that in addition to inducing CFU-GM expansion,

administration of CDDO-me caused a significant mobilization of myeloid and erythroid progenitors in the spleen and blood, but not to the same level as seen in mice receiving 2.5 μg/dose BID rhG-CSF (Figure 7). Also in contrast to rhG-CSF induced mobilization which resulted in a decrease of hematopoietic precursors in the bone marrow [17, 18], mice that received CDDO-me showed a significant increase in the number of myeloid, erythroid and high proliferative potential in the bone marrow (Figure 6).

In this study, we have shown that CDDO-me administration can have a large effect on the myeloid and myeloid progenitor cells. Mice administered with CDDO-me showed a significant increase in myeloid and myeloid progenitor cells in the spleen, bone marrow and blood compared to the mice that received vehicle control. Administration of CDDO-me expands the number of spleen and bone marrow hematopoietic progenitors. Myeloid and primitive, high proliferative potential progenitors were increased in the spleen and bone marrow of mice that received CDDO-me compared to mice that received vehicle control. We hypothesize that this may be due at least in part, to the increase in hematopoietic growth factor (G-CSF, GM-CSF, IL-3 and others) production due to CDDO-me administration, likely by significantly increasing the G-CSF level in mice that received CDDO-me as was previously described. The expansion and mobilization of myeloid and myeloid progenitor cells into the spleen and blood that was observed in the mice that received CDDO-me had been seen after administration of G-CSF, AMD3100 and others [4, 17, 19]. However, the retention of hematopoietic precursors in the bone marrow of mice that received CDDO-me but not rhG-CSF (Figure 6) suggests additional mechanisms are involved in the hematopoietic expansion and mobilization by CDDO-me. These results suggest that CDDO-me administration could have a critical effect in

hematopoietic progenitor expansion in the spleen and bone marrow as well as in the mobilization of these cells to the peripheral blood. To investigate the role of CDDO-me with regard to hematopoietic cytokines production, we explored the effect of CDDO-me on hematopoietic growth factor production in the serum from mice that received CDDO-me or vehicle control.

Determining the effect of CDDO-me on the hematopoietic growth factor production

A great number of cytokines and growth factors are involved in the regulation of myelopoiesis. Expression of various cytokine receptors on HSC could explain why some cytokines could be more efficient than others at expanding and/or promoting the recruitment of HSC into proliferation and expansion [20, 21]. Many cytokines have been shown to have negative or positive effects on *in vitro* stem cell expansion in mice and humans [20, 22]. To study the effect of CDDO-me on myeloid progenitor expansion, we first sought to determine the hematopoietic growth factor production in the serum of mice that received CDDO-me or vehicle control. This was achieved by injecting 120 µg/dose BID of CDDO-me for 7 days and collecting the blood by cardiac puncture (exsanguination). The cytokine production was assessed by clonogeneic assay, stimulating CFU-GM with serum from mice that received CDDO-me or vehicle control. The Luminex method was used to analyze hematopoietic cytokines that were found to be important for maintaining normal levels of hematopoietic progenitors in bone marrow, G-CSF, GM-CSF and IL-6, which were shown to be strongly involved in myelopoiesis [22, 23].

CDDO-me and Cytokines productions:

Clonogeneic assay

Serum from mice that were treated with CDDO-me or VC was added to the colony assay media in order to determine if it supported colony formation. Low dose of either IL-3 (0.31 ng/mL) or GM-CSF (0.31 ng/mL) and 10% of the serum from mice treated with CDDO-me or VC were used to stimulate the colony formation. G-CSF (1.5 ng/mL) was used as a positive control for CFU-GM. The result showed that CDDO-me treated mice serum significantly increased bone marrow CFU-GM compared to the VC treated mice serum. This result suggests that there are some growth factors in the CDDO-me treated mice serum which stimulated the expansion of CFU-GM. We suggest that CDDO-me induces serum cytokines production which significantly upregulated the CFU-GM and CFU-HPP expansion and mobilization. The result showed that colonies which were stimulated with low dose of GM-CSF in addition to the 10% mice serum that received CDDO-me appear to expand most of the colonies compared to low dose of IL-3. In both cases, there was a significant increase in CFU-GM (Figure 8).

Determining the cytokines production in serum of CDDO-me and VC treated mice by Lyminex assay.

Serum from mice that were treated with CDDO-me or VC was analyzed by Luminex using mice antibody bead kit, IL-6, GM-CSF and G-CSF to determine the level of these cytokines on the serum samples. The result showed a significant increase in

G-CSF serum level when comparing sera from mice treated with CDDO-me or VC. The level of cytokines IL-6 and GM-CSF in the serum samples of both mice that received CDDO-me and VC were found to be below the limits of detection of this assay (data not shown). The increase in G-CSF level in the serum confirms that CDDO-me administration caused a modification in cytokine production that could effect the HSC expansion and mobilization.

Our data indicates that CDDO-me has profound effects on the regulation of primitive hematopoietic progenitor cells. It stimulates the proliferation of these cells at least by increasing the production of G-CSF in the serum. G-CSF is mostly produced by monocytes/macrophages, fibroblast, and endothelial cells. G-CSF production can increase the production of neutrophils, accelerate maturation and differentiation of neutrophil precursors, enhance physiological activation of mature neutrophil, and promote growth, differentiation, and marrow release of hematopoietic progenitor cells (HSC). Any drug that causes a direct or indirect effect on the production of G-CSF will increase or decrease these biological actions. Our result showed that CDDO-me causes a significant increase of G-CSF level in the serum, therefore, CDDO-me (which is in clinical trial for cancer treatment) is a good candidate for cancer patients receiving myelosuppressive chemotherapary following induction or consolidation chemotherapy, for acute myelogenous chemotherapy, for myeloid reconstitution after HSCT, and for mobilization and collection of PBSC for transplantation severe chronic neutropenia. The result showed that IL-6 cytokines is below the level of detection for this assay (data not shown), many cytokines are significantly suppressed by CDDO or CDDO-me to

extremely low levels (IL-6, IFN-g, IL-12p70, and few others). Our data confirm that CDDO-me administration does not increase the level of IL-6 production in the serum.

CONCLUSION

The data presented in this chapter showed that the administration of CDDO-me significantly stimulated myeloid, erythroid and CFU-HPPs expansion in the spleen and bone marrow. In addition to inducing CFU-GM and CFU-HPPs expansion, administration of CDDO-me caused a significant mobilization of myeloid and erythroid progenitors in the spleen and blood. CDDO-me administration induced serum cytokines production (e.g. G-CSF), which significantly upregulated the CFU-GM and CFU-HPP expansion and mobilization into the spleen and blood. CDDO-me administration significantly increased myeloid cells (Gr-1+ CD11b+) in the spleen and BM.

The ability to find drugs that could expand numbers of human HSCs in vivo or in vitro would clearly be a huge boost to all current and future medical uses of HSC transplantation. Therefore, CDDO-me is a good candidate for HSC and HPC mobilization and/or promoting myeloid expansion. It would be interesting to investigate the effect of CDDO-me administration on growth factor and cytokine production other than G-CSF. Determining the mechanisms by which CDDO-me causes the expansion and mobilization of myeloid cells and myeloid progenitors could be important for future medical uses of CDDO-me alone or in combination with other drugs and/or cytokines or in BMT.

FIGURES AND LEGENDS

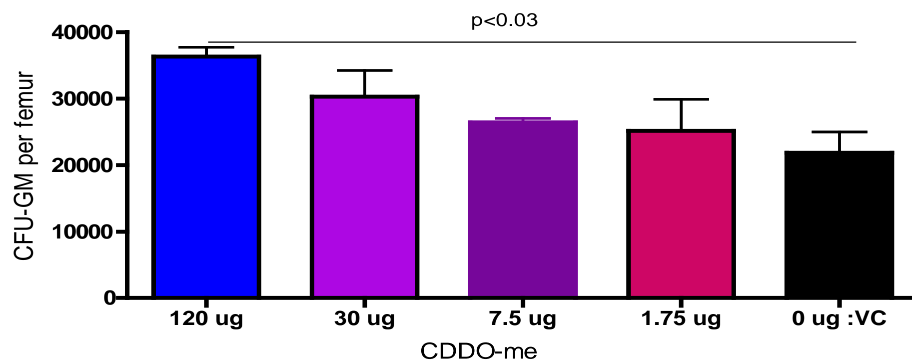


Figure 1. Dosing: Determination of the optimal dose of CDDO-me for myeloid progenitor's expansion. CDDO-me was given at doses ranging from 120 µg to 1.75 µg/dose BID, i.p, for 5 days. Bone marrow was collected at day 5 and processed for colony assays. Three mice per group were used.

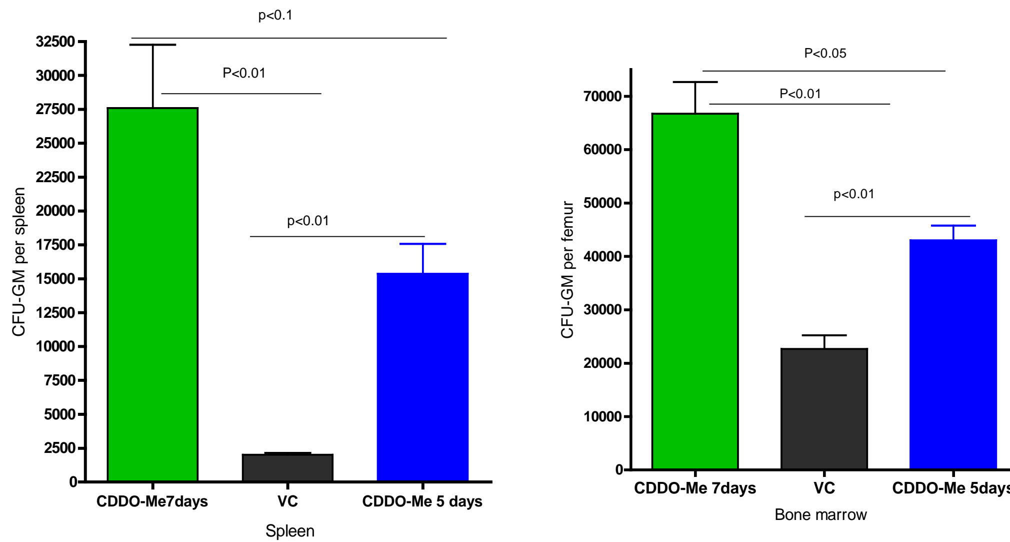


Figure 2. **Timing: CFU-GM myeloid progenitor expansion is CDDO-me time dependent**. Spleen and bone marrow cells compared at day 7 and day 5, CDDO-me (120 μg/dose) twice a day, given at time points ranging from 7 days to 1 day, i.p. Spleen and bone marrow cells were harvested and processed for colony assay. Statistical differences were determined by One-way ANOVA (Tukey) and p-value <0.05 was considered significant. The data are representative of two independent experiments with three mice per group per experiment.

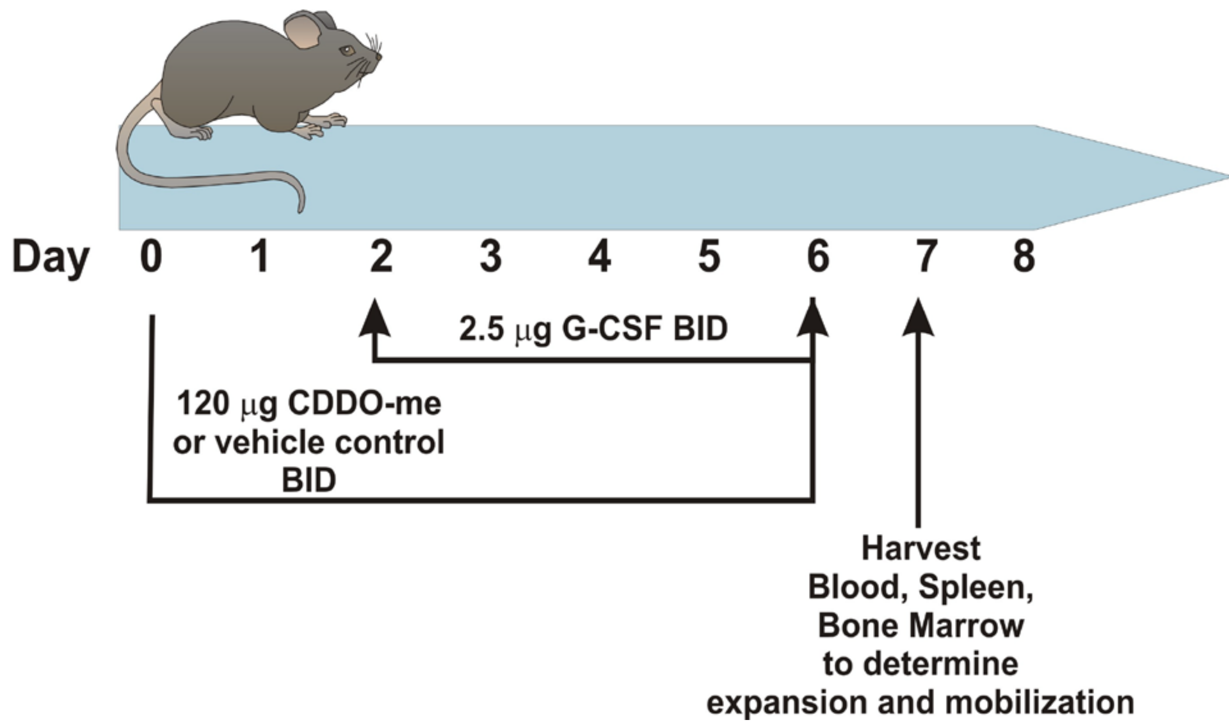


Figure 3. CDDO-me, vehicle control (VC) and G-CSF injection schedules.

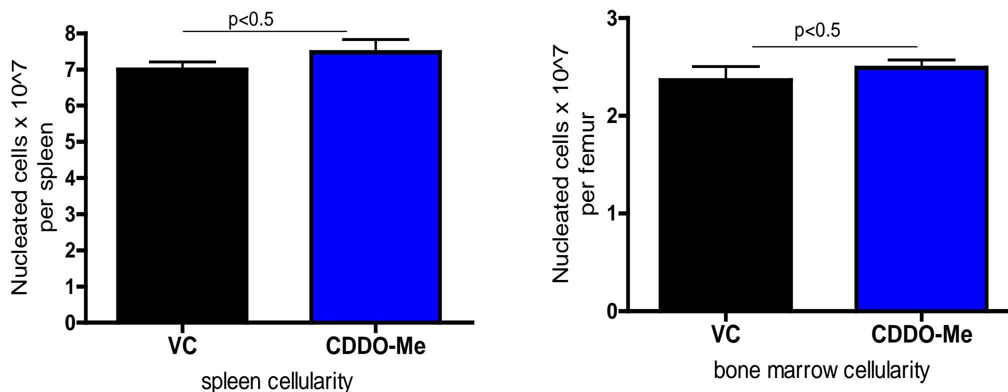
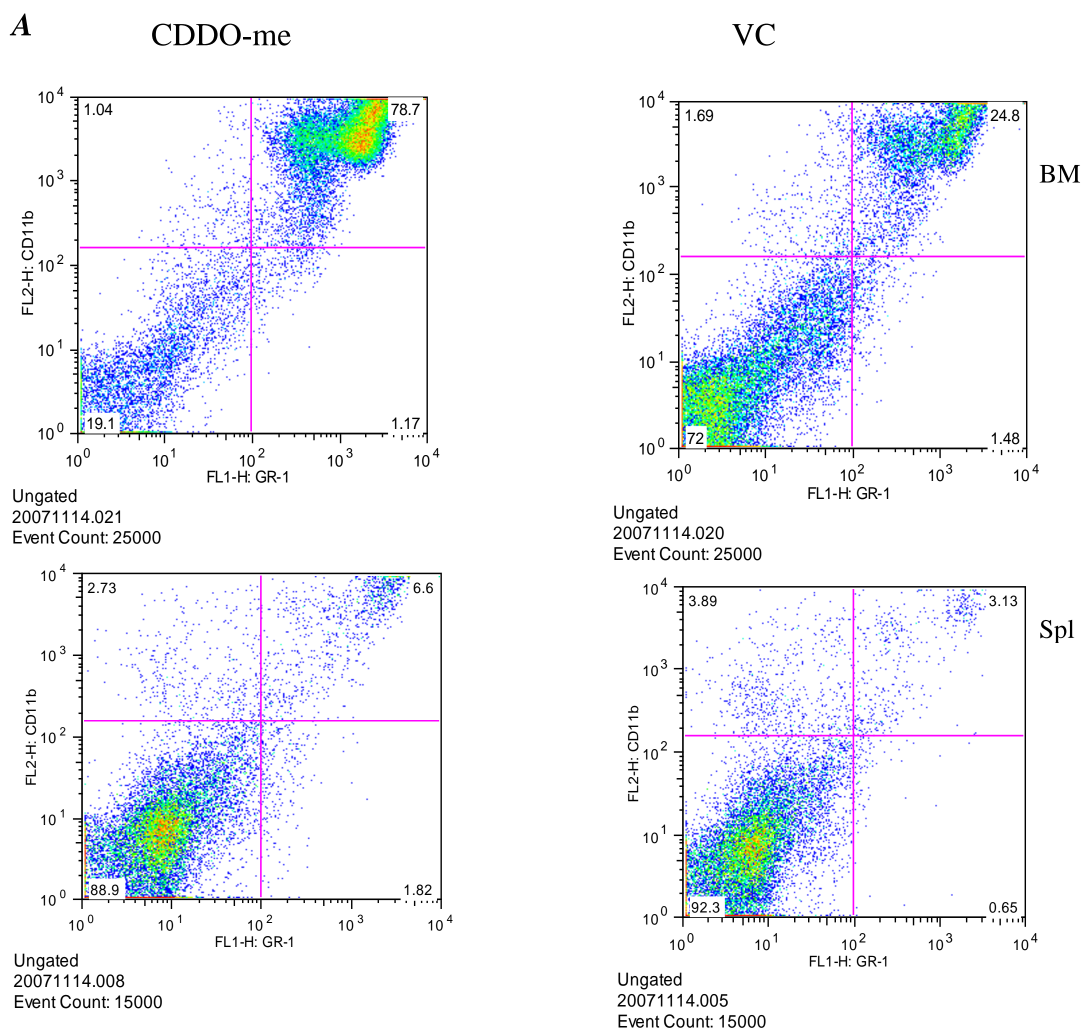


Figure 4. Effect of CDDO-me administration in naive mice cellularity. Mice were treated with vehicle control or CDDO-me (120 µg/dose) twice a day intraperitoneally (i.p) for 7 days. Bone marrow and spleen were collected at day 7, processed into single cell suspensions. Cell counts were performed on a Coulter Z1 cell counter (Coulter Electronic, Hialeah, FL). The data are representative of three independent experiments three mice per group per experiment. Statistical differences were determined by Student's t test and p-value <0.05 was considered significant.

A



B

	Frequency of Gr-1+ CD11b+	
Tissues	CDDO-me	VC

BM	78.7 %	24.8 %
Spleen	6.6 %	3.13 %

C

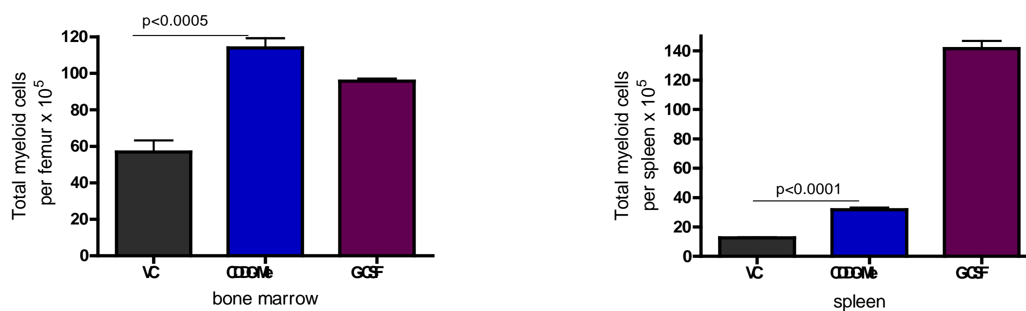
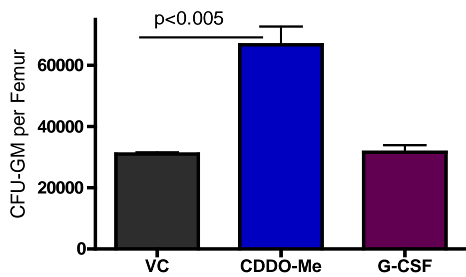
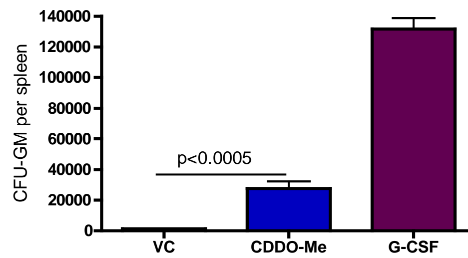


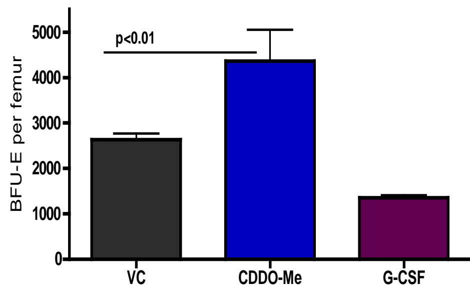
Figure 5. CDDO-me administration caused an increase in CD11b+/Gr-1+ cells in the spleen and bone marrow in naïve mice. CDDO-me was given at 120 µg/dose twice a day for 7 days via intraperitoneal (i.p) injection. Spleen and bone marrow were stained for CD11b and Gr-1. G-CSF (2.5µg/dose BID) was used as a control. *A*, Frequency CD11b+Gr-1+. *B*, table showing the frequency (%) CDDO-me or VC treated naïve mice. ***C***, total CDD11b+/Gr-1+ cells and the data is representative of three independent experiments with three mice per group per experiment. Statistical differences were

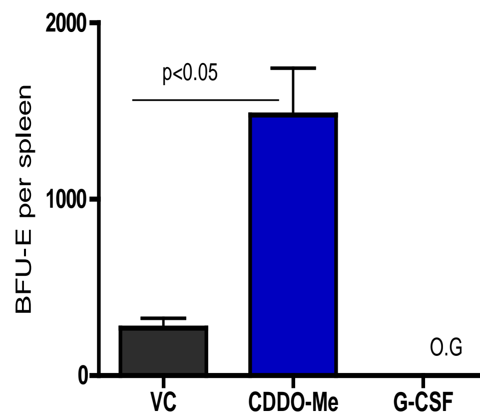
determined by One-way ANOVA (Tukey) and a p-value<0.05 was considered significant.

Bone_marrow *Spleen*









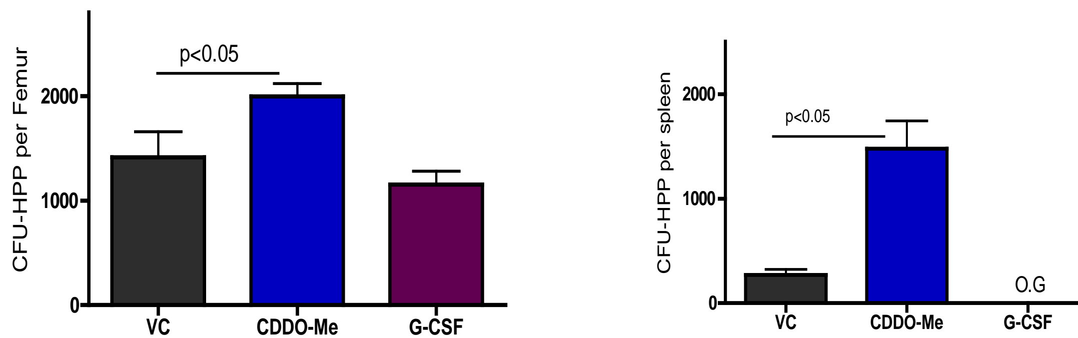


Figure 6. CDDO-me administrations caused significant increase of CFU-GM, BFU-e and CFU-HPP in the spleen and BM. Mice were treated with vehicle control or CDDO-me (120 µg/dose BID) a day intraperitoneally (i.p) for 7 days. Bone marrow and spleen were collected at day 7, processed into single cell suspensions and used for colony assays. Recombinant hG-CSF (2.5 µg/dose BID) was used as a control. The data is representative of three independent experiments with three mice per group per experiment. Statistical differences were determined by One-way ANOVA (Tukey) and p-value<0.05 is considered significant. O.G: over grown colonies.

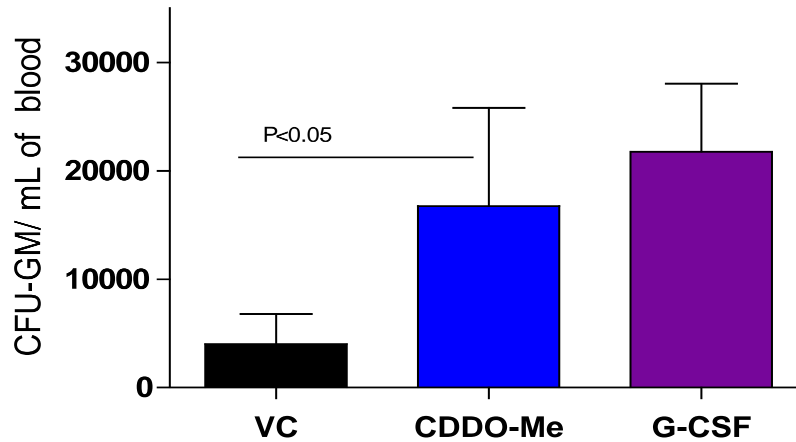


Figure 7. CDDO-me induced an expansion and a mobilization of CFU-GM cells in the blood. Mice were treated with vehicle control or CDDO-me (120 µg/dose) twice a day intraperitoneally (i.p) for 7 days. Blood was collected at day 7, processed into single cell suspensions and used for colony assays. The data is representative of CFU-GM from two independent experiments. Statistical difference was determined by one-way ANOVA and p-value <0.05 was considered significant.

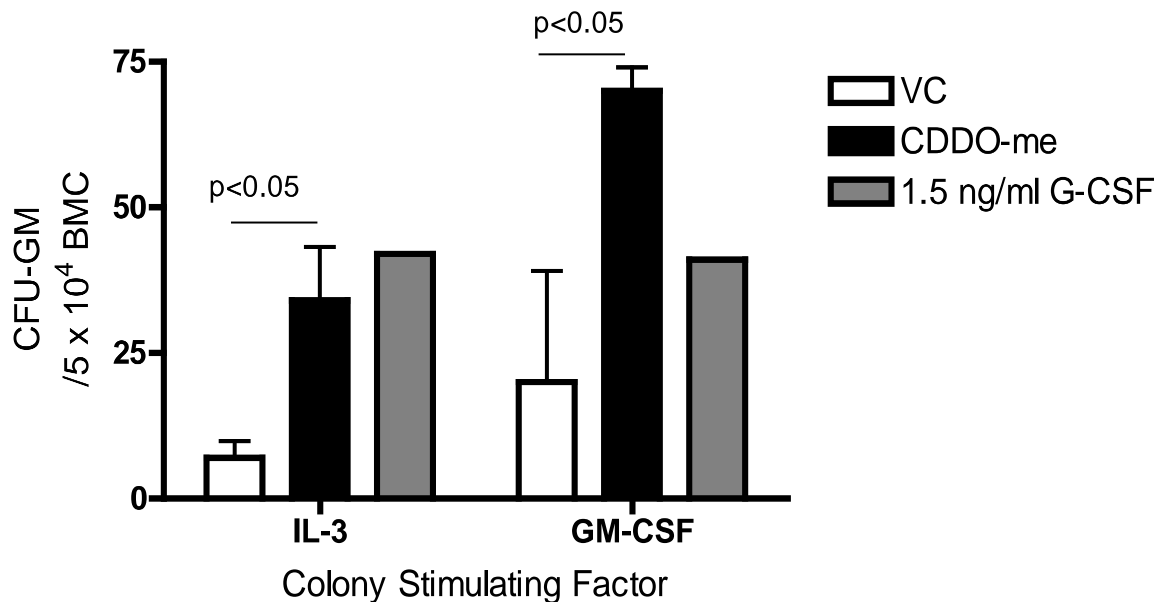


Figure 8: Serum from mice that received CDDO-me caused a significant expansion of bone marrow CFU-GM. Serum from mice that were treated with VC or CDDO-me (120 µg/dose BID) for 7 days, 10% of serum from mice that received CDDO-me or VC were added to the colony assay media which is stimulated with low dose of IL-3 (0.31 ng/mL) or GM-CSF (0.31 ng/mL). Bone marrow cells were plated in 35-mm Petri dishes at concentrations of $5x10^4$ nucleated cells per plate. RhG-CSF (1.5 ng/mL) was used as a positive control. Statistical differences were determined by one-way ANOVA and p-value<0.05 is considered significant. Three mice per group were used.

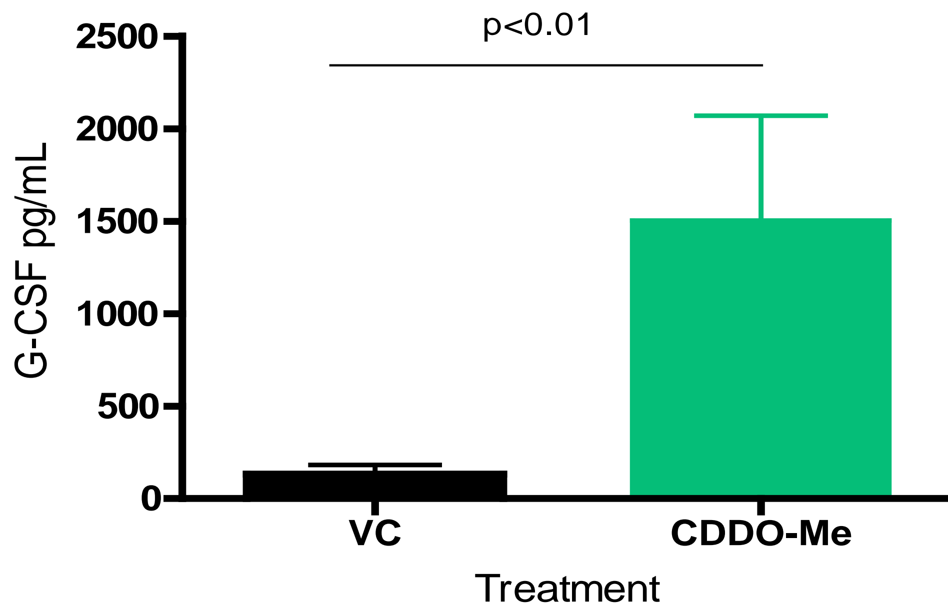


Figure 9. CDDO-me administration significantly increase the level of G-CSF in the serum. Serum from mice that were treated with VC or CDDO-me (120 µg/dose BID) for 7 days was quantified by Luminex technology assay using mice antibody bead kit G-CSF. The plate was analyzed within an hour using the Luminex 100 system. A set of standards were run simultaneously in the assay. The readout for the concentration of each cytokine was detected as median fluorescence intensity (MFI) by the instrument. The values were subsequently converted to pg/mL of cytokine.

REFERENCE LIST

1. Pelus, L.M. and S. Fukuda, *Chemokine-mobilized adult stem cells; defining a better hematopoietic graft.* Leukemia, 2008. **22**(3): p. 466-73.

2. Broxmeyer, H.E., et al., *Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist.* J Exp Med, 2005. **201**(8): p. 1307-18.

3. Nervi, B., D.C. Link, and J.F. DiPersio, *Cytokines and hematopoietic stem cell mobilization.* J Cell Biochem, 2006. **99**(3): p. 690-705.

4. Pulliam, A.C., et al., *AMD3100 synergizes with G-CSF to mobilize repopulating stem cells in Fanconi anemia knockout mice.* Exp Hematol, 2008. **36**(9): p. 1084-90.

5. Shishodia, S., et al., *A synthetic triterpenoid, CDDO-Me, inhibits IkappaBalpha kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through down-regulation of expression of nuclear factor kappaB-regulated gene products in human leukemic cells.* Clin Cancer Res, 2006. **12**(6): p. 1828-38.

6. Decaudin, D., et al., *Mitochondria in chemotherapy-induced apoptosis: a prospective novel target of cancer therapy (review).* Int J Oncol, 1998. **12**(1): p. 141-52.

7. Liby, K., et al., *The synthetic triterpenoids CDDO-methyl ester and CDDO-ethyl amide prevent lung cancer induced by vinyl carbamate in A/J mice.* Cancer Res, 2007. **67**(6): p. 2414-9.

8. Deeb, D., et al., *CDDO-me induces apoptosis and inhibits Akt, mTOR and NF-kappaB signaling proteins in prostate cancer cells.* Anticancer Res, 2007. **27**(5A): p. 3035-44.

9. Samudio, I., et al., *Inhibition of mitochondrial metabolism by methyl-2-cyano-3,12-dioxooleana-1,9-diene-28-oate induces apoptotic or autophagic cell death in chronic myeloid leukemia cells.* Mol Cancer Ther, 2008. **7**(5): p. 1130-9.

10. Konopleva, M., et al., *Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia.* Blood, 2002. **99**(1): p. 326-35.

11. Konopleva, M., et al., *The novel triterpenoid CDDO-Me suppresses MAPK pathways and promotes p38 activation in acute myeloid leukemia cells.* Leukemia, 2005. **19**(8): p. 1350-4.

12. Deeb, D., et al., *Oleanane triterpenoid CDDO-Me inhibits growth and induces apoptosis in prostate cancer cells by independently targeting pro-survival Akt and mTOR.* Prostate, 2009. **69**(8): p. 851-60.

13. Deeb, D., et al., *CDDO-Me inhibits proliferation, induces apoptosis, down-regulates Akt, mTOR, NF-kappaB and NF-kappaB-regulated antiapoptotic and proangiogenic proteins in TRAMP prostate cancer cells.* J Exp Ther Oncol, 2008. **7**(1): p. 31-9.

14. Hestdal, K., et al., *Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells.* J Immunol, 1991. **147**(1): p. 22-8.

15. Kusmartsev, S. and D.I. Gabrilovich, *Role of immature myeloid cells in mechanisms of immune evasion in cancer.* Cancer Immunol Immunother, 2006. **55**(3): p. 237-45.

16. Kress, C.L., et al., *Triterpenoids display single agent anti-tumor activity in a transgenic mouse model of chronic lymphocytic leukemia and small B cell lymphoma.* PLoS One, 2007. **2**(6): p. e559.

17. Christopherson, K.W., 2nd, S. Cooper, and H.E. Broxmeyer, *Cell surface peptidase CD26/DPPIV mediates G-CSF mobilization of mouse progenitor cells.* Blood, 2003. **101**(12): p. 4680-6.

18. Christopherson, K.W., et al., *CD26 is essential for normal G-CSF-induced progenitor cell mobilization as determined by CD26-/- mice.* Exp Hematol, 2003. **31**(11): p. 1126-34.

19. Liles, W.C., et al., *Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist.* Blood, 2003. **102**(8): p. 2728-30.

20. Matsunaga, T., et al., *Negative regulation by interleukin-3 (IL-3) of mouse early B-cell progenitors and stem cells in culture: transduction of the negative signals by betac and betaIL-3 proteins of IL-3 receptor and absence of negative regulation by granulocyte-macrophage colony-stimulating factor.* Blood, 1998. **92**(3): p. 901-7.

21. Matsunaga, T., et al., *Thrombopoietin promotes the survival of murine hematopoietic long-term reconstituting cells: comparison with the effects of FLT3/FLK-2 ligand and interleukin-6.* Blood, 1998. **92**(2): p. 452-61.

22. Metcalf, D., *Hematopoietic cytokines.* Blood, 2008. **111**(2): p. 485-91.

23. Cooper, S.H. and H.E. Broxmeyer, *Measurement of interleukin 3 and other hematopoietic cytokines, such as GM-CSF, G-CSF, M-CSF, erythropoietin, steel factor, and Flt-3 ligand.* Curr Protoc Immunol, 2001. **Chapter 6**: p. Unit 6 4.

CHAPTER 2

Effect of CDDO-me administration on myeloid recovery after congeneic hematopoietic stem cell transplantation (HSCT)

Abstract:

The objective of this chapter was to examine the toxicity of CDDO-me and the effect of this drug on t recovery of the immune system after BMT. These tests were done by determining the maximum tolerated doses and the lethal doses in naïve and bone marrow transplanted mice. Naïve and congeneic BMT model were used to study the CDDO-me toxicity and safety. This drug was tested at different doses ranging from 60 to 240 µg/dose BID. A murine congeneic BMT models were used to study the effect of CDDO-me on myeloid and myeloid progenitor cell recovery post-BMT. Complete blood count, cell count, flow cytometry analysis and clonogeneic assay methods were used to assess the recovery of myeloid and myeloid progenitor cells. The results showed that 240 µg/dose BID of CDDO-me is lethal dose for females C57BL/6 mice even in the absence of congeneic bone marrow transplantation (cBMT). CDDO-me at 120 µg/dose BID is well tolerated in pre-BMT, but it is a lethal dose post-BMT. CDDO-me at 60 µg/dose BID is well tolerated in pre and/or post-BMT in murine model. The result showed a significant increase of spleen CFU-GM in the mice that received CDDO-me (60 µg/dose BID) at day 14 post- BMT. We concluded that CDDO-me at 60 µg/dose BID pre and/ or post-BMT is a safe dose; CDDO-me at this dose had a positive effect on myeloid and myeloid progenitor cells recovery at day 14 post-BMT.

Introduction

Both CDDO-me and bone marrow transplantation (BMT) are used in the treatment of cancer. However, it is important to investigate the toxicity of CDDO-me and the effect of this drug on the immune system recovery after BMT (particularly myeloid recovery), to protect against opportunistic infections such as bacterial, fungal and viral pathogens [1, 2]. We hypothesized that administration of CDDO-me after congeneic hematopoietic stem cell transplantation (HSCT) would result in increased myeloid and myeloid progenitor cells. HSCT or BMT is a medical procedure consisting of transplanting HSC from a donor to a recipient. HSCs and hematopoietic progenitors cells (HPCs) are commonly used in bone marrow transplantation (BMT) to replace the hematopoietic system in patients with hematological disorders, such as asplastic anemia or in cancer patients who are undergoing high dose chemotherapy [3-5]. It has been shown that immune deficiency following the high dose of radiation or immunotherapy and BMT causes significant morbidity and mortality [6, 7]. Accelerating early recovery of innate immunity (granulocytes, monocytes/macrophages, and natural killer (NK) cells) can protect against opportunistic infections such as bacterial, fungal and viral pathogens [1, 2]. Consequently, significant efforts have been pursued to find more efficient means of accelerating immune recovery after BMT, whereas granulocyte/monocyte colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and other components have been used to promote granulocyte recovery and hematopoietic stem cell mobilization [8, 9]. Mobilization of stem cells by G-CSF has been shown to decrease donor NK cell function, the number of NK cell progenitors [9], and the ability of donor monocytes and dendritic cells to produce IL-12, which is important for Th1 immunity against bacteria, viruses

and fungi [9] . Our laboratory has shown that the administration of CDDO can inhibit the development of murine acute graft-versus-host disease (GVHD) and cause cell death in proliferating, but not resting lymphocytes in mixed lymphocytes culture [10, 11]. CDDO-me can eliminate tumor development and metastasis in immunocompetent mice. Both CDDO and CDDO-me have anti-inflammatory actions, inhibit cell proliferation and selectively induce apoptosis in some cancer cells such as human lung cancer cells [12-16]. CDDO-me pretreatment showed an increase in levels of antioxidant genes and suppression of interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin1β (IL-1β) and lipopolysaccharide (LPS)-induced cytokines expression [17, 18]. CDDO and its derivative CDDO-me are currently in phase I clinical trails for treatment of leukemia and solid tumors [14, 19, 20].

The objective of this chapter was to examine the toxicity of CDDO-me, by determining the maximum tolerated and the lethal doses in naïve and BMT mice and to investigate the effect of CDDO-me on myeloid and myeloid progenitor cell recovery post congeneic BMT. CDDO-me was tested at different doses ranging from 60 to 240 μg/dose BID. Murine congeneic BMT model was used to study the effect of CDDO-me on myeloid and myeloid progenitor cells. In this study, we first determined the toxicity of CDDO-me on female C57BL/6, Ly5.2 pre and post-BMT. In the second part, we looked at the effect of CDDO-me on myeloid and myeloid progenitor cell recovery after BMT. In addition to cancer prevention and treatment, CDDO-me significantly increased myeloid cells including precursor populations in vivo in the spleen. Our result showed that CDDO-me administration did not decreased hematopoietic recovery when injected to mice following sublethal total body irradiation.

MATERIALS AND METHODS

Animals

Female C57BL/6, Ly5.2 and Ly5.1 mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). Animals were kept in specific pathogen-free conditions. All animal protocols were approved and in vivo studies were performed at the University of Nevada, Reno (UNR). At the start of the experiments, mice were between eight and thirteen weeks of age. Experiments were conducted in accordance with UNR IACUC guidelines.

Reagents and Media

CDDO-me

CDDO-me was kindly provided by Reata Pharmaceutics, Inc and stored at 4ºC. CDDO-me was prepared in a solution of 10% dimethyl sulphoxide (DMSO) (Sigma, St Louis, MO), 10% Cremophor EL (Sigma), and 80% of a 0.9% sodium chloride solution, USP (Baxter Healthcare Corporation). CDDO-me was weighed and dissolved into DMSO, followed by the addition of Cremophor EL. Lastly, 0.9% sodium chloride solution was added and mixed for about one minute. The vehicle control (VC) was prepared under the same conditions as above without adding CDDO-me. The solutions were sterile filtered and stored at 4ºC for up to 7 days prior to use. The following concentrations of CDDO-me were tested for toxicity: 0.3mg/ml, 0.6mg/ml and 1.2mg/ml.

Cell preparation:

Spleens were harvested and teased apart into single cell suspensions under sterile conditions. Bone marrow cells were harvested by crushing a femur with a mortar and pestle in processing buffer consisting of 1% Fetal Bovine Serum (FBS) in PBS solution. The processed cells were run through a nylon mesh to remove the debris. Cell suspensions were centrifuged for 10 minutes 250 x gravity (g) at 4ºC, and the supernatant was discarded. The samples were resuspended in processing buffer. Cell counts were performed on a Coulter Z1 cell counter (Coulter Electronic, Hialeah, FL). Cell suspensions were kept on ice or at 4ºC prior to use for colony assay and flow cytometry analysis.

Complete blood count (CBC)

Blood samples were collected by cardiac puncture in microvette capillary blood collection tubes with EDTA (Sarsted, Germany). The CBC analysis was performed on Hemavet 950 (Drew Scientific, Oxford, CT).

Flow Cytometry analysis:

One million (1×10^6) bone marrow cells or splenocytes were centrifuged into a pellet and resuspended in 50µL of blocking buffer (1% Human AB serum in PBS), incubated for 15-20 minutes, then incubated with antibodies for 20-30 minutes at 4ºC and washed twice with staining buffer. The stained cells were fixed with 1% paraformaldehyde in PBS, and analyzed on a FACSCan flow cytometer (Becton

Dickinson, San Jose, CA) with CellQuest software. The following antibodies were used for cell staining: FITC-conjugated anti-mouse CD45.1 (Clone A20, Pharmagen), FITC-conjugated anti-mouse CD45.2 (clone 104, Pharmingen), PE-conjugated anti-mouse CD11b (clone M1/70, Pharmagen), PE-Cy5-conjugated anti-mouse Gr-1 (Clone 8C5, eBioSciences), PE-conjugated anti-mouse CD45R/B220 (Clone: RA3-6B2, Pharmingen), biotinylated anti-mouse IgM (Fisher) and streptavidin (SA-PE-Cy5, Pharmingen). The flow cytometric analysis was performed on Flowjo software (Tree Star Inc).

Colony assay

For colony forming unit-granulocyte/monocyte (CFU-GM) and burst forming unit-erythrocyte (BFU-E) assays, bone marrow cells and spleen cells were plated in 35-mm Petri dishes at concentrations of $5x10^4$ or $5x10^5$ nucleated cells per plate, respectively. Media consisted of FBS (15%), penicillin/streptomycin (50 IU/mL), 2-mercapto-ethanol ($5x10^{-3}$M) IMDM and methylcellulose (2.75%). Cells were added to the media, mixed, and 1mL was plated into each of three 35-mm Petri dishes. Colony formation was stimulated by 10ng/mL recombinant murine IL-3, 10ng/mL recombinant murine GM-CSF (PeproTech) and 5 U/mL recombinant human Epo (Amgen). Plates were incubated for 7 days at 37ºC in humidified atmosphere with 5% CO_2. Colonies were defined as an aggregate of more than 50 cells. Colonies that contained only red cells in single or multiple bursts were scored as BFU-E. Colonies that contained only white cells consisting of granulocytes and/or macrophages were scored as CFU-GM, and colonies that were a mix (white and red) were scored as colony-forming unit generating granulocytes, erythroblasts, macrophages and megakaryocytes (CFU-GEMM).

Bone marrow transplantation and toxicity

Ly5.2 mice received myeloablative doses (950 cGy) of total body irradiation (TBI) from a ^{137}Cs source. Irradiation was followed by an infusion of one million congeneic bone marrow cells (BMCs) from C57BL/6 (Ly5.1) donors into the tail vein (i.v) of C57BL/6 (Ly5.2) recipient mice. Recipient Ly5.2 mice received CDDO-me or vehicle control (VC) intraperitoneally (i.p) within the dose range of 60 µg to 240 µg twice a day before bone marrow transplantation (BMT) from day -5 to day -1 and/or before and after the BMT from day -5 to day 13. Mice were monitored daily, and weight monitored twice per week the first two weeks then every week. Mice were euthanized after a 20% weight loss was observed in addition to clinical signs of toxicity. All experiments were performed at least two or three times with 3 to 5 mice per group. Mice were monitored for toxicity and survival for 15 to 20 days.

Statistical Analysis

Statistical analysis was performed by, one way ANOVA analysis of mean, or Student's t test. Data were considered significant at $p<0.05$.

Results and Discussion

I- CDDO-me and toxicity:

CDDO and its derivates CDDO-Im and CDDO-me are synthetic triterpenoids confirmed to be multifunctional drugs and are effective agents in controlling cancer cell growth in preclinical model of leukemia, breast cancer(4T1 cells), pancreatic cancer (COLO357 and PANC1), colon cancer (SW-480) , and lung cancer (human non-small cell lung carcinoma (NSCLC cell lines) [19, 21-24]. CDDO-me is currently in phase I clinical trails for treatment of leukemia and solid tumors, which is why it is critical to investigate the effect of CDDO-me on the hematopoietic cells originating in the bone marrow and to determine the toxicity of CDDO-me after BMT in the murine model. We first looked at the toxicity of CDDO-me on B6 (Ly5.1) mice using a range of CDDO-me from 60 μg/dose to 240 μg/dose BID pre, post, and pre and post-BMT. CDDO-me toxicity in a mice model is defined by 20% weigh loss in addition to clinical signs of toxicity. Kress CL et al. [25] showed an evidence of kidney and liver toxicity using immunohistochemical analysis of tissue from mice that died during treatment with CDDO-Im. The toxicity effect was indicated by hemorragies, cytoplasmic eosinophilia and vascularization in the kidneys, hepatocellular necrosis and acidophilia in the liver [25]. These toxicity effects were not seen in mice that received low doses of CDDO-Im or VC.

a) CDDO-me at 120 μg/dose BID is well tolerated in pre-BMT in murine model.

First, we analyzed the toxicity effect of CDDO-me on bone marrow transplanted and naïve mice using a range of CDDO-me from 60 μg/dose to 240 μg/dose. For the bone marrow transplantation, mice were irradiated with a myeloablative dose (950 cGy), injected (i.v) via tail with one million congeneic bone marrow cells. The results presented in Figure 1 show that administration of a 240 μg/dose BID is lethal for female C57BL/6 mice even before bone marrow transplantation. These mice were scheduled to receive bone marrow transplantation after 5 days of CDDO-me injections. However, 60% of the mice that received CDDO-me at 240 μg /dose BID died before bone marrow transplantation. These mice were considered as naïve mice because they did not receive any bone marrow transplantation. 80% of the mice that received CDDO-me (240 μg/dose) BID died within 7 days after CDDO-me injection. The animal were euthanized after 20 % weight loss associated with other clinically signs such as toxicity related to kidneys and liver complications that were seen with CDDO-Im treatment [25]. CDDO-me at 120 μg/dose and 60 μg/dose BID is tolerated when given pre-BMT in the murine model. Mice that received CDDO-me at 120 μg/dose BID daily over 5 days, prior to receiving a myeloablative dose of TBI and congeneic BMT tolerated the regimen without a significant toxicity. 100% of the mice that received CDDO-me (120 μg/dose or 60 μg/dose BID) pre-BMT exhibited the same weight lost as the mice that received the vehicle control (VC) (Figure 1B). These two doses are safe and tolerable when given to mice for 5 days.

immediately prior to a congeneic BMT (Figure 1A). However, 240μg/dose BID is lethal even in the absence of a BMT.

b) CDDO-me at 60 μg/dose BID is well tolerated when given pre and post-BMT

We next investigated the toxicity effect of CDDO-me in mice that received bone marrow transplantation. Knowing that both 60 μg/dose and 120 μg/dose BID could be safely administrated prior to congeneic-bone marrow transplantation (pre-BMT), we gave CDDO-me at 60μg/dose or 120 μg/dose BID, intraperitoneally (i.p) pre and/or post-BMT. Mice were irradiated at 950 cGy and injected with one million congeneic bone marrow cells from Ly5.1 donors into the recipient Ly5.2 mice i.v. via the tail vein. The schedule and doses for CDDO-me, VC and BMT are represented in figure 2. The result presented in Figure 3 shows that CDDO-me administered at 120 μg/dose BID is a lethal dose post-BMT. All mice that received 120μg/dose BID post-BMT, and pre and post-BMT died eight days after bone marrow transplantation (post-BMT). Mice that received 60 μg/dose BID, pre-BMT, 60 μg/dose BID pre and post-BMT and the vehicle control displayed 100% survival with no major signs of CDDO-me toxicity (Figure 3).

II- Effect of CDDO-me on myeloid and myeloid progenitor recovery after BMT

Knowing that administration of CDDO-me (60 μg/dose BID) is safe in murine model, we next determined the effect of CDDO-me on bone marrow and spleen cells. This part was done by investigating the effect of CDDO-me on the myeloid and myeloid progenitor cell recovery post-BMT. Administration of 60 μg/dose BID of CDDO-me prior to and/or post congeneic-bone marrow transplantation was shown to be safe.

However, CDDO-me at a 120 µg/dose or 240 µg/dose twice a day were show to be lethal in the mice model after BMT. In all of the bone marrow transplantation studies, CDDO-me was used at 60 µg/dose BID intraperitoneally (i.p).

Effect of CDDO-me on the cellularity at Day 14 post-BMT

To determine the effect of CDDO-me on myeloid and myeloid progenitor recovery after BMT, we first wanted to look at the effect of CDDO-me on the bone marrow and spleen cellularity. Stimulation of C57BL/6 mice with 60 µg/dose BID by CDDO-me for 13 days resulted in a significant increase in nucleated cells in the spleen of the mice that received CDDO-me compared to the negative controls, which are composed of mice that received vehicle control (VC). These were prepared under the same conditions as CDDO-me solution without adding CDDO-me to the powder and to the naïve mice that did not received any treatment (Figure 4).

Complete blood count (CBC)

CDDO-me administration had no significant effect on complete blood count recovery (white blood cell (WBC), red blood cell (RBC) or platelet (PLT)) after bone marrow transplantation compared to mice that received vehicle control (Figure 5). These results demonstrate that CDDO-me administration to recipients with congeneic-bone marrow transplantation does not impair complete blood count at day 14 post-BMT. Based upon these results, we suggest that it is safe to administer CDDO-me (60 µg/dose, BID) after bone marrow transplantation in the mouse model. CBC parameters were not significantly affected by CDDO-me administration in murine model at day 14 post-BMT.

Effect of CDDO-me administration on donor engraftment and myeloid reconstitution:

Mice were treated with vehicle control or CDDO-me (60ug/dose) twice a day intraperitoneally from day 1 to day 13 post-BMT. CDDO-me administration after bone marrow transplantation caused a slight increase in total donor CD11b+/Gr-1+ cells on the spleen but not on the bone marrow at day 14 post-BMT, although this increase was not statistically significant.

The frequency of donor cells in the bone marrow and spleen following congeneic BMT was not significantly affected by CDDO-me administration compared to mice that received vehicle control (Figure 6).

CDDO-me administrations cause an increase of spleen CFU-GM at day 14 post-BMT.

CDDO-me (60µg/dose) or vehicle control was administrated twice a day for 13 days after BMT. Spleen and bone marrow cells were collected at day 14 post-transplantation and processed for colony assay. The results showed a significant increase of spleen CFU-GM in the mice that received CDDO-me (60 µg/dose BID) compared to the mice that received vehicle control. The spleen BFU-E was slightly increased but this increase was statistically insignificant. No major change was seen in bone marrow CFU-GM at day 14 post-BMT compared to mice that received CDDO-me and mice that received vehicle control (VC) (Figure 7). From this result, we concluded that the spleen is the major source of hematopoietic stem cells after BMT at day 14 and CDDO-me stimulation accelerated the myeloid recovery at day 14 post-BMT.

CONCLUSION

CDDO-me at 120 µg/dose or 60 µg/dose BID exhibited the same weight lost as the mice that received the vehicle control (VC). These two doses are safe and tolerable when given to mice for 5 days immediately prior to a congeneic BMT. However, 120µg/dose BID post-BMT, and pre and post-BMT is a lethal dose. CDDO-me at 240µg/dose BID is lethal dose even in the absence of a BMT. All mice died before the BMT.

Administration of CDDO-me prior to myelosuppression with subletal total body irradiation resulted in significant increase of spleen cellularity after 14 days recovery due to significant expansion of myeloid cells as well as increase on B cells (data not shown). CDDO-me administration to recipient congeneic-bone marrow transplantation does not reduce complete blood count at day 14 post-BMT. These results suggest that it is safe to administer CDDO-me (60 µg/dose, BID) after bone marrow transplantation and CBC parameters were not significantly affected by CDDO-me administration in murine model at day 14 post-BMT. The results show a significant increase of spleen CFU-GM in the mice that received CDDO-me (60 µg/dose BID) compared to the mice that received vehicle control. No major change was seen in bone marrow CFU-GM at day 14 post-BMT compared to mice that received CDDO-me and mice that received vehicle control (VC). These results, confirm that the spleen is the major source of hematopoietic stem cells after BMT at day 14. Our data showed that CDDO-me administration did not significantly affect hematopoietic recovery compared to the VC treated animal when CDDO-me was given to mice following sublethal total body irradiation. CDDO-me

stimulation accelerated the myeloid recovery at day 14 post-BMT by stimulating the myelopoiesis in vivo. This could be due, in part, to a significant increase in circulating G-CSF levels in CDDO-me treated mice.

FIGURES AND LEGENDS

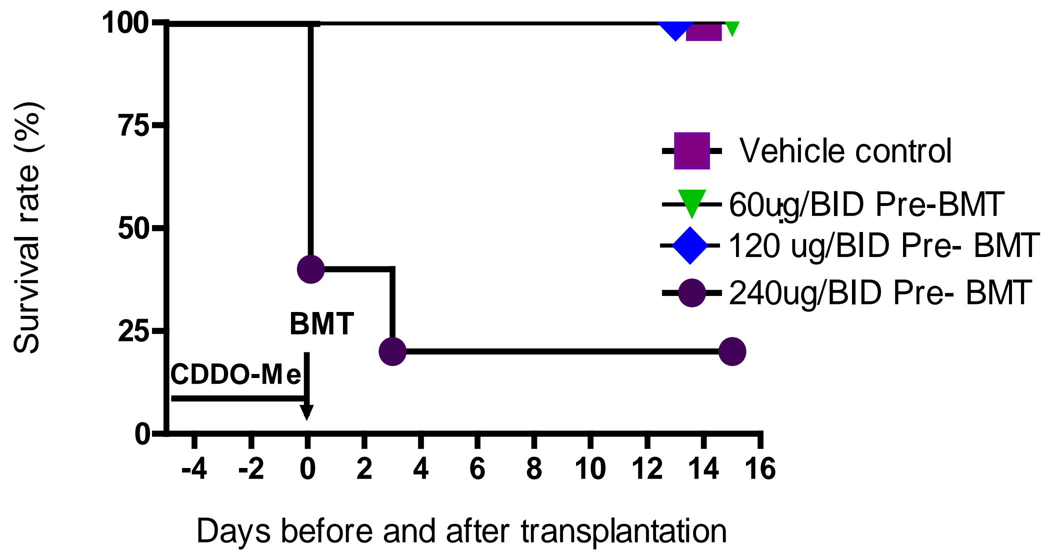


Figure 1*A*. 120 μg/dose BID of CDDO-me is tolerated in pre-BMT in murine model. CDDO-me was given at doses ranging from 60 μg/dose to 240 μg/dose, i.p pre-BMT over 5 days. Mice were irradiated at 950 cGy and were injected with one million congeneic bone marrow cells i.v. via tail. Five mice per group were used.

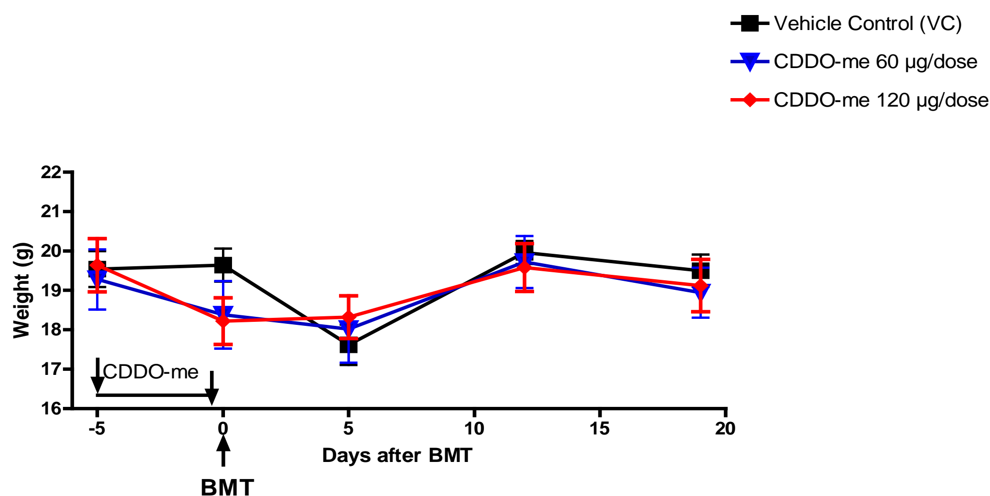


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Figure 1*B*. Weight loss in CDDO-me treated animals: CDDO-me was given at doses ranging from 60µg/dose to 120µg/dose BID, i.p pre-BMT during 5 days. Mice were irradiated at 950 cGy and were injected with one million congeneic bone marrow cells i.v. via tail. The weight was measured prior to CDDO-me injections, prior to BMT, 5 days post-BMT after that every week. The data represent two independent experiments with 5 mice per group per experiment.

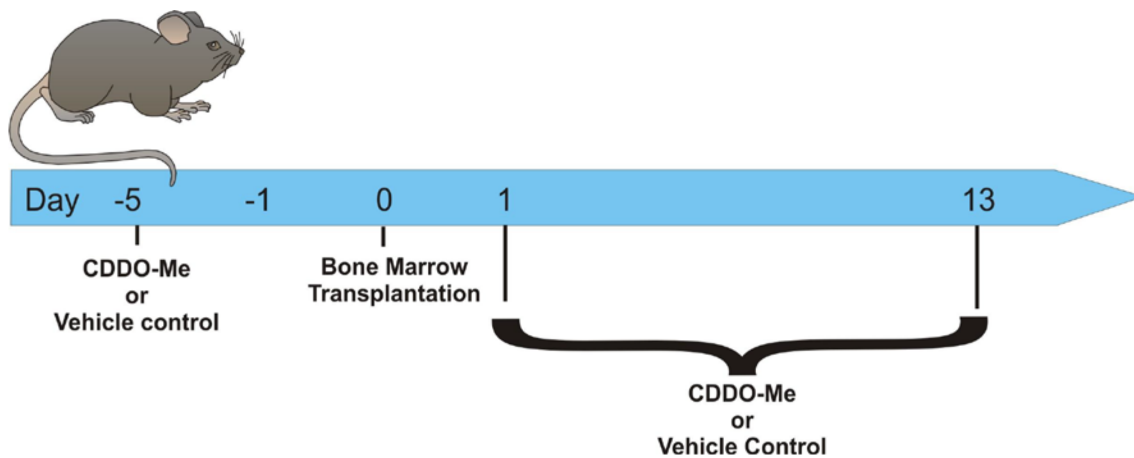


Figure 2. CDDO-me, vehicle control (VC) and bone marrow cells injection schedules

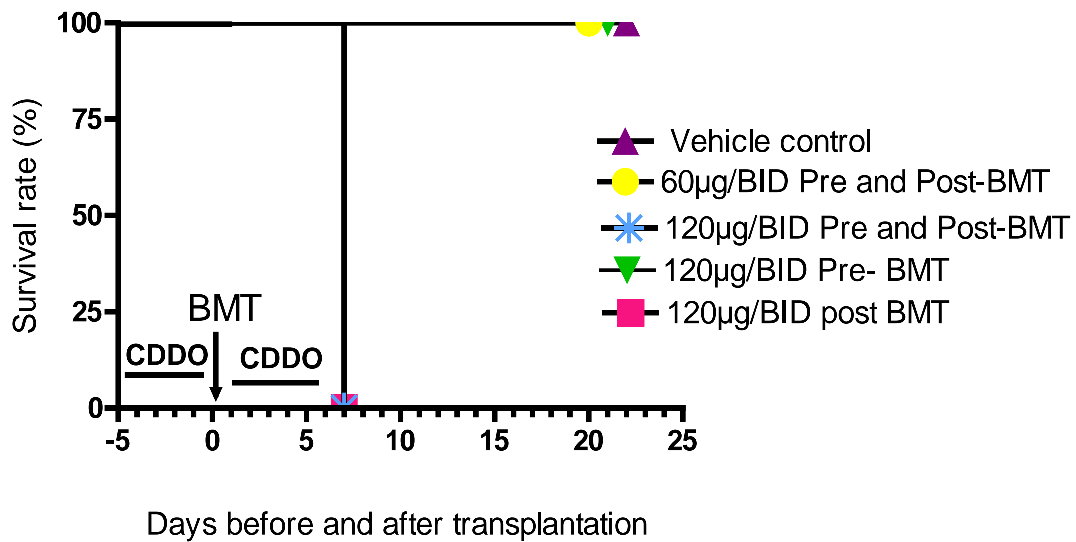


Figure 3. CDDO-me at 60µg/dose BID is tolerated in pre and/ or post-BMT mice. CDDO-me was given at doses ranging from 60 µg/dose to 120µg/dose BID, and i.p pre and/or post-BMT. Mice were irradiated at 950 cGy and injected with 1 million congeneic bone marrow cells i.v. via tail. Five mice per group were used.

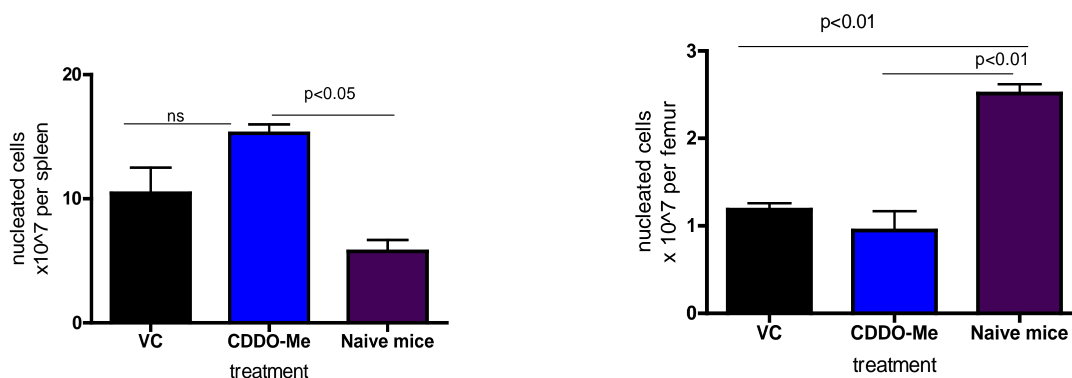


Figure 4. Cellularity at day 14 from mice that received bone marrow transplantation. Female C57BL/6 (Ly5.1) mice were irradiated at 950 cGy and injected with one million Ly5.2 congeneic bone marrow cells i.v. via the tail vein. Mice received CDDO-me (60 µg/dose, BID) for 13 days, i.p, post-BMT. Cells from the bone marrow and spleen were resuspended in processing buffer. Cell counts were performed on a Coulter Z1 cell counter (Coulter Electronic, Hialeah, FL). Statistical differences were determined by one-way ANOVA (Tukey). P-value<0.05 is considered significant and these data are representative of two independent experiments with three mice per group per experiment.

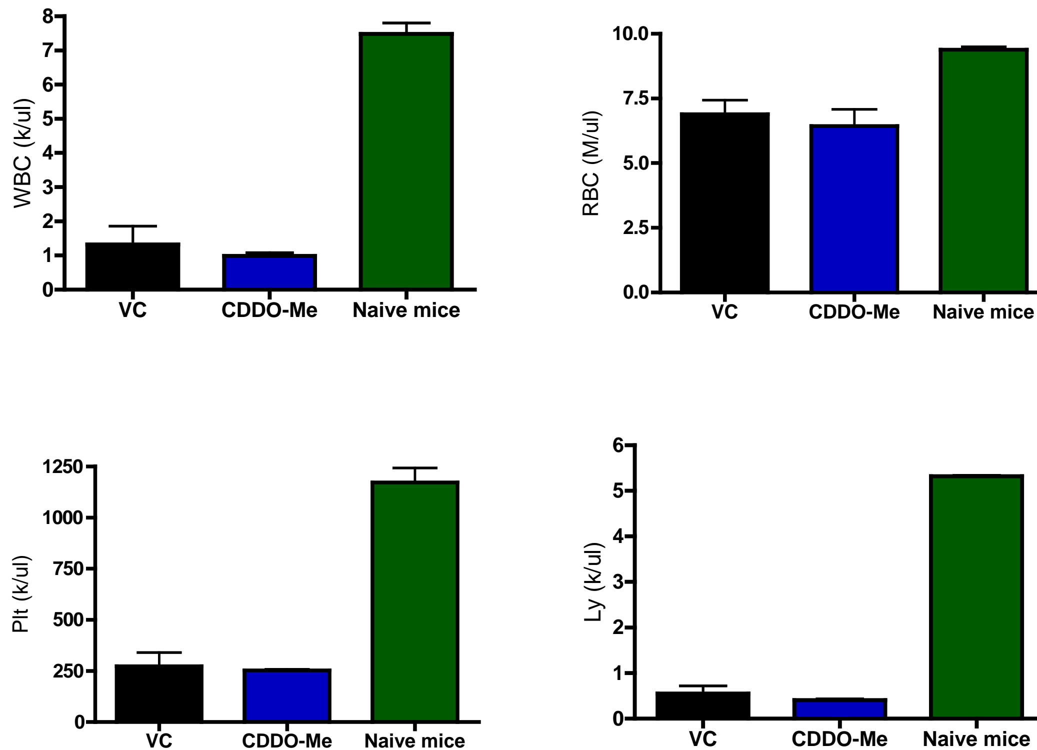


Figure 5. Complete blood count (CBC): Effect of CDDO-me administration on CBC (white blood cells, red blood cells and lymphocytes) recovery after bone marrow transplantation at Day 14. Mice were treated with vehicle control or CDDO-me (60 µg/dose) twice daily intraperitoneally from day +1 to day +13 post-BMT. VC: mice that received vehicle control, CDDO-me: mice that received CDDO-me and naïve mice: mice that did not receive any treatment. Blood samples were collected in microvette capillarity blood collection tubes with EDTA (Sarsted, Germany). The CBC analysis was performed on a Hemavet 950 (Drew Scientific, Oxford, CT). These data are representative of two independent experiments with three mice per group per experiment.

***A*-**Total donor CD11b+Gr-1+

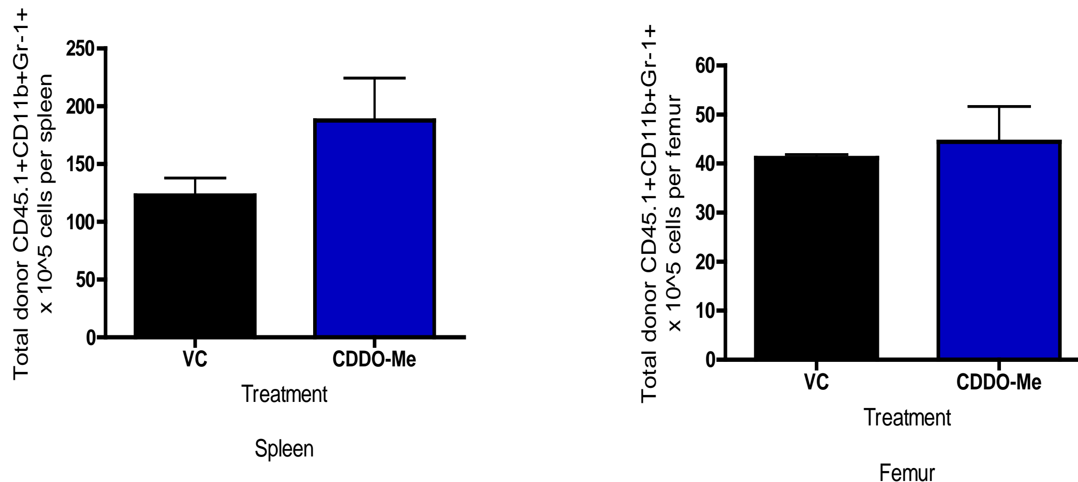
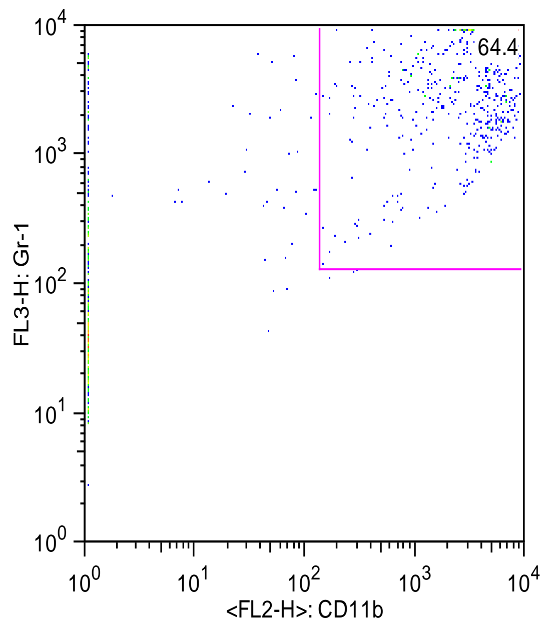
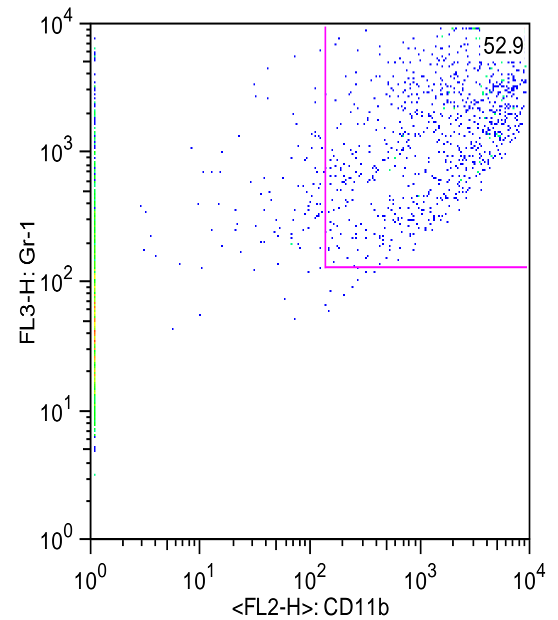


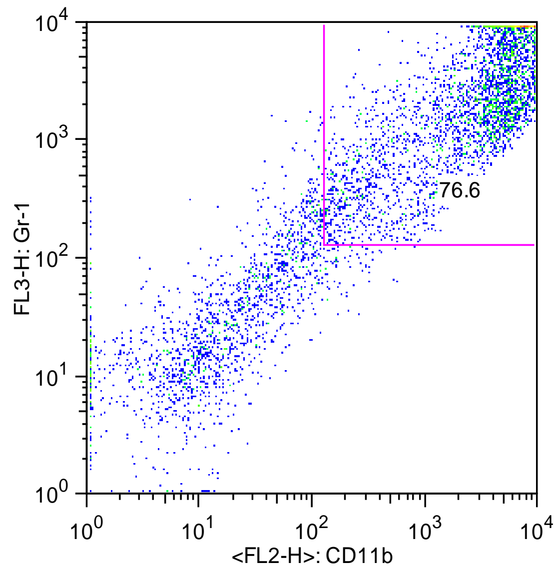
Figure 6*A*. Effect of CDDO-me administration on donor engraftment and myeloid reconstitution: Mice were irradiated at 950 cGy and injected with one million congeneic bone marrow cells i.v. via tail. Female C57BL/6, Ly5.1 were injected with CDDO-me (60µg/dose BID) or VC for 13 days post-BMT. Processed cells from bone marrow and spleen were re-suspended in processing buffer. Spleen and bone marrow cells were stained for CD45.1, CD11b and Gr-1 antibodies. Samples were run on BD FACScan and the data was analyzed by Flowjo software. Statistical differences were determined by student's t test. P-value<0.05 was considered significant. These data are representative of two independent experiments and the statistical difference is not significant in these experiments.

B **-**Spleen donor CD45.1+CD11b+Gr1+





C**-** Bone marrow (BM) donor CD45.1+CD11b+Gr-1+



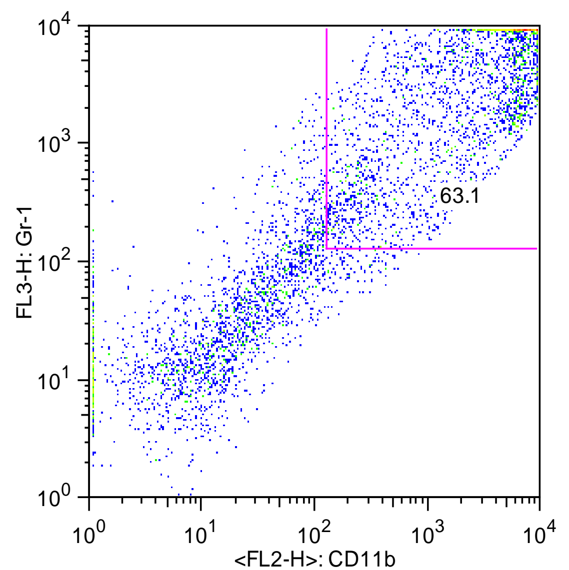


Figure 6*B* and 6*C*. CDDO-me administration caused a slight increase in the frequency of donor CD11b+/Gr-1+ cells on the spleen and bone marrow at day 14 post-BMT. B- Spleen CD45.1+CD11b+Gr-1+, in left mouse that received CDDO-me in right mouse that received vehicle control. **C-** Bone marrow CD45.1+ CD11b+Gr-1+, in left mouse that received CDDO-me in the right mouse that received vehicle control. CDDO-me (60µg/dose) or vehicle control was given twice a day during 13 days after BMT, spleen and bone marrow cells were stained for CD45.1, CD11b and Gr-1 antibodies. Samples are run on BD FACScan and the data was analyzed by Flowjo software.

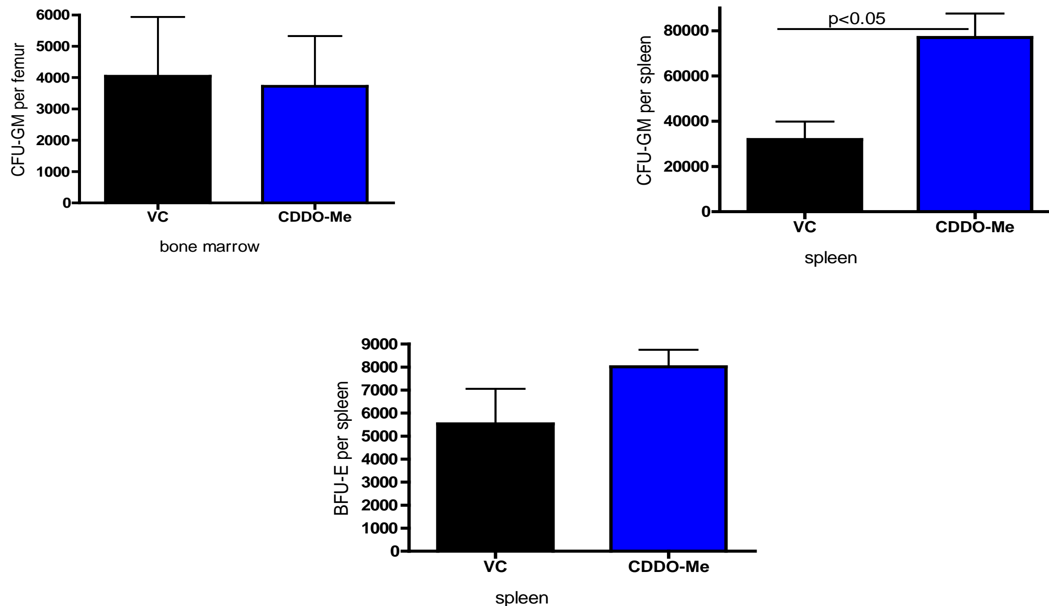


Figure7. CDDO-me administrations caused a significant increase in CFU-GM in the spleen at day 14 after congeneic BMT. Mice were treated with CDDO-me (60 µg/dose) or vehicle control twice a day over 13 days after BMT. Spleen and bone marrow were collected at day 14 post-transplantation and processed for colony assay. Statistical differences were determined by student’s t test. A p-value <0.05 is considered significant and these data are representative of two independent experiments.

REFERENCE LIST

1. *Guidelines for preventing opportunistic infections among hematopoietic stem cell transplant recipients.* Biol Blood Marrow Transplant, 2000. **6**(6a): p. 659-713; 715; 717-27; quiz 729-33.

2. Wesselkamper, S.C., et al., *NKG2D is critical for NK cell activation in host defense against Pseudomonas aeruginosa respiratory infection.* J Immunol, 2008. **181**(8): p. 5481-9.

3. Verfaillie, C.M., *Adult stem cells: assessing the case for pluripotency.* Trends Cell Biol, 2002. **12**(11): p. 502-8.

4. Verfaillie, C.M., *Optimizing hematopoietic stem cell engraftment: a novel role for thrombopoietin.* J Clin Invest, 2002. **110**(3): p. 303-4.

5. Glimm, H., et al., *Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID-beta2 microglobulin-null mice.* J Clin Invest, 2001. **107**(2): p. 199-206.

6. Devine, S.M., et al., *Recent advances in allogeneic hematopoietic stem-cell transplantation.* J Lab Clin Med, 2003. **141**(1): p. 7-32.

7. Yang, L., et al., *Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients.* Blood, 2005. **105**(7): p. 2717-23.

8. Bradstock, K.F., *The use of hematopoietic growth factors in the treatment of acute leukemia.* Curr Pharm Des, 2002. **8**(5): p. 343-55.

9. Miller, J.S., F. Prosper, and V. McCullar, *Natural killer (NK) cells are functionally abnormal and NK cell progenitors are diminished in granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cell collections.* Blood, 1997. **90**(8): p. 3098-105.

10. Sun, K., et al., *The synthetic triterpenoid, CDDO, suppresses alloreactive T cell responses and reduces murine early acute graft-versus-host disease mortality.* Biol Blood Marrow Transplant, 2007. **13**(5): p. 521-9.

11. Gao, X., et al., *Immunomodulatory activity of synthetic triterpenoids: inhibition of lymphocyte proliferation, cell-mediated cytotoxicity, and cytokine gene expression*

through suppression of NF-kappaB. Immunopharmacol Immunotoxicol, 2008. **30**(3): p. 581-600.

12. Deeb, D., et al., *CDDO-me induces apoptosis and inhibits Akt, mTOR and NF-kappaB signaling proteins in prostate cancer cells.* Anticancer Res, 2007. **27**(5A): p. 3035-44.

13. Gao, X., et al., *Synthetic triterpenoids inhibit growth and induce apoptosis in human glioblastoma and neuroblastoma cells through inhibition of prosurvival Akt, NF-kappaB and Notch1 signaling.* J Neurooncol, 2007. **84**(2): p. 147-57.

14. Liby, K., et al., *The synthetic triterpenoids CDDO-methyl ester and CDDO-ethyl amide prevent lung cancer induced by vinyl carbamate in A/J mice.* Cancer Res, 2007. **67**(6): p. 2414-9.

15. Ling, X., et al., *The novel triterpenoid C-28 methyl ester of 2-cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid inhibits metastatic murine breast tumor growth through inactivation of STAT3 signaling.* Cancer Res, 2007. **67**(9): p. 4210-8.

16. Deeb, D., et al., *CDDO-Me inhibits proliferation, induces apoptosis, down-regulates Akt, mTOR, NF-kappaB and NF-kappaB-regulated antiapoptotic and proangiogenic proteins in TRAMP prostate cancer cells.* J Exp Ther Oncol, 2008. **7**(1): p. 31-9.

17. Samudio, I., et al., *Guggulsterones induce apoptosis and differentiation in acute myeloid leukemia: identification of isomer-specific antileukemic activities of the pregnadienedione structure.* Mol Cancer Ther, 2005. **4**(12): p. 1982-92.

18. Tran, T.A., et al., *The synthetic triterpenoid CDDO-methyl ester modulates microglial activities, inhibits TNF production, and provides dopaminergic neuroprotection.* J Neuroinflammation, 2008. **5**: p. 14.

19. Konopleva, M., et al., *Novel triterpenoid CDDO-Me is a potent inducer of apoptosis and differentiation in acute myelogenous leukemia.* Blood, 2002. **99**(1): p. 326-35.

20. Liby, K., et al., *The synthetic triterpenoid CDDO-Imidazolide suppresses STAT phosphorylation and induces apoptosis in myeloma and lung cancer cells.* Clin Cancer Res, 2006. **12**(14 Pt 1): p. 4288-93.

21. Konopleva, M., et al., *Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia.* Cancer Cell, 2006. **10**(5): p. 375-88.

22. Kim, K.B., et al., *Identification of a novel synthetic triterpenoid, methyl-2-cyano-3,12-dioxooleana-1,9-dien-28-oate, that potently induces caspase-mediated apoptosis in human lung cancer cells.* Mol Cancer Ther, 2002. **1**(3): p. 177-84.

23. Ikeda, T., et al., *The novel triterpenoid CDDO and its derivatives induce apoptosis by disruption of intracellular redox balance.* Cancer Res, 2003. **63**(17): p. 5551-8.

24. Hyer, M.L., et al., *Apoptotic activity and mechanism of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic-acid and related synthetic triterpenoids in prostate cancer.* Cancer Res, 2008. **68**(8): p. 2927-33.

25. Kress, C.L., et al., *Triterpenoids display single agent anti-tumor activity in a transgenic mouse model of chronic lymphocytic leukemia and small B cell lymphoma.* PLoS One, 2007. **2**(6): p. e559.