CNTO 530 FUNCTIONS AS A POTENT EPO MIMETIC VIA UNIQUE SUSTAINED EFFECTS ON BONE MARROW PROERYTHROBLAST POOLS

Running title: *EPO mimetic CNTO 530 action modes*

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ABSTRACT

Anemia as associated with numerous clinical conditions can be debilitating, but frequently can be treated via administration of epoetin-alfa, darbepoietin-alfa or methoxy-PEG epoetin-beta. Despite the complexity of EPO-EPO receptor interactions, the development of interesting EPO mimetic peptides (EMPs) also has been possible. CNTO 530 is one such novel MIMETIBODY™ Fc-domain dimeric EMP fusion protein. In a mouse model, single-dose CNTO 530 (unlike epoetin-alfa or darbepoietin-alfa) bolstered red cell production for up to one month. In 5-fluorouracil and carboplatin-paclitaxel models, CNTO 530 also protected against anemia with unique efficiency. These actions were not fully accounted for by half-life estimates, and CNTO 530 signaling events therefore were studied. Within primary bone marrow erythroblasts, kinetics of STAT5, ERK, and AKT activation were similar for CNTO 530 and epoetin-alfa. p70S6K activation by CNTO 530, however, was selectively sustained. In vivo, CNTO 530 uniquely stimulated the enhanced formation of PODXL$^{\text{high}}$CD71$^{\text{high}}$ (pro)erythroblasts at frequencies multi-fold above epoetin-alfa or darbepoietin-alfa. CNTO 530 moreover supported the sustained expansion of a bone marrow- resident Kit$^{\text{neg}}$CD71$^{\text{high}}$Ter119$^{\text{neg}}$ progenitor pool. Based on these distinct erythropoietic and EPOR signaling properties, CNTO 530 holds excellent promise as a new EPO mimetic.
INTRODUCTION

As recombinant injectables, epoetin-alfa and darbepoietin-alfa are important therapeutics for anemia \(^1,^2\). Via its canonical dimeric single- transmembrane receptor (EPOR) \(^3^,^4\), and possibly a hypothesized accessory chain \(^5^,^6\), erythropoietin (EPO) also has been shown to confer significant cytoprotection for certain injured non-hematopoietic tissues \(^7\). This includes renal, cardiac, brain and retinal cells as damaged by ischemia, neurotoxins or UV irradiation \(^8^\text{-}^1^1\). However, epoetin-alfa can require frequent injections in anemia contexts, as well as relatively high dosing to cytoprotect non-hematopoietic cells \(^7\). To address these issues, several EPO-related erythropoiesis stimulating agents (ESAs) recently have been formulated and shown to possess one, or another, advantaged property. One orthologue is darbepoietin-alfa in which non-essential residues are mutated to provide for two additional N-linked glycosylation sites \(^1^3\). This can enhance darbepoietin-alfa’s in vivo half-life \(^1^4\), and EPO receptor occupancy \(^1^5\). Together, these properties can lessen dosing frequency \(^1^4\). A second example is provided by “Epo-Epo” in which EPO is configured as a peptide linked head-to-tail dimer \(^1^6\). For Epo-Epo, unique properties include enhanced secretion, and apparent advantaged in vivo activity following subcutaneous injection \(^1^7\). In addition, a pegylated epoetin-beta has been developed (CERA) that possesses a significantly extended half-life \(^1^8\), and likewise can be effectively utilized with less frequent dosing \(^1^9\).

A distinct approach to preparing ESAs involves the development of monoclonal antibodies which can bind, and activate the EPOR. In particular, Liu et al. \(^2^0\) recently described ABT007 as one such agent, and via crystallographic analyses provided evidence for unique EPOR domain interactions. In an alternate approach, initial success has also been indicated for small molecule ESAs in the form of HIF prolyl hydroxylase inhibitors \(^2^1\). As activated by hypoxia, HIFs (hypoxia inducible factors) act as heterodimeric HIF-ARNT PAS-domain transcription factors to promote the expression of several hypoxia response genes including \(Epo\) \(^2^2\). HIF degradation is regulated, in part, via prolyl hydroxylation \(^2^1\).
Proline hydroxylase inhibitors act to stabilize HIFs, and can substantially elevate endogenous EPO production and red cell formation\textsuperscript{22}.

In a third unique approach to ESA development, Jolliffe and coworkers\textsuperscript{23,24} previously applied phage display technologies to discover EPO mimetic peptides (EMPs) which despite a lack of sequence homology to EPO, bind and biologically stimulate the EPOR. Crystallographic analyses have revealed structural details of EMP1 – EPOR binding, and provided direct evidence for EPOR’s occurrence as a homodimeric receptor\textsuperscript{3,4}. In monomeric form, EMP activity in vivo, however, is limited\textsuperscript{25}.

Presently, we have characterized the activities of a unique ESA, CNTO 530, which is constructed as a dimeric EMP fused to a human IgG4 Fc scaffold. Analyses in murine models first reveal uniquely high and sustained erythropoietic activity for CNTO 530 as compared to epoetin-alfa, and darbepoietin-alfa. CNTO 530’s erythropoietic effects also were exhibited via advantaged amelioration of the anemia induced by 5-fluorouracil, or carboplatin plus paclitaxel. In ex vivo analyses using primary bone marrow erythroblasts, several common phosphoprotein signal transduction factors and target response genes were defined for CNTO 530 and epoetin-alfa. However, CNTO 530 more strongly and persistently stimulated p70S6K. In vivo, CNTO 530 uniquely enforced the enhanced formation of PODXL\textsuperscript{high}CD71\textsuperscript{high} erythroblasts, as well as the sustained expansion of a Kit\textsuperscript{neg}CD71\textsuperscript{high}Ter119\textsuperscript{neg} erythroid progenitor pool in bone marrow at levels several-fold above epoetin-alfa or darbepoietin-alfa. CNTO 530 therefore represents a highly effective ESA whose actions interestingly appear to depend, in part, on its unique engagement of the EPOR, together with the persistent stimulation of a defined cohort of targeted proerythroblasts.
MATERIALS AND METHODS

Mouse and rat models – For all in vivo and ex vivo analyses, mice used were strain C57BL/6 at age 8-12 weeks. Epoetin-alfa (EPO) (Eprex®-alfa) (Ortho Biotech, Raritan, NJ), darbepoietin-alfa (Aranesp®-alfa) (Amgen, Thousand Oaks, CA) and CNTO 530 injections were intraperitoneal in 0.2 mL saline. In tail vein injections, epoetin-alfa or CNTO 530 was administered in saline (100 μL injection volume). In 5-fluorouracil (5-FU) experiments, 5-FU was injected at 150 mg/kg (single intraperitoneal dose). In carboplatin and paclitaxel anemia models, female Sprague-Dawley CD rats at approximately 300 grams were used (Charles River Laboratories, Raleigh, NC). Carboplatin (50 mg/kg) (Polymed, Houston, TX) and paclitaxel (10 mg/kg) (Polymed, Houston, TX) were administered IP (approximately 60 minutes apart) on days 1, 8, and 15. Control rats received cremophore (Sigma, St. Louis, MO) and mannitol (Sigma-Aldrich) vehicles (1.0 mL/kg each). All procedures, and the use of all mice and rat models, were reviewed and approved by the institutional animal care and use committee of the Maine Medical Center Research Institute.

Hematological analyses – For peripheral blood samples, hematocrits, hemoglobin levels, and reticulocyte frequencies were determined using an Advia 120 hematology analyzer (Siemens Medical Solutions Diagnostics, Tarrytown, NY). Hematocrits and reticulocytes also were assayed independently via microcapillary centrifugation, and flow cytometry as described previously.

Primary erythroblast preparations – For ex vivo expansions, marrow cells were washed in IMDM and resuspended in 1 mL of phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA). Red cells were lysed, and progenitors were collected through 50% FBS in PBS. Cells were then plated at 1.8 x 10^6 cells/mL (7 mL per 100 mm dish) in StemPro-34 (Invitrogen) supplemented with 2.5 U/mL epoetin-alfa, 100 ng/mL SCF, 1 μM dexamethasone, 1 μM beta-estradiol, 75 μg/mL h-transferrin, (Sigma, St. Louis, MO), 0.5% BSA (Stem Cell Technologies, Vancouver, BC), 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin-G, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin-B (1XPSF) (Invitrogen) and 1.5 mM L-
glutamine (i.e., “SP34EX” medium). At 24 hours, 4 mL of fresh medium were added. At 48 hours, cells were collected, resuspended in 1 mL of conditioned medium, combined with 9 ml of fresh medium, and cultured for an additional 24 hours. Proerythroblasts then were purified as a Kit^{pos}CD71^{high}Ter119^{neg} cohort. Specifically, Lin^{pos} cells first were depleted from ex vivo cultures using biotinylated antibodies to CD5 (Ly-1), CD45R/B220, CDllb (Mac1), Ter119, and Ly6G (Gr1) (Stem Cell Technologies). Kit^{pos}CD71^{high}Ter119^{neg} cells then were retrieved at purities of > 99.9% via CD117 magnetic activated cell separation (Miltenyi Biotech, Auburn, CA). In analyses of bone marrow progenitor cell populations, femurs were isolated and thoroughly cleaned of all peripheral blood and tissue. Marrow was then extruded, gently dispersed in Iscove’s modified Dulbecco’s medium (IMDM) (Invitrogen), 2% FBS and passed through a 40 micron cell sieve. Cells were washed once in 0.1% BSA, IMDM prior to flow cytometric analyses.

**Flow cytometry** – In flow cytometry, 1x10^6 cells were washed, resuspended and incubated at 4°C in 0.2 mL PBS, 0.1% BSA and 1 μg rat IgG, and then with 1 μg of primary antibody for 45 minutes. Primary antibodies used were: biotin-anti-PODXL (R&D Systems, Minneapolis, MN), allophycocyanin (APC)-CD117, fluorescein (FITC)-CD71, and phycoerythrin (PE) Ter119 (BD Biosciences, San Jose, CA). Bound PODXL antibodies were detected using AlexaFluor-647 streptavidin (Molecular Probes, Eugene, OR). Nucleated erythroblasts were assayed by staining with DRAQ5 (10 μM; Alexis Biochemicals, San Diego, CA). Reticulocytes were stained with Retic-COUNT (BD Biosciences). Equivalent numbers of gated events were analyzed using a BD Biosciences FACScalibur flow cytometer.

**Signal transduction assays and western blotting:** Purified Kit^{pos}CD71^{high}Ter119^{neg} erythroblasts were isolated, washed and incubated at 8 x 10^5 cells/mL for 5.5 hours in 0.5% BSA, 50 μg/mL transferrin, 15 ng/mL insulin, 0.1 mM 2-mercaptoethanol in IMDM. Cells then were exposed to epoetin-alfa (2 U/mL) or CNTO 530 (0.5 μg/mL) for the time courses indicated, washed in 2°C PBS, and lysed initially in 0.2 mL 1% Igepal, 150 mM NaCl, 50 mM NaF, 2 mM Na₂EDTA, 0.1 mM NaVO₃, 1 mM dithiothreitol, 10 mM sodium pyruvate, 25 mM beta-glycerol phosphate, 10% glycerol, 50 mM HEPES (pH 7.5) plus 0.25
mg/mL phenylmethylsulfonylfluoride, 1x protease inhibitor and 1x phosphatase inhibitor cocktails (Sigma). 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 112.5 mM NaCl, 37.5 mM Tris-HCl (pH 7.4) then was added (0.15 mL). Cleared extracts were assayed for protein content, denatured, electrophoresed and blotted. Antibodies used were phospho-p70S6K and p70S6K (Cell Signaling, Danvers, MA, #9204 and #9202), phospho-AKT and AKT (Cell Signaling #9271 and #9272); phospho-STAT5 and STAT5 (Cell Signaling #9351 and #9352); and phospho-ERK1,2 and ERK1,2 (Cell Signaling #9106 and #9102). In chemiluminescence, HRP-conjugated antibodies (Jackson Immunoresearch, West Grove, PA) and Super-Signal West-Dura reagent (Pierce Biotechnology, Rockford, IL) were used. Signals were analyzed quantitatively using Image-J software (http://rsb.info.nih.gov/ij/).
RESULTS

Erythropoietic properties of CNTO 530 in steady-state and anemia models

To advance a characterization of CNTO 530’s in vivo biological properties, basic dose-response studies first were performed. Primary analyses were in adult mice at steady-state, and included comparisons to both epoetin-alfa (EPO), and darbepoietin-alfa. Specifically, erythropoietic activity was examined based on increases in hematocrits following dosing with CNTO 530, darbepoietin-alfa or epoetin-alfa. CNTO 530 and darbepoietin-alfa were as single doses. Epoetin-alfa was administered at 1 and 24 hours (to compensate for epoetin-alfa’s shorter half-life) \(^{14,29}\). Analyses revealed an ability of CNTO 530 to persistently increase hematocrits by day 8 to \(>15\) points (Figure 1A). This differed from response profiles for epoetin-alfa and darbepoietin-alfa in which increases in hematocrits peaked at somewhat lower levels at day 4, and subsequently decreased by day 8. Extended time course experiments next were performed using CNTO 530, epoetin-alfa and darbepoietin-alfa each at near-maximal doses (i.e., 0.15 \(\mu\)g, 1.5 U and 0.012 \(\mu\)g per gram-mouse, respectively). Here, single dosing of CNTO 530 was observed to provide significant increases in hematocrits for up to 25 days (Figure 1B). For epoetin-alfa and darbepoietin-alfa, increases in red cell production persisted for approximately 10 to 12 days (Figure 1B). These experiments established CNTO 530 as a potent ESA with apparently unique biological properties which are not well accounted for by in vivo half-life estimates (approximately 40 hours for CNTO 530 \(^{30}\), 8 to 48 hours for darbepoietin-alfa \(^{31,32}\); and 6 to 24 hours for epoetin-alfa \(^{29,33}\)).

Given CNTO 530’s capacity to promote erythropoiesis in a sustained fashion, its performance in 5-fluorouracil (5FU) and paclitaxel plus carboplatin induced anemia models next was assessed (Figure 2). In 5-FU experiments, mice received 150 mg/kg, and were then dosed with either CNTO 530 or darbepoietin-alfa at day 4.5 at doses of 0.3 \(\mu\)g / g-rat and 0.02 \(\mu\)g / g-rat, respectively. 5-FU-induced anemia proved to be lessened in severity by CNTO 530 (e.g., by 7.5 hematocrit points at day 15.5), but not significantly so by darbepoietin-alfa (Figure 2A).
Next, in paclitaxel plus carboplatin experiments, rats were dosed with these chemotherapeutic agents on days 1, 8 and 15. On day 16 (a point at which hematocrits fell to approximately 25%) (see below), CNTO 530 or darbepoietin-alfa was administered, each at two doses as 1.0 and 3.0 ug / g-rat (CNTO 530) or 0.03 and 0.3 ug / g-rat (darbepoietin-alfa). By day 28, both darbepoietin-alfa and CNTO 530 provided > 5 g/dL advantage in hemoglobin levels (Figure 2B upper panel). At day 35, 42 and 56, however, CNTO 530 when dosed at 3.0 ug/ g-mouse uniquely continued to provide marked advantages in hemoglobin levels. As analyzed in parallel, 5 to >10 point advantages in hematocrits also were realized at these time points due to CNTO 530 dosing (Figure 2B lower panel). Therefore, anemia in this model was selectively lessened by high-dose CNTO 530.

**CNTO 530 stimulation of signal transduction pathways in primary bone marrow erythroblasts**

Based on CNTO 530’s potent erythropoietic activities, and unique configuration as an IgG4 Fc- fused homodimeric EMP, analyses next focused on CNTO 530- stimulated signal transduction events in primary proerythroblasts. Via an initial basic analysis, the abilities of CNTO 530 vs epoetin-alfa to support bone marrow CFUe formation first were compared. Specifically, low doses of each ESA required for essentially matched sub-maximal CFUe stimulation first were defined (i.e., 0.08 U/mL EPO and 0.08 μg/ml CNTO 530. Activities of these and equivalent fold-increased doses of each ESA then were assessed. Interestingly, as doses increased, CNTO 530 more readily stimulated the formation of 8-16 cell CFUe colonies (Figure 3A).

In subsequent signal transduction experiments, primary bone marrow-derived erythroblasts were used which were isolated as a highly EPO responsive Kit<sup>pos</sup>CD71<sup>high</sup>Ter119<sup>neg</sup> CFUe-like cohort. Here, such upstream signaling events as induced by CNTO 530 vs epoetin-alfa were analyzed in parallel. This included analyses of p70S6K, AKT, STAT5 and ERK1,2 activation. In particular, Kit<sup>pos</sup>CD71<sup>high</sup>Ter119<sup>neg</sup> proerythroblasts were expanded and purified. Cytokines then were withdrawn, and cells were incubated
for a 5.5 hour interval in 0.5% BSA, 50 μg/mL transferrin, 0.1 mM 2-ME, 10 μg/mL insulin in IMDM. Proerythroblasts then were exposed to either CNTO 530 (0.5 μg/mL) or epoetin-alfa (2U/mL). At 0, 10, 20, 40 and 80 minutes, lysates were prepared and analyzed by western blotting for phospho- activation of the above signal transduction factors (Figure 3 B-D). For P-S473-AKT, P-Y694-STAT5, and P-T202/Y204-ERK1,2, CNTO 530- stimulated activation profiles largely paralleled those generated by epoetin-alfa (Figure 3 B-D). For p70S6K, however, its activation by CNTO 530 at late time points interestingly persisted as compared directly to EPO. To confirm this interesting result, adjusted doses of CNTO 530 and EPO were defined that stimulated p70S6K T421/S424 phosphorylation at comparable levels. Blots were then probed for ERK1,2 T202/Y204 phosphorylation levels. These analyses of a co-processed and ECL-developed blot again confirmed differentials, here, with comparably decreased ERK1,2 activation for CNTO 530 relative to p70S6K (see supplemental Figure S-1).

Within bone marrow, CNTO 530 drives the formation of PODXL$^{high}$CD71$^{high}$ erythroblasts, and persistently expands a Kit$^{neg}$CD71$^{high}$Ter119$^{neg}$ erythroid progenitor pool

Previously, epoetin-alfa has been demonstrated to stimulate a rapid release of reticulocytes from marrow into peripheral blood $^{36}$. We therefore first examined effects of epoetin-alfa and CNTO 530 on this possible response in two-hour time-frame experiments. Epoetin-alfa proved to stimulate a significant efflux of reticulocytes to blood at two hours to levels that were approximately 180% over levels elicited by CNTO 530 (Figure 4A). This provoked the notion that late-stage nucleated erythroid progenitor cells also might be affected, and this was studied by co-staining for DRAQ5 positivity among CD71$^{high}$ peripheral blood cells (again at a two hour interval). Notably, a significant efflux of these progenitors also was observed in response to epoetin-alfa, but again was lesser in response to CNTO 530 (with epoetin-alfa-induced levels 238% over CNTO 530) (Figure 4B). Results therefore are consistent with the notion that epoetin-alfa, more than CNTO 530, may rapidly stimulate an emptying of a late pool of red cell progenitors from marrow.
Effects of CNTO 530 on erythropoiesis within bone marrow per se next were analyzed, and further were compared directly to epoetin-alfa and darbepoietin-alfa. To limit effects due to differing half-lives, epoetin-alfa was administered at both 1 and 24 hours at a dose (2U /g-mouse) which by day 4 supported reticulocyte (and red blood cell) formation at levels highly similar to those afforded by CNTO 530 (at 0.1 𝜇g/g-mouse), and darbepoietin-alfa (at 0.012 𝜇g/g-mouse) (Figure 5A). Under these balanced dosing conditions, marker expression among developing bone marrow erythroid cells was analyzed, including Podocalyxin (PODXL) as a marker of EPO-induced erythropoiesis. Interestingly, CNTO 530 first was observed to support clear increase in PODXL expression intensities among CD71^high^ cells within bone marrow (Figure 5B). CNTO 530 also was observed to promote increases in frequencies of a PODXL^high^CD71^high^ erythroblast cohort (Figure 5C). In particular, and as analyzed in time-course experiments, CNTO 530 at day 2 was modestly yet significantly more effective than epoetin-alfa and darbepoietin-alfa in expanding this progenitor pool. At day 4, this advantage increased to approximately 4-fold. At day 6, this PODXL^high^CD71^high^ progenitor pool remained elevated as selectively supported by CNTO 530 (12% positivity) and was 6-fold above epoetin-alfa and darbepoietin-alfa- stimulated levels (which were essentially background by day 6 post-dosing). PODXL expression among earlier stage CD71^pos^Ter119^neg^ and CD71^high^Ter119^pos^ erythroid progenitors also was assessed (Figure 5D). Enhanced effects of CNTO 530 on PODXL expression was realized within each bone marrow progenitor pool.

Findings further prompted analyses of CNTO 530 vs. darbepoietin-alfa effects on the expansion of sub-populations of erythroid progenitors within bone marrow. These included Kit^pos^CD71^high^Ter119^neg^, Kit^neg^CD71^high^Ter119^neg^, and Kit^neg^CD71^high^Ter119^pos^ cohorts (designated here as sequential stages E1, E2 and E3) (see Figure 6A). In particular, mice were dosed with CNTO 530 (single 0.2 ug/g-mouse injection), darbepoietin-alfa (single 0.015 𝜇g/g-mouse injection) or saline as a baseline (or steady-state) control. E1 and E2 cells were assayed by gating off Ter119^pos^ cells, and analyzing frequencies of Kit^pos^ vs Kit^neg^ CD71^high^ erythroid progenitors. E3 cells were later stage Ter119^pos^CD71^high^ erythroblasts (and were uniformly Kit^neg^). At steady-state, stage-E3 cells represented approximately 20% of nucleated
marrow cells, while E1 and E2 progenitors were relatively infrequent and occurred at ~1% and ~1.3%, respectively (Figure 6B). At day 2.5 post ESA dosing, darbepoietin-alfa and CNTO 530 each markedly modulated stage-E2 cell frequencies, with 10- and 13- fold increases realized, respectively. E1 progenitor pools remained low, and were comparably unaffected (Figure 6C, left panel). Perhaps most strikingly, at day 4.5 post ESA administration, the expansion of stage-E2 progenitor cells proved to be sustained uniquely in response to CNTO 530 (Figure 6C, right panel). In primary flow cytometry analyses, this effect was visibly obvious for a CD71<sup>high</sup> Ter119<neg> population of bone marrow erythroid progenitors. While these progenitors have not yet been tagged and tracked, it is considered likely that they possess high potential for populating the adult erythron. By comparison, levels of stage-E3 cells were not so markedly affected by CNTO 530, or darbepoietin-alfa (data not shown).
DISCUSSION

For CNTO 530, several unique properties and apparently distinct action modes, presently are defined that merit discussion. These include, first, CNTO 530’s notable erythropoietic activity as demonstrated at steady-state and within two chemotherapeutic agent- induced anemia models. Second, CNTO 530’s capacities to activate known EPO/EPOR signal transduction pathways are defined, together with several distinguishing signaling effects. Finally, a third property of significant interest involves CNTO 530’s unique in vivo abilities to enhance the formation of Podocalyxin - positive erythroid progenitors, and to also promote the sustained expansion of a defined (pro)erythroblast pool within bone marrow.

In basic concentration- dependent, and time- course experiments, CNTO 530 efficiently promoted red cell production for up to 25 days following a single injection at sub-maximum dosing (see Figure 1). This amplification of the adult erythron was more robust than that provided by epoetin-alfa or darbepoietin-alfa. In part, this may reflect differential half-lives which for CNTO 530 recently has been shown to approximate 40 hours. By day 10, however, >95% of CNTO 530 is predicted to become unavailable. This therefore points to additional mechanistic explanations for CNTO 530’s potency (see below). Here, it also should be considered that on a molar basis, an approximate ten-fold higher mass of CNTO 530 over epoetin-alfa is required to achieve maximal bioactivity. This may reflect properties involved with marrow entry, potential EPOR on- and/or off- rates, and/or other as yet undefined features. As an IgG4 Fc fusion protein, CNTO 530 is readily prepared and purified. Requirements for higher mass dosing therefore are not disadvantageous. In its single dose format, CNTO 530 also proved to be uniquely advantageous in limiting the anemia induced by 5-fluorouracil, or paclitaxel plus carboplatin. In particular, and at essentially matched doses, CNTO 530 but not darbepoietin was observed to lessen the severity of anemia incurred following 5-FU mediated myelo-depletion (see Figure 2A). In the case of an optimized paclitaxel/carboplatin model, however, anemia was not so much affected at these doses by either darbepoietin or CNTO 530. When doses were increased to approximately 10-fold over maximal steady-state doses, however, CNTO 530 (but not darbepoietin-alfa) interestingly was capable of significantly
lessening this anemia in severity and duration (see Figure 2B). Why this dosing difference exists between effects in a 5FU (mouse) model vs paclitaxel/carboplatin (rat) model presently is unclear. Nonetheless, the unique ameliorating effects of CNTO 530 in each model are apparent. In future studies, it will be of significant interest to establish CNTO 530’s effectiveness in additional genetically-based anemia models (eg, thalassemia and/or sickle cell). Phase I clinical trials for CNTO 530 likewise indicate initially promising erythropoietic effects.

In initial characterizations of CNTO 530’s molecular action modes, we demonstrated this EMP’s ability to competitively bind to the EPOR, and to stimulate the growth and survival of an EPO-dependent cell line, UT7epo. Presently, we have used primary bone marrow derived KitposCD71highTer119neg erythroblasts to analyze CNTO 530 signal transduction factor activation responses, and have compared these responses directly to those modulated by epoetin-alfa. In the activation of P-S473-AKT, P-Y694-STAT5, and P-T202/Y204-ERK1,2, signal transduction factor analyses demonstrated similar time-course responses for CNTO 530 and epoetin-alfa. For p70S6K, however, CNTO 530 interestingly proved to selectively enforce a prolonged stimulation (see Figure 3B). In addition, a significant direct differential in the relative stimulation of p70S6K vs. ERKs by CNTO 530 vs. EPO was observed (see supplemental Figure S-1). p70S6K lies downstream of mTOR (especially mTOR- C1). However, p70S6K activation also can be regulated by PDK1,2; PKC; PP2A and PTP1b phosphatases, and ubiquitination. Which pathway might be engaged by CNTO 530 to sustain S6K activation is presently undefined, but is of significant interest. Functionally, S6K can exert diverse effects on cell growth, development, homeostasis and protein translation, and therefore comprises an interesting effector for future investigations of CNTO 530’s action mechanisms. With regards to ERK1,2 increased activation via a PY-mutated EPOR-HM allele interestingly has been observed and has been functionally linked to perturbed proerythroblast differentiation events. How interactions of this homodimeric EPO mimic peptide with the EPOR might differentially sustain this response also is of interest, especially in the context of a conformation-mediated model for the activation of dimeric EPOR plus JAK2 complexes.
In vivo, CNTO 530 further first was observed to selectively enhance the formation of Podocalyxin-positive CD71\textsuperscript{high} erythroid bone marrow progenitor cells. This was obvious as early as day-2 post-dosing, and subsequently was reinforced at days 4 and 6 (see Figure 5C). At day 6, this effect of CNTO 530 on PODXL\textsuperscript{pos} progenitors expansion in marrow, in fact, was realized at levels six-fold beyond those promoted by either epoetin-alfa or darbepoietin-alfa. Using primary bone marrow erythroblasts and a global profiling approach, we recently discovered Podxl as a major EPO response gene\textsuperscript{27}. PODXL is a transmembrane sialomucin and CD34 orthologue\textsuperscript{49}. In renal podocytes, PODXL is thought to function in maintaining diaphragm slits\textsuperscript{50}. In contrast, in hematopoietic stem cells\textsuperscript{26} and potentially in certain cancers (e.g. prostate and certain leukemias)\textsuperscript{51,52}, PODXL may act as an adhesion and/or cell migration factor. It therefore might be speculated through correlation that PODXL, as persistently induced by CNTO 530 may affect the residency of erythroid progenitors in marrow.

A second effect as discovered via extended analyses of Kit\textsuperscript{pos}CD71\textsuperscript{high}Ter119\textsuperscript{neg}, Kit\textsuperscript{neg}CD71\textsuperscript{high}Ter119\textsuperscript{neg} and Kit\textsuperscript{neg}CD71\textsuperscript{high}Ter119\textsuperscript{pos} cell formation (“E1”, “E2” and “E3” progenitors) involved CNTO 530’s uniquely sustained effects on stage-E2 progenitor expansion within bone marrow. Specifically, when E1-E3 progenitor cell pools were assayed in bone marrow following CNTO 530 or darbepoietin-alfa dosing, CNTO 530 uniquely supported the enhanced expansion of stage-E2 10-fold over those stimulated by maximal dose darbepoietin-alfa (see Figure 6). Here, it is proposed that this unique activity of CNTO 530 in persistently stimulating this erythroid progenitor pool at least in part account for its potent and sustained activity as an EPO mimetic peptide. Precisely, how CNTO 530 exerts this effect presently is less clear. Previously, select ligand-receptor interactions within discrete niches have been described for activated B-cells in a BLyS signaling context\textsuperscript{53} and for T-cell development in IL7 signaling contexts\textsuperscript{54}. Potential bone marrow niche sequestration of CNTO 530 therefore represents one possibly enhanced action mode. Potential effects on macrophage-dependent erythroblast island formation, alternatively, are possible. Erythroblast island associated events, however, are thought to predominantly affect late-stage development, including enucleation\textsuperscript{55}. Understanding specific bases for CNTO 530’s unique in vivo properties therefore will require further investigation. The present investigations nonetheless
demonstrate CNTO 530 to comprise a unique and potent ESA with significant promise for prospective clinical utility in the treatment of anemia.
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AUTHORSHIP CONTRIBUTIONS - All authors contributed to bench investigations, and manuscript construction. Lead roles assumed by individual authors include the following: P. Sathyanarayana: phosphoprotein analyses (with D. Marshall) and gene profiling; E. Houde: In vivo CNTO 530, EPO, Aranesp dosing experiments and phosphoprotein analyses (with P. Sathyanarayana); C. Emerson and A. Pradeep: Analyses of ESA effects on erythroid progenitor pools in vivo; D. Marshall: Phosphoprotein analyses (with P. Sathyanarayana) and carboplatin/paclitaxel model and experiments (with A. Volk and D. Makropoulos); A. Volk and D. Makropoulos: Carboplatin/paclitaxel experiments (with D. Marshall); D.M. Wojchowski and P. Bugelski: Design and direction of all investigations, and lead authors of this manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. CNTO 530, epoetin-alfa and darbepoietin-alfa dose-dependent effects on red cell production.  

**A** In dose-response studies, the capacity of CNTO 530 to stimulate red cell production in wild-type C57BL/6 mice was analyzed, and was compared to dose responses for epoetin-alfa (EPO), and darbepoietin-alfa. CNTO 530 and darbepoietin-alfa were as single IP injections, while epoetin-alfa (EPO) was injected at 1 and 24 hours (as indicated by arrows). Doses are as microgram, or unit (U, EPO) per mouse gram-weight. Hematocrits are mean values +/- SE (n=5).  

**B** Time-course analysis of CNTO 530- induced red cell, and reticulocyte production – Mice were injected with CNTO 530, epoetin-alfa (EPO) or darbepoietin-alfa at near maximal doses (0.15 μg/g-mouse, 1.5 U/g-mouse, and 0.012 μg/g-mouse, respectively). Responses in hematocrits and reticulocytes then were determined on days 4, 8, 12, 16 and 24, and are graphed as mean values +/- SE (n=4).

Figure 2. Efficient CNTO 530 lessening of the anemia induced by 5-fluorouracil, or paclitaxel plus carboplatin.  

**A** Anemia was induced via single 5-FU injection (150mg/kg). On day 4.5, CNTO 530 (0.3 ug / g-mouse), darbepoietin-alfa (0.02ug /g-mouse) or saline then was administered. Hematocrits were determined at the indicated time points. Results for two independent experiments are shown (means +/- SE , n=4 for each experiment). (**, p≤0.001; *, p≤0.024)  

**B** In Sprague-Dawley rats, anemia was induced via dosing on days 1, 8 and 15 with 10 mg/kg paclitaxel (pac) plus 50 mg/kg carboplatin (carbo). On day 16, CNTO 530 or darbepoietin-alfa then was administered and effects on hemoglobin levels and hematocrits were determined over a 50 day interval. Dosing for CNTO 530 was at 1 ug and 3ug / g-rat, and for darbepoietin-alfa at 0.03 ug and 0.3 ug / g-rat. Mean values +/- SD are graphed (n= 8 for controls, for pac/carbo, for pac/carbo plus CNTO 530, and for pac/carbo plus darbepoietin-alfa). The untreated group is indicated as “NT” (not treated). Upper and lower panels illustrate representative effects on hemoglobin levels and, hematocrits.
Figure 3. CNTO 530 activates known EPO signal transduction pathways in primary bone marrow proerythroblasts, and induces sustained p70S6K activation.  A] CNTO 530 and EPO dose-dependent support of CFUe formation – To initially examine ex vivo effects on CFUe progenitors, adult murine bone marrow cells were plated in methyl cellulose colony forming assays in the presence of varied concentrations of CNTO 530 (0.08, 0.25, 1.0, μg/mL) or EPO (epoetin-alfa) (0.08, 0.25, 1.0 U/mL) for direct comparison. At 50 hours of culture, frequencies of 8-16 cell CFUe were determined, values are mean +/- SE and are means of 3 35mm culture dishes for each dose. B-E] CNTO 530 and EPO modulation of p70S6K, AKT, STAT5 and ERK1,2 in primary bone marrow pro-erythroblasts - Primary erythroid progenitor cells from adult bone marrow were expanded in SP34EX medium. At 72 hours, Kit^posCD71^highTer119^neg^ proerythroblasts were then isolated. Cytokines were withdrawn and cells (at 1 x 10^6 cells/mL) were incubated for 5.5 hours in IMDM, 0.5% BSA, 15 ng/mL insulin, and 0.1 mM 2-ME. Cells next were exposed in parallel to either CNTO 530 (0.5 μg/mL), or EPO (2U/mL) for 0, 10, 20, 40 and 80 minutes. Levels of activated phospho-T421/S424-p70S6K [A]; phospho-S473-AKT [B]; phospho-Y694-Stat5 [C]; and phospho-T202/Y204-ERK1,2 [D] then were determined. Quantitation was by Image-J analysis, and was normalized based on levels of total p70S6K, AKT, STAT5 and ERK1,2. Values are percent maximal signals for CNTO 530, and EPO.

Figure 4. Shortly following administration, a rapid release of reticulocytes and nucleated DRAQ5^pos^ erythroid progenitors is stimulated by EPO (epoetin-alfa), but less so by CNTO 530. Mice (n=3 per treatment group) were administered CNTO 530 (0.15 μg/g-mouse), epoetin-alfa (EPO) (2 U/g-mouse) or 0.9% saline (to account for background staining levels) via tail vein injections. At two hours, peripheral blood samples were analyzed for levels of TO^pos^ DRAQ5^neg^ reticulocytes (panel A); and for DRAQ5^pos^CD71^high^ progenitors (panel B). (Note base line levels of CD71(+) TO (-) and CD71(+) DRAQ5 (+) cells were 1.2% and 1.4%, respectively).

Figure 5. CNTO 530 enhances the formation of a PODXL^high^CD71^high^ bone marrow erythroid progenitors.  A] To investigate possible differential effects of CNTO 530, and epoetin-alfa or
darbepoietin-alfa on bone marrow erythroid progenitor cell populations, mice were administered each at
doses which at day 4 provided near maximum, and highly comparable levels of reticulocyte and red cell
production. Near equivalency in reticulocyte production at day 4 was observed post dosing with CNTO
530 (0.15 μg per g-mouse), epoetin-alfa (EPO) (2 U/g-mouse at 1 and 24 hours), or darbepoietin-alfa
(0.012 μg/g-mouse). Values for each injected mouse are shown together with mean values (horizontal
bars). B] For bone marrow - resident erythroid progenitors, CNTO 530 induced significant increases in
the intensity (i.e., cell surface density) of PODXL expression. Here, this is shown in representative flow
cytometric analyses which compare PODXL expression levels post- CNTO 530 vs. EPO dosing, and
CNTO 530 vs. darbepoietin-alfa. C] At days 2, 4 and 6, frequencies of PODXL$^{\text{high}}$CD71$^{\text{high}}$ progenitors
within bone marrow were determined by flow cytometry. Upper panels illustrate representative flow
cytometry analyses, and demonstrate a clear advantage for CNTO 530 in stimulating the formation of
this PODXL$^{\text{high}}$CD71$^{\text{high}}$ erythroid progenitor cell pool, especially at days 4 and 6. Mean frequencies (+/-
SE) of such PODXL$^{\text{high}}$CD71$^{\text{high}}$ progenitors are graphed as analyzed in n=3 mice post CNTO 530, EPO,
or darbepoietin-alfa injections. (EPO was administered at 1 and 24 hours). D] Gating on, or off,
Ter119$^{\text{pos}}$CD71$^{\text{high}}$ erythroid progenitors revealed that CNTO 530’s effects on enhanced PODXL
expression was exerted among not only later stage Ter119$^{\text{pos}}$ cells, but also their CD71$^{\text{high}}$Ter119$^{\text{neg}}$
progenitors. Values are means +/- SE (n=3).

Figure 6. CNTO 530 uniquely induces the sustained expansion of a bone marrow - resident
Kit$^{\text{neg}}$CD71$^{\text{high}}$Ter119$^{\text{neg}}$ “E2” progenitor pool. A] Based in part on recent ex vivo analyses of
murine bone marrow erythroid progenitor pools$^{27,35,56}$ a course of stepwise development can be defined
as a Kit$^{\text{pos}}$ CD71$^{\text{high}}$Ter119$^{\text{neg}}$ → Kit$^{\text{neg}}$ CD71$^{\text{high}}$Ter119$^{\text{neg}}$ → Kit$^{\text{neg}}$ CD71$^{\text{high}}$Ter119$^{\text{pos}}$ cell progression (i.e.
stages “E1” → “E2” → “E3”. B] At steady-state, stage E3 bone marrow progenitors were predominant
as assayed via co-staining for Kit, CD71 and Ter119 markers, and gating-off RBCs and reticulocytes. E1
and E2 progenitors were assayed by gating off Ter119$^{\text{pos}}$ cells, and on Kit$^{\text{pos}}$ vs Kit$^{\text{neg}}$ CD71$^{\text{high}}$ cells.
Each symbol in each set represents the value for an independently treated and analyzed mouse.
C] CNTO 530 uniquely and markedly expands “stage E2” bone marrow progenitors – C57BL/6 mice were dosed with either CNTO 530 (0.2 ug / g-mouse) or darbepoietin (0.015 ug / g-mouse). At days 2.5 and 4.5, levels of E1, E2 and E3 progenitors were then determined via flow cytometry. Graphed values (upper panel) are means +/- SE (n=3). In lower panels, primary flow cytometry data illustrate CNTO 530 expansion of stage E2 erythroid progenitors within bone marrow (here at day 4.5 post dosing with either CNTO 530, darbepoietin, or saline).
**FIGURE 2**

(A) EXP. 1

- CNTO 530 Darbepoietin
- CNTO 530
- Darbe
- Saline

Hematocrit

Days post 5FU Injection

(B) EXP. 2

- CNTO 530 Darbepoietin
- CNTO 530 (3)
- NT
- CNTO 530 (1)
- Darbe (0.3)
- Darbe (0.03)
- Saline

Hgb (g/dL)

Days post Initial Taxol / Carbo Injection

- Taxol / Carbo
- Darbepoietin

Hematocrits

Days post Initial Taxol / Carbo Injection
FIGURE 3

(A) CFU<sub>e</sub>, CNTO 530

(B) phospho-p70S6K

(C) phospho-AKT

(D) phospho-STAT5

(E) phospho-ERK1,2
(A) CD71 (high) Reticulocytes, % gated events

EPO  CNTO 530

p=0.0001

(B) CD71 (high) DRAQ5 Pos. cells, % gated events

EPO  CNTO 530

p=0.0001

FITC-CD71 versus DRAQ5

1.9%  0.8%

EPO  CNTO 530
Figure 5

(A) peripheral blood

Reticulocytes, % total

2 U/g-m
0.1 ug/g-m
0.012 ug/g-m

Saline EPO CNTOS50 Aransep

(B) FITC-CD71

PODXL(bright)

EPO CNTO-530

PODXL

Darbepoetin

PODXL Expression, relative fluorescence intensity

(C) PCTO high cells, % gated events

CD71 pos

PODXL pos

Day 0 Day 2 Day 4 Day 6

p = 0.002

p = 0.005

(C) Control CNTO 530 EPO Darbepoetin

Day 2 Day 4 Day 6

Fitc-CD71

APC-PODXL

(D)

GATED ON CD71 high Ter 119 neg CELLS

GATED ON CD71 high Ter 119 pos CELLS

PODXL pos CD71 high

PODXL pos CD71 high

Day 0 Day 2 Day 4 Day 6

CNTO 530 EPO Darbepoetin
Figure 6

(A) Kit, CD71, Ter119

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<tr>
<td>E3</td>
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(B) E1, E2, E3 Frequencies, % nucleated cells

(C) CNTO 530  Darbepoietin

<table>
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<tr>
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(D) Exp. 1  Exp. 2

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Exp. 1: 1.5%  CNTO 530 17.0%  Darbepoietin 3.6%

Exp. 2: 1.6%  CNTO 530 17.5%  Darbepoietin 3.4%

p<0.0001 **
CNTO 530 functions as a potent EPO mimetic via unique sustained effects on bone marrow proerythroblast pools

Pradeep Sathyanarayana, Estelle Houde, Deborah Marshall, Amy Volk, Dorie Makropoulos, Christine Emerson, Anamika Pradeep, Peter J. Bugelski and Don M. Wojchowski