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Bi-functional prodrugs of 5-aminolevulinic acid and butyric acid increase erythropoiesis in anemic mice in an erythropoietin-independent manner

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ABSTRACT

Anemia is a major cause of morbidity and mortality worldwide resulting from a wide variety of pathological conditions. In severe cases it is treated by blood transfusions or injection of erythroid stimulating agents, e.g., erythropoietin (Epo), which can be associated with serious adverse effects. Therefore, there is a need to develop new treatment modalities. We recently reported that treatment of erythroleukemic cells with the novel the bi-functional prodrugs of 5-aminolevulinic acid (ALA) and butyric acid (BA), AN233 and AN908, enhanced hemoglobin (Hb) synthesis to a substantially higher level than did ALA and BA individually or their mixture. Herein, we describe that these prodrugs when given orally to mice induced histone deacetylase inhibition in the kidneys, bone marrow and spleen, thus, indicating good penetrability to the tissues. In mice where anemia was chemically induced, treatment with the prodrugs increased the Hb, the number of red blood cells (RBCs) and the percentage of reticulocytes to normal levels. The prodrugs had no adverse effects even after repeated treatment at 100–200 mg/kg for 50 days. The lack of increased levels of Epo in the blood of mice that were treated with the prodrugs suggests that AN233 and AN908 affected the Hb and RBC levels in an Epo-independent manner. Taken together with our previous studies, we propose that the prodrugs increase globin expression by BA inhibition of histone deacetylase and elevation heme synthesis by ALA. These results support an Epo-independent approach for treating anemia with these prodrugs.

1. Introduction

Anemia is a common, and sometimes the major, clinical symptom of a wide variety of pathological conditions (Means, 1999; Knight et al., 2004; Thomas and Thomas, 2005; Weiss and Goodnough, 2005). Its severity is determined by the number of red blood cells (RBC) and their hemoglobin (Hb) content. The main treatments for anemia include supplementation with iron and vitamins, blood transfusion and injection of erythropoiesis-stimulating agents, based on erythropoietin (Epo) - the major growth factor for RBC formation. Epo is produced principally in the kidneys, and its level is controlled by tissue oxygen tension (Daghman et al., 1999). In the bone marrow, it promotes survival, proliferation and maturation of erythroid progenitors and precursor cells, thus leading to the elevation of RBC mass and Hb levels (Chateauvieux et al., 2011). Epo treatment involves bolus administration of supper physiological doses that may cause serious thromboembolic and cardiovascular events, as well as mortality of patients (Bohlius et al., 2009; Bennett et al., 2012; Kelada and Marignol, 2014). Blood transfusions alleviate that anemia symptoms bear the risks of blood-borne pathogens, potential toxins, immuno-modulating factors and severe allergic reaction (Glaspy, 2014).

The risks and cost associated with the currently available treatments raise the need for safe alternative approaches. Histone deacetylase inhibitors (HDACIs) such as butyric acid (BA) activate expression of the globin genes, and induce differentiation of erythroid cells (Ikuta et al., 1998; Canh Hiep et al., 2012; Steliou et al., 2012). Previously, we described novel prodrugs that upon esterase-dependent metabolic breakdown, release the HDAC inhibitory BA and aldehyde(s) (Aviram et al., 1994; Nudelman et al., 2005). These type of prodrugs blocked by esterification, are activated by cellular esterases to release the active drug(s), (Bardos et al., 1969). The BA prodrugs were shown to possess anticancer activity as well as cardioprotective activity (Kessler-Icekson et al., 2012; Tarasenko et al., 2012a; Tarasenko et al., 2012b). AN10, AN7 and AN40, induced the synthesis of Hb in the human leukemic cell line K562, as well as in primary cultures of human erythroid precursors (Rephaeli

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et al., 2000; Nudelman et al., 2001). The BA prodrug, pivaloyloxymethyl butyrate (AN9) was demonstrated to display safety in a Phase I clinical study (Patnaik et al., 2002).

We have also synthesized and studied a novel prodrug, AN233, of 5-aminolevulinic acid (ALA) and BA. ALA, that is synthesized from glycine and succinyl Co-A by ALA synthase, is the first and rate-limiting step of the heme synthetic pathway. By circumventing this step, exogenously supplied ALA can accelerate heme production (Malik et al., 1988). Heme is a central component of Hb, myoglobin, cytochromes, catalase and peroxidase. In addition, heme increases Hb production by enhancing globin mRNA translation (Liu et al., 2007; Chiabrando et al., 2014). Combination treatment with ALA and BA of the human leukemic cell line K562 enhances Hb synthesis to a substantially higher level than does each compound separately (Kawasaki et al., 1998). We have previously shown that AN233 was superior to ALA in induction of protoporphyrin-IX synthesis and photodynamic therapy toxicity to cancer cells (Berkovitch et al., 2008; Feuerstein et al., 2011; Berkovitch-Luria et al., 2012a). In K-562 cells, AN233 was significantly more effective than ALA, BA or their mixture, in increasing the expression of globin mRNAs and proteins (Berkovitch-Luria et al., 2012b).

We now report that AN233 and a novel bi-functional prodrug of BA and ALA, AN908, can ameliorate anemia in mice by increasing Hb and RBC production in an Epo-independent manner. The results suggest that these prodrugs have the potential to correct anemia.

2. Materials and methods

2.1. Synthesis of the prodrugs

The chemical structure and the hydrolytic metabolites of AN233 and AN908 are shown in Table 1. The synthesis of AN233 and AN908 was previously reported (Berkovitch et al., 2008).

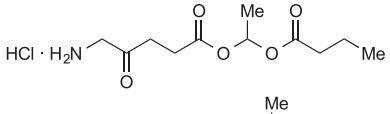
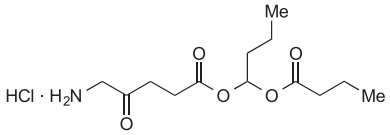
2.2. In vivo studies

The animal experiments were conducted according to the NCI Laboratory Animal Care Guidelines with the approval of the Tel Aviv University Committee for Animal Experimentation and the Israel Ministry of Health (the permit number M-15-070).

Male Balb-c mice (Harlan Laboratories, Jerusalem, Israel) were kept in the Institutional Animal Facility at a controlled air-flow and temperature and on a 12/12 h dark/light regime with free access to standard food and tap water.

Anemia was induced by three ip. injections of cycloheximide and chloramphenicol (60 mg/kg each, Sigma), 2 injections prior, and a third injection on day 6 or 7 after the initiation of the treatment with the prodrugs or Epo. The prodrugs and Epo were administered five times a week; the prodrugs - at 100–200 mg/kg orally (po) by gavage and human Epo (40,000 IU/mL from Ortho Biotech, Janssen, PA, US) at 200 U/kg was given subcutaneously (sc).

Table 1
Chemical structure and metabolic products of AN233 and AN908.

Prodrugs	Structure	Hydrolysis products
AN233		ALA, BA, acetaldehyde
AN908		ALA, BA, butyraldehyde

2.3. Determination of hematological parameters

Blood drawn from the tail vein of the mice was used for Hb determination by staining with the Drapkin's reagent (Sigma). Absorption at 540 nm was read using the spectrophotometer (Synergy III, Biotech Instruments, Inc. Germany). For determination of additional parameters, blood was drawn in anticoagulant containing tubes from the eyes of mice slightly anesthetized by inhalation of isoflurane. In addition to Hb, the numbers of RBC, hematocrit and white blood cells (WBC), in whole blood of 7 mice of each treatment group was analyzed (System KX-21, system corp., Japan) by AML Laboratory (Animal Department, Herzlia Medical Center, Israel).

2.4. Quantification of blood reticulocytes

Reticulocytes, defined as CD71 (transferrin receptor) positive RBCs (Dalko et al., 2013), were quantified following staining with anti-CD71-FITC antibody (Bio Legend, San-Diego, CA, US) at 4 °C for 30 min and analysis on a flow cytometer (Gallous, Beckman Coulter Inc., USA). Data were calculated using the Kaluza®Flow analysis software (Beckman Coulter Inc.).

2.5. Serum Epo levels

Epo in the serum of mice was measured using mouse ELISA kit (Quantikine), according to manufacturer's instructions (R & D Systems, Minneapolis, MN, US).

2.6. Quantification of histone acetylation and HDAC expression in mice

Non anemic mice were treated with a single oral dose of 400 mg/kg of AN908, AN233 or the vehicle. The kidneys, bone marrows and spleens were harvested after 3 h, homogenized, and their histones purified (Rephaeli et al., 2006). The protein content in the purified histones was determined with the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples of 10 μg/15 μL protein were loaded on a 15% SDS gel and were subjected to Western blot analysis using rabbit anti-acetylated H4, Lysin-12 (1:800) and anti-rabbit anti-total H3 (1:1000, both from Cell Signaling, USA) followed by the secondary IgG IRDye 680DX antibody (LI-COR Biosciences). Each detected band was quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). The fold increase of acetylated histone was determined by the ratio of the band intensity (normalized to the loading controls) obtained from prodrugs treated to that of vehicle treated mice.

In another experiment, described in Fig. 3, crude extracts of kidneys were homogenized in a cold cell lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1.0% Triton X-100 and a protease-inhibitor cocktail (Calbiochem, USA). After 30 min of incubation on ice, the samples were centrifuged at 12,500 $\times g$ at 4 °C, the supernatants were aliquoted and stored at −80 °C. For Western blot analysis the samples were thawed, tissue lysates (25 μg protein/20 μL) were subjected to a 15% SDS gel and analyzed by Western blot using antibodies against HDAC1, HDAC2, actin, pan-acetylated H3 and total H3. Each detected band in the extracts was quantified as described above.

2.7. Statistics

Differences between the experiment groups were tested by analysis of variance and Student's *t*-test. Results are expressed as mean ± SEM < 0.05 was considered statistically significant.