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Nonclinical evaluation of the potential for mast cell activation by an erythropoietin analog

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ABSTRACT

The erythropoietin analog peginesatide was withdrawn from marketing due to unexpected severe anaphylactic reactions associated with administration of the multi-use formulation. The adverse events occurred rapidly following the first ever administration of the drug with most affected patients becoming symptomatic in less than 30 min. This is most consistent with an anaphylactoid reaction due to direct activation of mast cells. Laboratory evaluation was undertaken using rat peritoneal mast cells as the model system. Initial studies showed that high concentrations of the formulated drug as well as formulated vehicle alone could cause mast cell degranulation as measured by histamine release. The purified active drug was not able to cause histamine release whereas the vehicle filtrate and lab created drug vehicle were equally potent at causing histamine release. Individual formulations of vehicle leaving one component out showed that histamine release was due to phenol. Dose response studies with phenol showed a very sharp dose response curve that was similar in three buffer systems. Cellular analysis by flow cytometry showed that the histamine release was not due to cell death, and that changes in light scatter parameters consistent with degranulation were rapidly observed. Limited testing with primary human mast cells showed a similar dose response of histamine release with exposure to phenol. To provide *in vivo* confirmation, rats were injected with vehicle formulated with various concentrations of phenol *via* a jugular vein cannula. Significant release of histamine was detected in blood samples taken 2 min after dosing at the highest concentrations tested.

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Introduction

Patients on hemodialysis secondary to kidney failure often require secondary treatment to maintain erythrocyte production. Where the failure is due to iron deficiency, the treatment is with an approved iron supplement. In other cases, direct stimulation of erythropoiesis is needed. There are several FDA approved products that are forms of human erythropoietin. These products have a long clinical track record and a well defined safety profile. On March 27, 2012, a new drug was approved for direct stimulation of erythropoiesis. This drug, peginesatide (Omontys®), is a synthetic peptide that does not have structural homology with human erythropoietin. Peginesatide consists of two identical 21 amino acid peptides covalently linked through sarcosine to a polyethylene glycol (PEG) molecule of ~40 kD. Each peptide has an internal disulfide bond between residues 6 and 15. The intended pharmacological action is stimulation of the erythropoietin receptor (EPOR) to increase production of erythrocytes.

Following the initiation of marketing of this drug, adverse event reports were received of anaphylactic type reactions. The reactions occurred in approximately 0.2% of patients with approximately 1/3 requiring serious medical intervention and with fatal reactions in 0.02% of patients (FDA website, 2013). These reactions occurred rapidly (<30 min) following administration of the first dose of the drug. Peginesatide adverse events were correlated with exposure of new groups of patients in dialysis clinics (Bennett et al., 2014). The drug was administered intravenously *via* an injection port on the dialysis machine during active dialysis. The drug was withdrawn from the market on February 23, 2013 by the manufacturer. A total of about 25,000 patients were treated with peginesatide prior to its withdrawal (FDA website, 2013).

A more detailed evaluation of individual case reports suggested a clinical picture consistent with an anaphylactoid reaction due to direct activation of mast cells. Anaphylactoid reactions represent non-IgE mediated activation of mast cells and/or basophils resulting in the release

Abbreviations: API, active pharmaceutical ingredient; HBSS, Hanks Balanced Salt Solution; hMCs, human mast cells; MCs, mast cells; MU, multiuse; MUV, multiuse vehicle; PMCs, peritoneal mast cells.

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of histamine, leukotrienes and other chemical mediators of inflammation. While anaphylactoid reactions are rare, many compounds have been shown to have the potential to induce them including radiographic contrast media, quinolone antibiotics, vancomycin, non-steroidal anti-inflammatory drugs (NSAIDS) and opiates (Farnam et al., 2012).

Mast cells are typically found in tissues rather than circulation and are present in normal human heart, particularly the atrial appendage (Sperr et al., 1994) and aorta (DeBruin et al., 2015). Their numbers increase between 1.7 and 6-fold in association with a variety of cardiac pathologies, including those observed in association with renal failure (reviewed in Janicki et al., 2015; DeBruin et al., 2015). Importantly, cardiac mast cells have been reported to play a role in fibrosis and formation of atherosclerotic plaques (Janicki et al., 2015). However, cardiac mast cells are difficult to isolate and while some differences exist between rodent and human mast cell sources (reviewed in Bischoff, 2007) we chose to perform laboratory experiments using purified rat peritoneal mast cells and human mast cells from humanized mice to test the hypothesis of anaphylactoid reaction and to determine whether a specific causative agent could be identified.

Materials and methods

Chemicals

The chemicals used to recreate the pharmaceutical vehicle were obtained from Sigma Chemical Corporation (St Louis, MO) and were of the highest grade available. Cell culture reagents were obtained from Life Technologies (Carlsbad, CA). The mast cell assay reagents Compound 48/80, teicoplanin (Sugimoto et al., 2000) and octyl β-glucoside were obtained from Sigma Chemical Corporation (St Louis, MO).

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee, Center for Drug Evaluation and Research, FDA, and carried out in an AAALAC-accredited facility.

Rats

Female Sprague-Dawley rats (10–12 weeks old) for mast cell donation were purchased from Taconic (Hudson, NY) and were doubly housed. Double cannulated rats used for the *in vivo* studies were also purchased from Taconic and were implanted with jugular vein and femoral vein cannulas by Taconic. Maintenance of the cannulas was performed according to the protocol provided by Taconic. The cannulated rats were single housed. All rats were fed Certified Purina Chow #5002 (Ralston Purina Co., St Louis, MO) and water *ad libitum*.

Mice

Female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were group housed in individually ventilated cage racks, fed autoclaved LabDiet (St. Louis, MO) formula #5K52, and provided autoclaved acidified water *ad libitum*.

Mouse humanization methodology and testing

Humanization surgery was performed essentially as previously described (Melkus et al., 2006) with differences noted as follows. Bone marrow ablation was completed using a single dose of either Busulfan at 30 mg/kg, i.p. or Treosulfan at 2 g/kg i.p., 24 h prior to i.v. CD34+ stem cell infusion. Isolated CD34+ stem cells were either maintained in culture overnight or frozen and stored in liquid nitrogen and then administered either following implant surgery or three weeks post-surgery. Total number of CD34+ cells injected ranged from 2.2–2.4 × 10^5. Monitoring of humanization began approximately eight weeks after surgery and was assessed using whole blood every 3–4 weeks for over 20 weeks. Humanization analysis by flow cytometry employed RBC Lysis Buffer (Biolegend, San Diego, CA) according to manufacturer instructions. Cells were blocked using TruStain FcX and Human TruStain FcX (Biolegend, San Diego, CA) and stained using monoclonal antibodies against murine CD45 (clone 30-F11), human CD45, CD3, CD19, CD33, CD4 and CD8 (clones HI30, UCHT1, HIB19, WM53, RPA-T4, and RPA-T8, respectively; Biolegend, San Diego, CA). Samples were acquired and analyzed using a FACS Aria III flow cytometer and FACS Diva software (Becton, Dickinson & Co, Franklin Lakes, NJ). CountBright™ absolute counting beads (Life Technologies, Grand Island, NY) were used to determine absolute human cell numbers in addition to the percentage of human cells present. Mice were euthanized and splenocytes and bone marrow cells were isolated using standard techniques.

Rat mast cell isolation

Rat peritoneal mast cells were isolated using a method adapted from Shanahan et al., 1985. Briefly, an untreated rat was euthanized by CO_2 asphyxiation using IACUC approved methodology. The peritoneal cavity was injected with ice cold Hanks Balanced Salt Solution (HBSS) supplemented with 1% heat-inactivated horse serum (HBSS/HS) and 1000 IU/ml of sodium heparin. After 15 min, the peritoneal cells were aspirated and centrifuged at 120 × *g* for 5 min. Cells were resuspended in 2 ml HBSS/HS and layered over 8 ml of a mixture of 50% Percoll and 50% RPMI 1640 with 10% horse serum. This was centrifuged 30 min at 300 × *g*. Purified rat peritoneal mast cells (PMCs) were recovered in the pellet and washed twice in HBSS/HS. Purity was >95% as assessed by toluidine blue staining.

Human mast cell isolation

Mast cells were isolated from mice with an engrafted human immune system. Human mast cells (hMCs) were isolated from combined spleen and bone marrow cell suspensions using Miltenyi Biotec (San Diego, CA) Fc-block, anti-human CD117-PE antibody (clone A3C6E2), followed by anti-PE microbeads (kit 130-048-801) according to manufacturer instructions. Cells were sorted using a MidiMACS magnetic separator and LS columns. The positive and negative fractions were washed and counted. A post-enrichment flow cytometric analysis was run to determine the percent hMC however, the antibody used to enrich for hMC appears to partially block binding of alternate antibodies used for phenotyping hMC. Therefore, toluidine blue was used to identify hMC and flow cytometry staining was used to identify the other cell types present (Supplemental Fig. 1). The purity of hMC across four mouse isolations from two original tissue donors was 55 ± 9% as assessed by toluidine blue staining. The remaining cells were primarily human hematopoietic precursors and murine neutrophils. Less than 0.6% of cells present following hMC enrichment were murine mast cells.

Histamine release assay

Purified rat or human MCs were resuspended at a concentration of 5 × 10^5/ml in HBSS/HS. Test or control solutions in a volume of 90 μl were placed in wells of a round-bottomed 96 well plate. Where needed, volume was made up to 90 μl with HBSS/HS. A volume of 10 μl of cell suspension was added and mixed. The plate was incubated for 30 min at 37 °C. The plate was centrifuged for 5 min at 120 × *g* and then 50 μl of the supernatant was transferred to a different well. The cell pellet was solubilized by adding 5 μl of a 5% solution of the detergent octyl β-glucoside. The plate was then sealed and frozen at −30 °C for analysis of histamine release by LC–MS (Chimalakonda et al., 2015). Total available histamine was calculated as the sum of the histamine measured in half the supernatant well plus the amount of histamine released in the well with the other half supernatant plus cell pellet. Percent release was calculated at two times the half supernatant value divided by the