

The potential utility of iron oxide nanoparticles for the prophylaxis of skin inflammation in a mouse model of psoriasis

Ankit Shah¹, Edward Cedrone¹, Chelsea L. Sanders², Donna Butcher³, Alexandra M. DeFrancesco³, Kasey L. DeGrange³, Simone Difilippantonio², Elijah F. Edmondson³ and Marina A. Dobrovolskaia^{1*}

¹Nanotechnology Characterization Laboratory, Cancer Research Technology Program,

²Animal Research Technical Support, ³Pathology and Histology Laboratory, Frederick National Laboratory for Cancer Research, sponsored by the National Cancer Institute, Frederick, MD 21702, USA

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Abstract

Several studies have demonstrated that immunosuppressive effects accompany systemic administration of some iron oxide nanoparticles (IONPs). In our earlier study, conducted using human peripheral blood mononuclear cells, we showed that therapeutic formulation of IONPs, Feraheme, approved for clinical use in the United States for the treatment of iron deficiency in chronic kidney disease, suppresses the function of activated T-cells in vitro via a mechanism involving mitochondrial damage. Here we report an in vivo study demonstrating that topical application of Feraheme prior to disease onset decreases the development of skin lesions in the mouse model of chemically induced psoriasis.

Keywords:

Nanoparticles, in vivo, preclinical, T-cells, psoriasis, inflammation, immunosuppression, Feraheme, iron oxides

Introduction

Psoriasis is a human skin disorder characterized by signs of both chronic inflammation and autoimmunity. It affects 125 million people worldwide.(1) Despite well-established clinical signs, the mechanism underlying this disease is incompletely understood. It is currently debated in the literature whether psoriasis is a primary autoimmune disease leading to the skin inflammation or the autoimmunity is secondary to the chronic inflammation.(2, 3) Recently, another term, autoinflammation, was coined to describe psoriasis.(4) Regardless of the debate regarding terminology and classification of this disease, T-cells are widely recognized as primary players in its pathogenesis and considered as the main therapeutic target. (5, 6) Currently available treatments include systemically administered biologics (e.g., recombinant proteins and antibody therapeutics inhibiting cytokines TNF α , IL-17A, IL-12, and

IL-23) and small molecules (e.g., cyclosporin and methotrexate); phototherapy, as well as orally and topically administered steroids and non-steroid anti-inflammatory drugs (e.g., hydrocortisone and thalidomide analog apremilast).(7)

Nanoparticles can be used for the delivery of a variety of therapeutic agents, including those intended for the therapeutic intervention of inflammatory and autoimmune diseases.(8-10) Of interest are the nanoparticles which possess intrinsic immunosuppressive properties. Such properties may result in undesirable immunosuppression and, associated with it, safety concerns. On the other hand, such nanoparticles may be beneficial when the inhibition of the immune system is desirable, for example in the treatment of autoimmune and inflammatory conditions. Such particles, therefore, may serve as active pharmaceutical ingredients (APIs) and provide therapeutic benefit without loading of a particular drug.

*Corresponding author: Marina A. Dobrovolskaia, Nanotechnology Characterization Laboratory, Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD, USA. E-mail: marina@mail.nih.gov

Several earlier studies have reported that clinical formulation of iron oxide-based imaging agent Resovist suppresses the function of immune cells in mice. (11-13) In one study, a single intravenous (IV) dose of Resovist resulted in an attenuation of delayed-type hypersensitivity reactions, lower levels of blood cytokines (IFN γ , IL6, and TNF α), a decrease in the number of tissue-infiltrating T-cells, and footpad swelling.(12) In other studies, Resovist suppressed T-cell-dependent antibody response.(11, 13) Recently, we reported that clinical IONP formulation Feraheme, approved for clinical use in the United States (U.S.) for the treatment of iron deficiency in patients with chronic kidney disease, suppresses proliferation and cytokine production by activated primary human T-cells in vitro via a mechanism involving mitochondrial damage.(14) IL-17 was one of the cytokines in which production by activated T-cells was reduced by Feraheme.(14) Since this cytokine is a known therapeutic target for the treatment of psoriasis,(6) we hypothesized that Feraheme might be useful in reducing psoriatic skin inflammation. Here we report the results of an in vivo study demonstrating that topical application of Feraheme inhibited the development of skin lesions in a mouse model of chemically induced psoriasis.

Materials and Methods

Reagents

Clinical-grade Feraheme (AMAG Pharmaceuticals, Waltham, MA) and Aldara cream were obtained from a National Institute of Health pharmacy. An over-the-counter formulation of hydrocortisone cream with aloe vera was purchased from a Giant pharmacy. BrdU kits for the assessment of T-cell proliferation were from Millipore (EMD Millipore, Billerica, MA). Vacutainer tubes for blood collection were from BD (Franklin Lakes, NJ). Fetal bovine serum (FBS), and penicillin/streptomycin were from HyClone (Logan, UT). RPMI-1640 was from Invitrogen/Life Technologies (Carlsbad, CA). Ficoll-Paque™ Premium was from GE Healthcare (Piscataway, NJ).

In vitro study design

Healthy volunteer blood was collected under the National Cancer Institute (NCI) at Frederick Protocol OH99-C-N046. Blood was drawn into

BD vacutainer tubes containing Li-heparin as the anticoagulant, then kept at room temperature and used within two hours after collection. Primary T-cells were isolated from the freshly drawn blood by the RosetteSep™ Human T-cell Enrichment Cocktail from STEMCELL Technologies, Inc., (Cambridge, MA) as described previously.(14) In our earlier experiments, we evaluated a range of concentrations from 0.01 to 3 mg/mL and found that despite a dose-response relationship, some of these concentrations are more optimal for cells from individual donors.(14) Therefore, we used the concentrations which show the optimal response in the cells from individual donors (14). The donors selected for the current in vitro study demonstrated maximum response at the concentration 1 mg/mL, therefore the data presented in this manuscript was generated using Feraheme at the concentration 1 mg/mL. The IL-17 in culture supernatants was detected using the Q-Plex™ kit (Quansys Biosciences, Logan, UT) as was also described earlier.(14) Data plotting and statistical analysis were performed using GraphPad Prism 7 software.

In vivo study design

All animal studies were approved by the Animal Care and Use Committee of NCI-Frederick and all animal care was in accordance with institutional guidelines. Female and male A/J mice at 6 weeks of age were obtained from Charles River. In one study, herein referred to as prophylactic mode, Feraheme was applied 2-hours after application of Aldara cream. PBS and hydrocortisone cream were used as the negative control and positive controls, respectively, and were applied at the same time as Feraheme. We verified that Aldara cream is quickly absorbed into the skin within the first 30 minutes of treatment. The treatment with Aldara and then, 2-hours later, Feraheme or controls were repeated for 5 days. The animals were euthanized on Day 6 of the study. In another study, herein referred to as therapeutic mode, first Aldara cream was administered daily for 5 days. After that, Feraheme or controls (PBS and hydrocortisone) were administered on a daily basis for the next 5 days. The animals were euthanized on Day 11 of this study. The age of mice was 8–10 weeks old. Each treatment groups included five males and five females. The fur on the back of animals was shaved to form 1 cm \times 1 cm treatment area.

The Aldara cream (62.5 mg per treatment area per animal), Feraheme (30 μ L of the stock formulation per treatment area per animal) and hydrocortisone cream (62.5 mg per treatment area per animal) were applied using a syringe without needle top. Animals were singly housed with e-collars (Kent Scientific) to avoid removal of the cream after application. The utility of Aldara cream to establish a model of psoriasis has been described before,^(15, 16) and verified by us prior to the initiation of studies described herein.

Pathology Evaluation

Skin was evaluated at necropsy for any gross lesions in the region of treatment and graded for erythema and flaking; erythema was scored

using a scoring table with red taints excluding animals in the Feraheme treatment group due to the brown and yellow color of Feraheme, which altered skin color and prevented accurate evaluation. Sections of skin were fixed in 10% formalin for 48–72 hours, embedded in paraffin, and 5- μ m sections were routinely processed for H&E staining. Dermatitis, hyperkeratosis, and acanthosis were scored independently on a scale from 0 to 4 as follows: 0- none; 1- minimal; 2- mild; 3- moderate; 4- marked. The cumulative score (erythema plus scaling plus thickening) served as a measure of the severity of inflammation and is reported herein as total psoriasis score (scale 0–12).

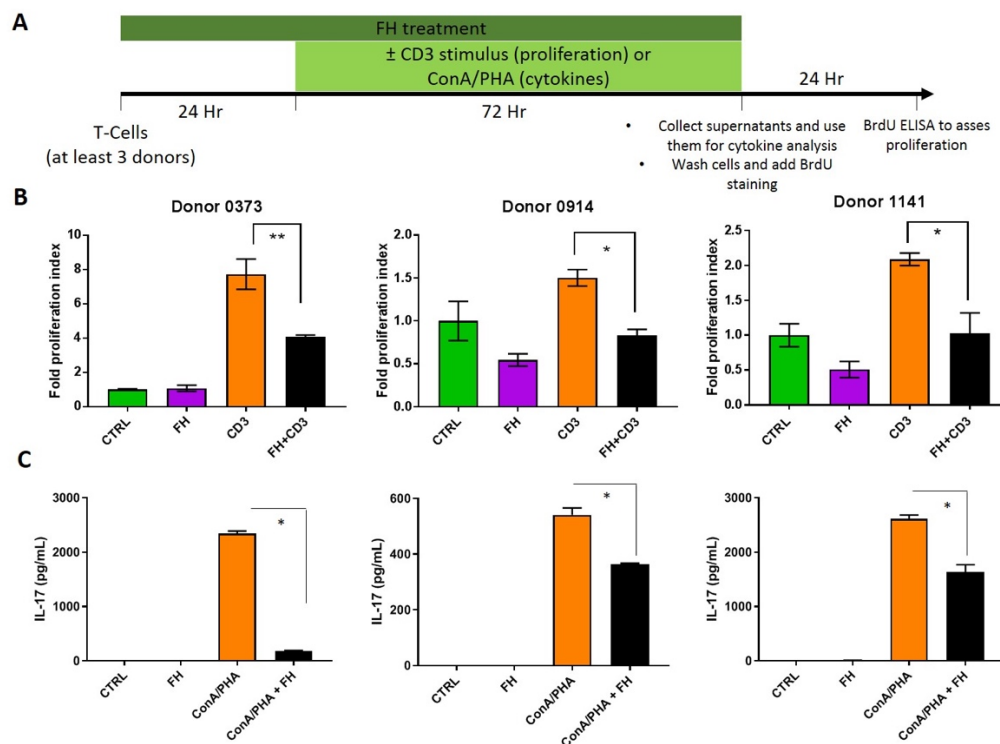


Figure 1. Feraheme inhibits human T-cells activation in vitro. Human T-cells were isolated from fresh whole blood of three donors. (A) experiment outline showing the timeline of treatments with a various agent (Feraheme, and agonists of T-cell activation, α -CD3 antibody or Con-A/PHA) (B) T-cell proliferation induced by α -CD3 antibody was measured by BrdU assay as described in materials and methods. (C) Supernatants were collected at the end of the study from Con-A/PHA treated cells and analyzed by ELISA to detect IL-17. Each bar shows the mean of a duplicate response and a standard deviation ($N=2$). CTRL = control (PBS); FH = Feraheme; CD3 = anti-CD3 antibody (also mentioned as α -CD3 in this legend); ConA/PHA = combination concanavalin A and phytohemagglutinin-M. The effect of a treatment was considered statistically significant with $p \leq 0.05$ (*) or $p \leq 0.01$ (**).

Immunohistochemistry

Immunohistochemistry to detect CD4 positive cells was performed on a BondRX autostainer (LeicaBiosystems) with the

following conditions: antigen retrieval with EDTA for 10 minutes, application of anti-CD4 antibody (eBioscience #13-9766) diluted at 1:250, secondary antibody Rabbit anti-rat mouse adsorbed (Vector Laboratories), and

LeicaBiosystems' Bond Polymer Refine Detection Kit (#DS9800) with omission of the Post Primary Reagent. H&E and CD4 IHC slides were digitized with an Aperio ScanScope

XT (Leica) at 200X. CD4 positive cells were quantified in the dermis using automated cell detection algorithms on Aperio whole slide images.

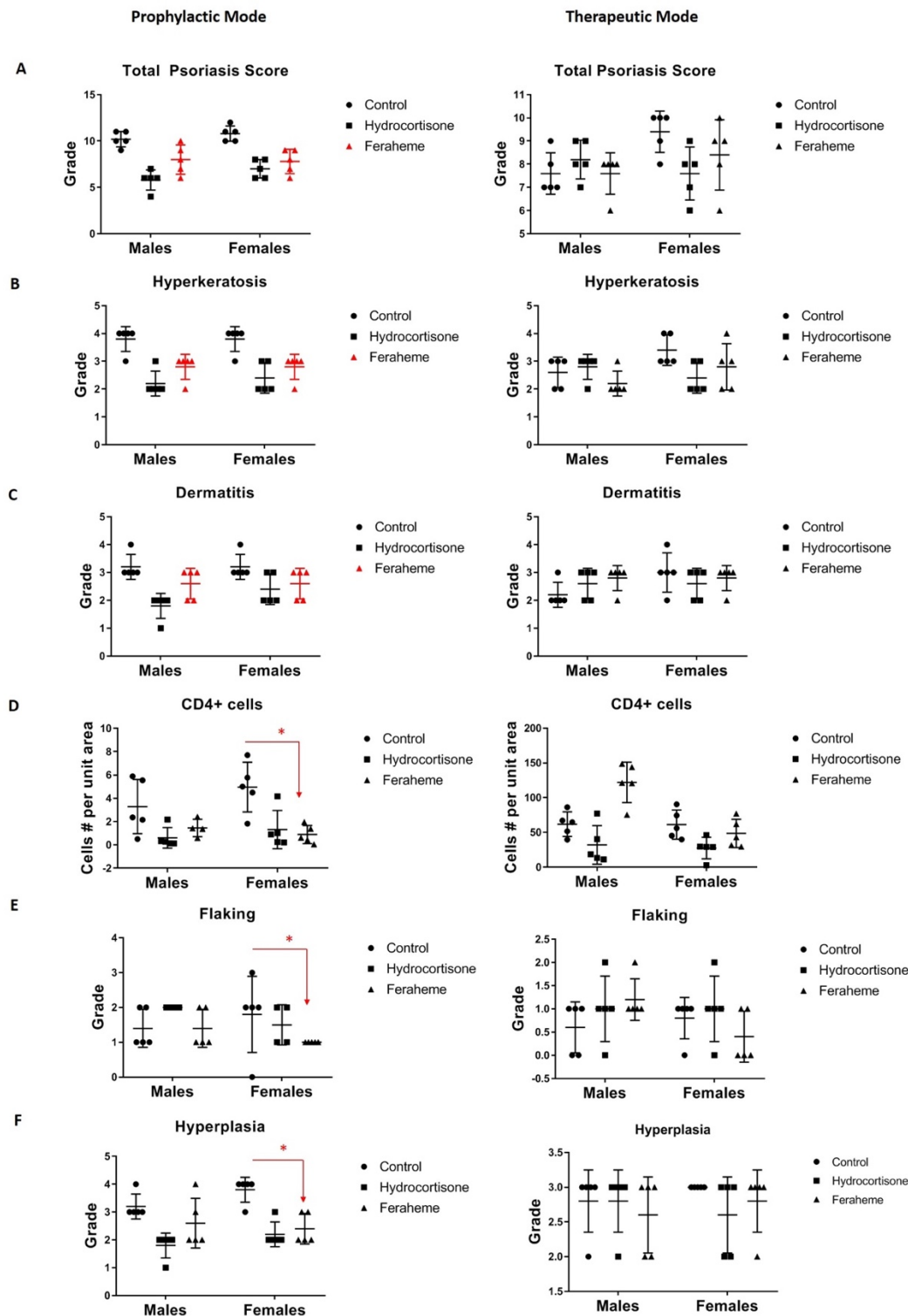


Figure 2. Effects of the topical Feraheme application on skin lesions in the mouse model of psoriasis in vivo. Animal studies were conducted in A/J mice as described in materials and methods. Each point on the graph represents the score from a single animal. The statistically significant ($p < 0.05$) difference is highlighted in red.

Positive controls for CD4 IHC included mouse spleen and stained appropriately. Negative controls included omission of the primary anti-CD4 antibody and replacement with nonspecific antibody from the same species and of the same isotype; negative isotype controls were performed on mouse spleen tissue and skin tissue and no staining was observed. All test slides, control slides, digital images, and IHC quantifications were reviewed by a board-certified veterinary pathologist (E.F.E).

Results

Immunosuppressive action of Feraheme against activated T-cells was verified *in vitro* using human primary T-cells. The cells were treated with Feraheme for 24 hours and then activated by either antibody specific to the T-cell receptor (CD3) or the co-treatment of mitogenic agents concanavalin A (con-A) and plant lectin, phytohemagglutinin M (PHA-M) (Figure 1A). Feraheme was kept in cultures until the end of treatments with agonists. The proliferative response and production of IL-17 were detected (Figure 1 B and C). Pre-treatment of cells with Feraheme reduced both α -CD3-triggered proliferation (Figure 1B) and IL-17 secretion induced by Con-A/PHA-M treatment (Figure 1C).

Both gross pathology and histology confirmed induction of psoriatic skin lesions by Aldara creme in the preliminary study (data not shown). In the prophylactic mode of the *in vivo* study, topical application of Feraheme resulted in a reduction in total psoriatic score, hyperkeratosis and dermatitis in both males and females (Figures 2 A-C). In the same study, a statistically significant ($p < 0.05$) reduction in skin flaking, hyperplasia and the number of CD4+ T-cells were observed in females only (Figures 2 D-E).

These effects were comparable to that of the positive control hydrocortisone (Figure 2, left column). In the therapeutic mode of the study, no such significant changes were observed in the Feraheme treated group (Figure 2, right column). However, a trend in reduction of the total score and hyperkeratosis in both genders, and CD4+ cell count and skin flaking in females, was observed (Figure 2, right column). Hydrocortisone treatment was more efficient in females than in males (Figure 2, right column).

An increase in the number of infiltrating T-cells was noticed in the Feraheme treatment group in males only (Figure 2 E). We also performed the analysis of dendritic cells using CD11c as a marker. However, the results of this analysis were inconclusive due to the low number of cells detected in all tissues (data not shown).

Discussion

The *in vitro* results of our study demonstrate immunosuppressive properties of Feraheme *in vitro* as evident by the inhibition of the proliferation and IL-17 production by mitogen-activated T-cells. This finding is in agreement with earlier studies.(14, 17) The observed effect is attributed to the release and accumulation of iron ions in the cells with subsequent changes in mitochondrial function (14). It is currently unknown whether the ion release occurs outside or inside the T-cells (14). The finding from the prophylactic mode of our *in vivo* study suggests that Feraheme can inhibit the development of skin inflammation triggered by the repeated administration of TLR7 agonist imiquimod, which is an active ingredient in the Aldara cream. In this arm of the study, Feraheme resulted in a statistically significant reduction in the total score, hyperkeratosis, and dermatitis in both males and females. It also reduced skin flaking, hyperplasia and the number of infiltrating CD4+ T-cells in females. These effects were comparable to that of hydrocortisone, specially formulated for topical applications. We speculate that the underlying mechanism involves the inhibition of T-cell function similar to that observed *in vitro* with purified T-cells. However, other mechanisms could not be ruled out. When, however, the inflammation was already established after the full week treatment with Aldara cream, the efficiency of the topically applied Feraheme was much weaker. In the therapeutic mode of the study, Feraheme did not induce dramatic changes in the inflammation score. The likely reason for the lower efficacy of Feraheme in this mode is a stronger inflammation which could not be overcome by the topical administration of the aqueous formulation. While most nanoparticles do not penetrate an intact skin barrier, there is sufficient evidence that the alteration of skin by UV-damage, burns and inflammation disrupts the intact skin barrier, and promotes nanoparticle penetration into the skin.(18) We did not study the degree

of Feraheme penetration into the skin damaged by the Aldara-triggered inflammation. However, we hypothesize that the penetration, even if occurred, was not optimal and could be improved by additional efforts to optimize Feraheme for the topical application. We, therefore, suggest that formulating Feraheme into a cream, a patch or a lotion may improve its efficacy in the therapeutic mode. Overall, the effects observed in this study suggest that Feraheme may find a potential utility in the treatment of the local areas of skin affected by the inflammation. Moreover, it may provide a unique treatment option in the area of nail psoriasis. The only currently available option for the topical treatment of nail psoriasis is the application of poly-urea-urethane (Nuvail formulation by Cipher) on the surface of affected nails to decrease the nails fragility.(19)

The incorporation of Feraheme particles into a polyurethane may be beneficial for the treatment of this form of psoriasis, and less painful than the injection of methotrexate into nail recently reported as an alternative to systemic use of anti-inflammatory therapies.(20) Our findings suggesting the potential utility for the topical application of Feraheme in psoriasis is in line with the earlier study demonstrating that topical application of Feraheme prevents the formation of bacterial biofilm on teeth and reduces tooth decay.(21) Future research focused on optimizing the Feraheme formulation for topical administration and testing it in other models, more closely resembling human psoriasis (e.g., pigs or genetically engineered mouse models) is needed to verify our findings.

Conclusion

The IONPs formulation Feraheme has a potential utility to decrease skin inflammation. It is desirable to further improve its efficacy, which would include reformulation of these particles into a cream, lotion or patch to increase skin penetration.

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Contributions of Authors:

A.S and E.C. performed in vitro experiments with human blood. S.D. and C.L.S. conducted animal work. E.F.E. analyzed histology and gross pathology data. D.B performed immunohistochemistry analysis of tissue sections. A.M.D. and K.L.D. performed gross lesions evaluation and processed tissues samples. M.A.D conceived and designed the study, analyzed data and wrote the manuscript. All authors contributed to the manuscript preparation.

Conflict of Interests

The authors declare no conflicts of interest. For signed statements, please contact the journal office: editor@precisionnanomedicine.com

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