HHS Public Access

Author manuscript

Nat Chem Biol. Author manuscript; available in PMC 2012 September 01.

Published in final edited form as:

Nat Chem Biol. ; 8(3): 311–317. doi:10.1038/nchembio.790.

Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase

Tracy L. Keller[1,*], **Davide Zocco**[1], **Mark S. Sundrud**[2], **Margaret Hendrick**[1], **Maja Edenius**[1], **Jina Yum**[3], **Yeon-Jin Kim**[3], **Hak-kyo Lee**[4], **Joseph F. Cortese**[8], **Dyann Wirth**[7,8], **John David Dignam**[6], **Anjana Rao**[2], **Chang-Yeol Yeo**[1,3,*], **Ralph Mazitschek**[5,8,*], and **Malcolm Whitman**[1,*]

[1]Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA 02115, USA

[2]Department of Pathology, Harvard Medical School and Immune Disease Institute, Children's Hospital, Boston, MA 02115, USA

[3]Department of Life Science, Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, 120-750, Korea

[4]Genomic Informatics Center, Hankyong National University, Ansung, Kyongi-do, 456-749, Korea

[5]Center for Systems Biology, Massachusetts General Hospital, Boston, MA 02114, USA

[6]Department of Biochemistry and Cancer Biology, University of Toledo College of Medicine, Toledo, Ohio 43614

[7]Harvard School of Public Health, Boston MA02115

[8]Infectious Disease Initiative, The Broad Institute of Harvard and MIT, 7 Cambridge Center, Cambridge, Massachusetts 02142, USA

Abstract

[*]Address Correspondence to: Tracy L Keller, PhD, Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA, 02115, USA, tkeller@hms.harvard.edu. Chang-Yeol Yeo, Department of Life Science, Division of Life and Pharmaceutical Sciences, Ewha, Womans University, Seoul, 120-750, Korea, cyeo@ewha.ac.kr. Ralph Mazitschek, Center for Systems Biology, Massachusetts General Hospital, Boston, MA 02114, USA., rmazitschek@mgh.harvard.edu. Malcolm Whitman, Department of Developmental Biology, Harvard School of Dental Medicine, Boston, MA, 02115, USA, mwhitman@hms.harvard.edu.

Author contributions: TLK and MW conceived of the idea for the study, designed, directed and interpreted experiments, performed the experiments central to target identification and enzymological characterization, and wrote the manuscript. RM conceived of, designed and synthesized critical chemical compounds for use in these studies, performed and interpreted experiments, and helped prepare the manuscript. CYY designed, directed and interpreted experiments performed in Korea, performed and interpreted the experiments involving proline rescue of HF-mediated effects on the AAR pathway and antifibrotic effects, and edited the manuscript. DZ performed and interpreted experiments central to EPRS knockdown and HF-sensitization, proline rescue of HF antifibrotic efects, and edited the manuscript. MSS planned and performed the immunology experiments, analyzed and interpreted data, and edited the manuscript. MH, ME, JY, YJK and HKL performed experiments, MH contributed Fig. 2c, and JFC carried out the experiment in Supplementary Figure 17. DW supervised the malaria experiments. JDD provided vital reagents, direction and technical expertise for the EPRS enzyme assays and edited the manuscript. AR designed, directed and interpreted the immunology experiments and edited the manuscript.

Competing Financial Interests

T.K., M.W., R.M., M.S., and A.R. have patent applications pending related to the potential therapeutic use of halofuginone and its derivatives.

Febrifugine, one of the fifty fundamental herbs of traditional Chinese medicine, has been characterized for its therapeutic activity whilst its molecular target has remained unknown. Febrifugine derivatives have been used to treat malaria, cancer, fibrosis, and inflammatory disease. We recently demonstrated that halofuginone (HF), a widely studied derivative of febrifugine, inhibits the development of Th17-driven autoimmunity in a mouse model of multiple sclerosis by activating the amino acid response pathway (AAR). Here we show that HF binds glutamyl-prolyl-tRNA synthetase (EPRS) inhibiting prolyl-tRNA synthetase activity; this inhibition is reversed by the addition of exogenous proline or EPRS. We further show that inhibition of EPRS underlies the broad bioactivities of this family of natural products. This work both explains the molecular mechanism of a promising family of therapeutics, and highlights the AAR pathway as an important drug target for promoting inflammatory resolution.

Plant bioactives are both historically important therapeutics and a valuable source of new drugs [1]. Approximately one third of the top 20 drugs on the market today are derived from natural products [2], the majority of these being derived from plants. The plant alkaloid febrifugine (FF) (**1**, Fig. 1) is the active ingredient found in the roots of Blue Evergreen Hydrangea, *Dichroa febrifuga* Lour [3]. During the roughly 2000 years of its therapeutic usage, the molecular mechanism of febrifugine in animal tissues has remained unknown. Historically recognized for its antiprotozoal activity, this herbal extract was used as an antimalarial remedy in traditional Chinese medicine. Halofuginone (HF) (**2**), a racemic halogenated derivative of febrifugine, was synthesized in a search of a less-toxic form of this plant bioactive [4]. In the last two decades, HF has gained attention, and progressed to phase 2, clinical trials for its potential as a therapeutic in cancer and fibrotic disease [56789]. Our previous work shows that HF potently inhibits the differentiation of pro-inflammatory Th17 cells, *in vitro* and *in vivo*, through activation of the nutrient-sensing amino acid response (AAR) pathway [10].

An important subset of natural product bioactives regulates highly conserved stress response pathways that are control points in cellular metabolism, such as the AMPK and TOR pathways, to confer therapeutic benefits in mammalian cells [2111213]. Less studied than the mTOR and AMPK pathways, the AAR pathway is conserved throughout eukaryotes as a cytoprotective response to nutrient limitation[14]. Amino acid restriction results in the accumulation of uncharged tRNAs that bind to and activate the protein kinase GCN2 (Supplementary Results, Supplementary Fig. 1). GCN2 activation results in its autophosphorylation, as well as phosphorylation of the translational initiation factor eIF2α, a shared component of multiple cellular stress response pathways that are collectively referred to as the integrated stress response[15] (ISR). Phosphorylation of eIF2α leads to a transient reduction in the initiation of mRNA cap-dependent translation, with a concomitant increase in the translation of a subset of mRNAs, including the mRNA encoding the transcription factor ATF4 [16]. Increased levels of ATF4 result in the activation of a set of genes that mediate the adaptation of cells to a stress environment, among them is the gene encoding the transcription factor C/EBP homologous protein (CHOP) [14]. In eukaryotic cells, the AAR and mTORC1 pathways both play a role in sensing nutrient status and activating cellular programs that mitigate restriction in the supply of environmental amino acids. These metabolic stress pathways are differentially activated, however, eliciting distinct sets of

transcriptional responses and biological effects [17,18,19]. In contrast to the AAR, the mTORC1 pathway is inhibited by amino acid restriction, and is thought to sense amino acid levels directly [20]. AAR pathway activation, on the other hand, is triggered by the intracellular accumulation of uncharged tRNAs that can result from insufficiency of any amino acid, or from the inhibition of any of the aminoacyl tRNA synthetases[15].

Metabolic sensor pathways that respond to changes in environmental levels of energy metabolites and nutrients, such as the AMPK, TOR, and AAR pathways, have become important drug targets in cancer [21], inflammatory diseases [22,23], and autoimmune disease [24,25]. Our recent work shows that HF inhibits the development of disease in a mouse model of Th17-driven multiple sclerosis[10] by activation of the AAR pathway. Despite extensive interest in the broad therapeutic potential of febrifugine-derived compounds, their development for clinical use has been hindered by the lack of knowledge regarding their molecular mechanism of action. We now show that febrifugine and its derivatives activate the AAR by directly inhibiting the prolyl tRNA synthetase activity of glutamyl-prolyl tRNA synthetase (EPRS). We show that febrifugine derivatives compete with proline for the prolyl tRNA synthetase (PRS) active site, causing the accumulation of uncharged $tRNA^{pro}$, and mimicking reduced cellular proline availability. We further show that addition of exogenous proline reverses a broad range of HF-induced cellular effects, indicating that EPRS-inhibition underlies the therapeutic activities of febrifugine derivatives.

RESULTS

HF limits proline-utilization during translation *in vitro*

We previously demonstrated that HF activates the AAR pathway in T cells[10], fibroblasts, and epithelial cells[26], and that AAR pathway activation selectively inhibits the development of pro-inflammatory Th17 cells[10]. In intact cells, amino acid incorporation into tRNA can be limited either by inhibiting the enzymes responsible for tRNA charging, or by decreasing the intracellular levels of amino acid through effects on transport, synthesis, or catabolism. To distinguish between these possibilities, we tested the effects of HF and febrifugine in a cell free *in vitro* translation system (rabbit reticulocyte lysate, RRL) where amino acid availability for translation can be controlled directly. Both HF and FF inhibited the translation of luciferase RNA in RRL; supplementation of RRL with excess amino acids established that only proline restores translation inhibited by HF (Fig. 2a). The activities of FF and of HF as antimalarials [27] and of HF as an inhibitor of Th17 cell differentiation [10] are enantiospecific. Only the 2R,3S isomer of HF (2), which matches the absolute configuration of FF, exhibits biological activity. Consistent with these observations, the 2S,3R isomer of HF also has no activity in the RRL assay (Fig. 2b). Additionally, HF-derivatives that lack activity in cell-based assays (MAZ1310 (3) and MAZ1442 (4) have no activity in the RRL assay (Fig. 2b). These data suggest that the ability of FF and HF to inhibit proline utilization is functionally linked to the bioactivities of these compounds.

To confirm that HF/FF-inhibition specifically targets the utilization of proline in translation, we examined how these compounds affected the translation of a pair of glutamate-rich (24 of 118 amino acids), synthetic polypeptides that differ only with respect to the presence of prolyl residues. We synthesized DNAs encoding two epitope-tagged polypeptides. The first

DNA, designated Propep, encodes a proline dipeptide, and the second DNA, called Nopropep, encodes a proline-free peptide. HF and FF prevent translation of Propep, but have no effect on the translation of Nopropep (Fig. 2c), establishing that proline utilization is the sole target for the inhibitory effect of these compounds on translation in RRL. Since the synthetic peptides are rich in glutamate, the unimpaired translation of Nopropep in the presence of HF/FF argues strongly against the inhibition of the glutamyl-tRNA synthetase activity of EPRS under these conditions.

Next, we directly examined the effects of HF on prolyl-tRNA charging in the RRL system. RRL were supplemented with ^{14}C-Pro or ^{35}S-Met in the presence or absence of HF, and total tRNA was isolated (Fig. 2d). HF inhibited the incorporation of ^{14}C-Pro, but not ^{35}S-Met, into tRNA at doses comparable to those necessary to inhibit translation, indicating that inhibition of amino acid utilization by HF is specific for proline. Moreover, addition of EPRS purified from rat liver to RRL substantially reduces the sensitivity of in vitro translation by RRL to HF inhibition (Supplementary Fig. 2), establishing that EPRS is the critical target for inhibition of translation by these compounds in RRL.

HF is a competitive inhibitor of purified ProRS

To directly examine the mechanism of HF-mediated inhibition of EPRS, we tested the effect of HF on $tRNA^{Pro}$ charging using the ectopically expressed and purified prolyl tRNA synthetase domain of EPRS (ProRS). Preliminary analysis of the inhibition kinetics indicated that the apparent K_i of HF for ProRS was similar to the concentration of ProRS enzyme in the tRNA charging assay. In this circumstance, a substantial fraction of the total inhibitor is bound to the enzyme during the assay, and therefore Michaelis-Menten analysis is not applicable. Alternative analytic approaches for tight binding inhibitors have been described[28], and are summarized in Copeland [29]. In this approach the modality of inhibition can be determined from the relationship between the IC_{50} of the inhibitor and varying substrate concentrations. For a tight binding competitive inhibitor, this relationship is given by: $IC_{50}= K_i (1+[S]/K_m)+0.5*E_t$, where E_t is the concentration of active enzyme in the reaction (determined by the method described by Copeland [29], Supplementary Fig. 4). Competitive inhibition is reflected in a linear relationship (with positive slope) between IC_{50} and [S]; the K_i can be derived from the slope of the plot of IC_{50} vs. $[S]/K_m$. This relationship is plotted for HF as inhibitor, and proline as substrate in Fig. 2A, using IC_{50} values determined at proline concentrations between 20 and 480 nM (Supplementary Fig. 5), and $K_{m(Pro)}$ determined as shown in Supplementary Fig. 4. These data fit the model predicted for a tight binding inhibitor, acting competitively with proline. The slope of the resulting line yields a K_i for HF of 18.3 nM+/− 0.5. The HF derivative MAZ1310, had no inhibitory effect on ProRS activity (Supplementary Fig. 3), consistent with its lack of activity in RRL (Fig. 2b) and biological assays [10].

To demonstrate direct binding of HF to ProRS, we have used a [^{3}H]-labeled derivative of HF ([^{3}H]-HFol, [^{3}H]-**5**) that has been developed in our laboratory and which we named halofuginol (HFol) (**5**). HFol, in contrast to the previously reported diastereomer derived from febrifugine (**6**)[30], retains potency similar to the parent compound for the inhibition of EPRS (Supplementary Fig. 7). [^{3}H]-HFol bound directly to immobilized ProRS, this binding

was competed by HF and FF, but not by MAZ1310 (Fig. 3a), and is competed by proline (Fig. 3b). If HF indeed acts in cells by inhibition of EPRS activity, we reasoned that reducing EPRS levels would sensitize cells to the action of HF. We therefore used siRNA-mediated knockdown to reduce EPRS levels in lung fibroblasts, which have high endogenous levels of EPRS and are relatively resistant to the effects of HF (in comparison to MEFs). Reduction of EPRS levels significantly sensitized these cells to AAR activation following HF treatment, as indicated by induction of GCN2 autophosphorylation (Fig. 3c) and induction of the AAR-response genes CHOP (Fig. 3d). Similar sensitization of induction was seen for additional AAR-response genes asparagine synthetase and alanine aminotransferase (Supplementary Fig. 8). These data establish that EPRS is the critical target through which HF activates the AAR pathway.

HF binds to ProRS in an ATP-dependent manner

The competition kinetics between HF and proline for ProRS binding strongly predict that HFol binds directly to the enzyme active site of ProRS, and competition for [^{3}H]-HFol to immobilized ProRS by proline is consistent with this hypothesis (Fig. 3b). 5-O-[N-(L-prolyl)-sulfamoyl]adenosine (ProSAd, **7**), a sulfamoyl analog of the charging reaction intermediate prolyl adenylate [31], very potently (10 nM) inhibited the binding of [^{3}H]-HFol to ProRS, supporting the interpretation that HF binds within the catalytic pocket (Fig. 4a). A higher concentration (50 nM) of an alanyl adenlylate analog, AlaSAd, partially inhibited [^{3}H]-HFol binding to ProRS, possibly reflecting the ability of ProRS to accomodate non-cognate aminoacyl adenylates such as alanine in the enzyme active site [32].

We next examined the effect of ATP on [^{3}H]-HFol binding to ProRS. ATP was essential for [^{3}H]-HFol binding (Fig. 4b), indicating that the mode of HF binding to the catalytic pocket is distinct from that of the reaction intermediate prolyl adenylate, since ATP and prolyl adenylate are expected to be mutually exclusive in the catalytic pocket [33]. The adenylylation reaction catalyzed by tRNA synthetases occurs through hydrolysis of the high-energy phosphate bond between the alpha and beta phosphates [34]. AMP-CPP, an ATP analog that is non-hydrolyzable at the alpha-beta phosphate linkage, supports [^{3}H]-HFol binding in place of ATP, indicating that hydrolysis of the alpha-beta phosphate does not have a role in ATP-stimulated [^{3}H]-HFol binding (Fig. 4b). Similar results with ATPγS suggest that utilization of the γ phosphate of ATP is also unlikely to be important for [^{3}H]-HFol binding to ProRS (Fig. 4b). AMP at 2mM does not detectably facilitate [^{3}H]-HFol binding, however, indicating that the triphosphate component of ATP is important for HF interaction with ProRS. These data indicate that HF/HFol inhibits ProRS by binding to a portion of the catalytic site that includes, at least in part, the proline-binding pocket. The ATP requirement for HF binding suggests that HF is not acting as simply to mimic prolyl adenylate in the catalytic site, but rather requires an ATP-induced conformational change in the enzyme that enables inhibitor binding. Although we have not ruled out the possibility that ATP binds allosterically to ProRS somewhere outside the catalytic pocket, structural analyses of *T.thermophilus* ProRS and other tRNA synthetases provide no support or precedent for this notion [33].

Proline addition reverses the biological effects of HF

The ability of proline to rescue the effects of HF on translation *in vitro* (Fig. 2), and the fact that HF inhibits competitively with respect to proline in the purified enzyme assay (Fig. 3) suggested that proline supplementation in intact cells might specifically reverse the effects of HF. We therefore examined whether proline supplementation antagonized HF-activation of the AAR pathway in intact cells. Consistent with this idea, stimulation of GCN2 phosphorylation by HF/FF in fibroblasts was abrogated by the addition of 2 mM proline (Fig 5a). Addition of proline also prevented HF-dependent activation of AAR pathway components downstream of GCN2 phosphorylation, including CHOP induction (Fig. 5b) and eIF2α phosphorylation (Supplementary Fig. 9), indicating that proline utilization is the principal target for HF action in intact cells as it is in RRL. As expected, these downstream AAR responses to HF were dramatically reduced in GCN2$^{-/-}$ fibroblasts ((Fig 5b). The mTOR pathway, like the AAR, acts as a cellular sensor for amino acid availability, but, unlike the AAR, mTOR signaling is not blocked by inhibition of tRNA synthetase activity. HF-treatment of T cells and fibroblasts activates the AAR pathway without concomitant inhibition of mTORC1 signaling (Supplementary Fig. 10). We conclude that HF is not exerting a direct effect on mTORC1 signaling, consistent with a model in which HF acts to limit tRNA charging rather than altering amino acid levels in intact cells. To exclude the possibility that proline blocks the action of HF by preventing its uptake or accumulation in intact cells, we used an anti-HF antibody in an ELISA assay (described in Supplementary Methods) to directly measure intracellular HF levels in the presence or absence of excess proline. The intracellular accumulation of HF was not affected by proline addition (Supplementary Fig. 11), supporting our interpretation that proline reverses the effect of HF on AAR activation by enhancing intracellular proline utilization.

We previously have shown that HF selectively inhibits Th17 cell differentiation, and that media-supplementation with mixtures of amino acids reverses these effects[10]. Comparison of the effects of non-essential versus essential amino acid pools established that only non-essential amino acids restore Th17 cell differentiation, or prevent eIF2α phosphorylation in the presence of 10 nM HF (Fig. 6a, Supplementary Fig. 14). Testing of individual non-essential amino acids established that only proline rescued Th17 differentiation in HF-treated T cells (Fig. 6a). Consistent with the competitive inhibition of proline utilization by HF seen with purified enzyme (Fig. 3), increasing concentration of proline increased the IC_{50} for HF inhibition of Th17 differentiation (Supplementary Fig. 12) We also tested whether a structurally unrelated tRNA synthetase inhibitor, the threonyl tRNA synthetase inhibitor borrelidin [28], could recapitulate the effects of HF on Th17 differentiation. Borrelidin inhibited Th17 cell differentiation with a selectivity identical to that which we have previously reported for HF [10](Supplementary Fig. 13). Like HF, the effects of borrelidin on Th17 differentiation were reversed by addition of an excess of the cognate amino acid, in this case threonine (Fig. 6b). These results demonstrate that either of two structurally unrelated tRNA synthetase inhibitors exerts a highly selective effect on effector T cell differentiation.

The ability of HF to inhibit tissue remodeling *in vivo* is evidenced by its potent suppression of tissue fibrosis [57] and tumor progression [6]. As an antifibrotic agent, HF inhibits the

overproduction and deposition of extracellular matrix (ECM) components, such as Type I collagen and fibronectin, both *in vivo* and in cultured fibroblasts. We found that HF inhibits, and proline supplementation restores, mRNA levels of *Col1A1*, *Col1A2*, and *S100A4* in mouse embryo fibroblasts (MEFs) (Fig. 6c). S100A4, which is produced and secreted from tumor-activated stromal cells, is implicated in fibrosis and tumor metastasis, as well as in tissue invasion by synoviocytes during rheumatoid arthritis[3536]. Expression of mRNA encoding the AAR-responsive factor CHOP was stimulated by HF, concomitant with inhibition of the expression of ECM genes. Consistent with prior reports [57], HF-treatment of cells for 24 hours dramatically inhibited the production of secreted Type I procollagen and the production of fibronectin, at doses that did not significantly change ^{35}S-methionine incorporation into total protein, (Fig. 6d). HF-mediated inhibition of these ECM proteins, like the HF-induced modulation of gene transcription, was reversed by the addition of 2 mM proline to the media (Supplementary Fig. 15).

In addition to the proline rescue of HF-mediated effects on AAR pathway activation, HF-mediated inhibition of Th17 cell differentiation, and HF-mediated antifibrotic effects, HF-inhibition of *P. falciparum* growth in red blood cells is reversed by the addition of proline. The addition of 5x proline to the amino acid containing media of red blood cells that are infected with the Dd2 strain of *P. falciparum* increased the effective IC_{50} of HF and febrifugine by roughly 7 and 5-fold, respectively, but did not affect the IC_{50} of an unrelated antimalarial amodiaquine (Supplementary Fig. 17). Whereas further work is necessary to establish whether HF acts by targeting *P.falciparum* prolyl-tRNA synthetase activity, these data indicate that HF does act specifically on the utilization of proline during *P.falciparum* growth, and indicates that this activity accounts for the antimalarial effects of HF.

DISCUSSION

We have shown that febrifugine-family compounds act competitively with proline as potent inhibitors of the tRNA charging activity of EPRS, and we propose that this constitutes a single primary mechanism for their reported biological activities. Although EPRS is an essential component of the protein synthetic-machinery, HF-inhibition of prolyl-tRNA charging evokes a highly specific cluster of biological effects, at doses that do not have a global effect on protein synthesis. These observations are consistent with HF-activation of a metabolic sensor, in this case AAR pathway activation, rather than with an HF-mediated blockade of proline incorporation into cellular protein. In fibroblasts, for example, HF enhances expression of the AAR protein CHOP, while selectively inhibiting production of ECM proteins – fibronectin, which is not rich in proline, as well as proline-rich collagen. Systematic analysis of mRNA-specific translational regulation during cytoprotective responses to different stresses will be essential to understand how stress pathway activation yields specific biological responses. It is significant to note that even though GCN2 phosphorylation is a hallmark of AAR signaling and constitutes the known signal transducer of the canonical AAR pathway, cells lacking GCN2 nonetheless respond to amino acid limitation by making specific changes in gene expression and mRNA splicing[3718]. These observations clearly indicate that cellular amino acid restriction or the chemical inhibition of tRNA charging can activate a metabolic sensor pathway, which includes primary components that have yet to be described.

It is clear that a key component of HF's therapeutic impact stems from activation of the AAR pathway, but whether or not any of the observed effects of HF can be attributed to EPRS-induced changes that are unique to proline metabolism, or to non-canonical activities of EPRS are interesting open questions. In particular, EPRS has been shown to participate in the interferon-γ-activated inhibitor of translation (GAIT) complex, in myeloid cells, to suppress expression of a posttranscriptional regulon of proinflammatory genes [38]. Whether HF might impact this non-canonical function of EPRS merits investigation.

HF and its family members act as proline restriction mimetics, by binding to EPRS and blocking prolyl-tRNA charging. In doing so, they act as molecular probes that highlight the AAR pathway's contribution to the potent, but unexplained, cellular and organismal benefits of caloric and nutrient restriction. Dietary restriction (DR) with adequate nutrition is the most robust intervention known for the systemic prevention of age-related diseases in animals and extension of lifespan across species [3940,41]. Amino acid restriction is a subset of DR, but many of the cellular changes that correlate with tissue and organism longevity can be reproduced by amino acid limitation alone [42,43]. DR has numerous benefits for the aging organism, which include a reduction of inflammation and oxidative stress. Inflammation is a common denominator in age-associated pathologies, such as metabolic syndrome, cardiovascular disease, cancer, and insulin resistant diabetes [39]. Dietary restriction of amino acids alone has been shown to increase insulin sensitivity in mice [43]. The molecular basis for DR's healthspan-increasing benefits is poorly understood, but metabolic sensor pathways that trigger organismal adaptation to diminished nutrient supply are central to this process [40,41]. We show here that, in the absence of true nutritional deficit, febrifugine-derived compounds block EPRS activity to send intracellular signals indicative of proline limitation, activating the AAR pathway and thereby reproducing a key component of the beneficial effects of caloric restriction.

Changes in the level of intracellular amino acids and the signaling pathways that detect these changes have an emergent role in the maintenance of immune and tissue homeostasis [2212]. Amino acid levels have been shown to regulate the inflammatory vs. tolerogenic state of cellular populations that are key to this process, in cell types such as plasmacytoid dendritic cells (pDC's), polymorphonuclear leukocytes (PMN) [44], and macrophages [45,46]. Amino acid-degrading enzymes such as arginase and indoleamine 2,3 dioxygenase (IDO) that catabolize L-arginine and L-tryptophan, respectively, exemplify metabolic sensor pathway adaptation for cellular regulation of the inflammatory/tolerogenic phenotype [47]. Amino acid-degradation has two functional outputs – the production or destruction of immunologically active amino acid metabolites, and the detectable nutrient limitation produced by amino acid-catabolism accompanied by a metabolic stress pathway response. In the case of L-arginine, which is itself a substrate for nitric oxide synthase (NOS), arginine degradation both depletes the substrate pool for pro-inflammatory nitric oxide (NO) and activates the AAR pathway [23]. It is now recognized that microorganisms can exploit this pathway to enhance their survival. Pathogens upregulate their arginases to deplete host arginine pools, suppressing the host innate immune response and inhibiting T cell proliferation [11]. The immunosuppressive enzyme IDO plays a complex regulatory role in a number of physiological and pathophysiological settings that include maternal-fetal tolerance, infection, allergy, autoimmune disease, and transplantation by mediating tryptophan

catabolism. Several immunoregulatory cells populations utilize the induction of IDO expression to activate anti-inflammatory or tolerogenic programs [11]. IDO-expressing pDCs inhibit effector T cell responses, activate Tregs, and can attenuate pro-inflammatory responses that manifest in chronic disease syndromes [23]. Critical mediators of peripheral tolerance, Tregs have been shown to induce the expression of enzymes that degrade at least five different amino acids in skin grafts and in DCs. Inhibition of the mTOR pathway consequent to amino acid limitation reinforced the tolerogenic signaling loop by induction of the Treg-specific transcription factor forkhead box P3 (FoxP3) [12]. To the emerging story of the adaptation of intracellular amino acid regulation for immune homeostasis, we recently added the observation that differentiation of pro-inflammatory Th17 cells is potently inhibited by activation of the AAR pathway [10]. We now show that HF suppresses pathologic inflammation by its action as a primary inhibitor of EPRS and an amino acid-restriction mimetic. These observations provide an additional example of a natural product therapeutic that acts by leveraging a metabolic stress response, in this case the amino acid response (AAR), for modulation of immune and inflammatory responses.

The therapeutic application of amino acid restriction-mimetics to a disease tissue environment provides a novel means of reprogramming key immunoregulatory cells to a tolerogenic/anti-inflammatory phenotype. Here we show that HF inhibits the prolyl-tRNA synthetase activity of EPRS and thereby activates the AAR to elicit antimicrobial and immunoregulatory responses in tissues. In light of the prominent role of inflammatory disease in age-related pathologies, amino acid-restriction mimetics may provide vital molecular probes for the study of health- and lifespan extension in humans. Our new understanding of the molecular mechanism of action of febrifugine and its derivatives, combined with the ability of these compounds to selectively inhibit Th17 cell differentiation *in vivo*, makes possible the design of a new family of therapeutic compounds with improved pharmacological properties for the treatment of a variety of serious illnesses, including multiple sclerosis, scleroderma, and rheumatoid arthritis.

METHODS

Statistical Analysis

Statistical analysis and graphing were performed using Prism Software (Graphpad).

cDNAs

Full length human EPRS was provided by Paul Fox (Cleveland Clinic). The 6xHis tagged ProRS cDNA was provided by Karin Musier-Forsyth (Ohio State).

In vitro translation assays

Effects of HF, HF derivatives, and added amino acids on cell free protein translation were assayed in rabbit reticulocyte lysate (RRL) according to the manufacturer's instructions (Promega), with the exception that the standard amino acid mix provided was diluted 5-fold for assays. In some experiments RRL was preincubated at 30 degrees with another RNA (hALK4) to deplete endogenous charged tRNAs. Luciferase activity was measured using a luciferase assay kit (Promega). To assay tRNA charging in RRL, 25 μl RRL was incubated

with 1 μCi ^{14}C Pro or ^{35}S Met, 1 μg added bovine tRNA, and 2 μM puromycin (to prevent utilization of charged tRNAs for protein translation). After 20 minutes, total tRNA was extracted using a miRVANA small RNA isolation kit (Ambion), and incorporated radioactivity measured in a scintillation counter. Sequence of Propep and Nopropep peptides is provided in Supplementary Methods.

siRNA knockdowns

For knockdown of EPRS, human lung fibroblast cells (IMR90) were transfected with siRNAs against EPRS and control (ON-TARGETplus SMART pool L-008245-00-0005 for human EPRS and ON-TARGETplus non-targeting Pool D-001810-10-05 as control; Thermo Scientific) using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen) described in detail in Supplementary Methods. For qPCR experiments, cells were left for 2 hours in serum-free media and then treated with HF for 6 hours in serum-free media, before harvesting RNA with Trizol gene expression quantitated by quantitative RT-PCR using the Roche UPR Light-Cycler system[48]. For Western blot experiments, cells were left 2 hours in media without serum and treated with HF for another 2 hours (in serum-free media) before harvesting the protein lysates with RIPA buffer plus protease and phosphatase inhibitors. SiRNA sequences are provided in Supplementary Methods.

Assay of ProRS activity

The prolyl tRNA synthetase domain of human EPRS (ProRS) was expressed in E.coli with a 6-his tag and purified as described[31]. Purified enzyme was visualized as a single band by Laemmli gel electrophoresis/Coomassie staining. Enzymatic activity was assayed using incorporation of ^{3}H Pro (94 Ci/mmol, Perkin-Elmer) into the tRNA fraction essentially as described [49], except that the charged tRNA fraction was isolated by rapid batchwise binding to Mono Q sepharose [50] (GE Healthcare) (described in Supplementary Methods) and quantitated by liquid scintillation counting. In preliminary experiments, the assay was established to be linear over the time (6′) and conditions used (30 degrees, 2 mM ATP, 1 μg/μl total tRNA purified from rabbit liver). For all kinetic assays, the concentration of active enzyme in the reaction (determined by inhibitor titration as described in Copeland[29]) was 40 nM. Similar inhibition by HF was seen using the human ProRS domain purified from bacteria and full length EPRS purified from rat liver [49].

Assay of HF binding to ProRS

To examine HF binding to ProRS, ^{3}H HF was synthesized by American Radiochemicals (St.Louis) by reduction of halofuginone with $NaB^{3}H_4$ at the ketone (Fig. 1). 100 nM ^{3}H HF was incubated with ProRS immobilized on Ni-NTA beads (Pierce) in 50 mM Tris pH 7.5, 5 mM MgCl2, 20 mM Imidazole, 1 mM DTT, 10% glycerol, and nucleotides, proline, or HF derivatives at room temperature for 10 minutes as indicated. After incubation, beads were washed twice with ice-cold wash buffer (50 mM Tris 7.5; 20 mM Imidazole; 1 mM DTT, 20% Glycerol). ^{3}H HF bound after washing was measured by liquid scintillation counting. Ni-NTA beads incubated with E.Coli extracts lacking tagged ProRS were used as a background reference in all experiments.

Proline rescue of HF in T cells

To study proline rescue of HF effects in T-cells, primary murine CD4+ CD25− T cells were isolated from spleens and peripheral lymph nodes of wild-type C57B/6 mice (Jackson laboratories), and T cell activation, differentiation, treatment with HF, and amino acid supplementation was performed as described[10]. In some experiments, T cell cultures were supplemented with excess individual amino acids as described above and previously[10]. All FACS data was acquired on a FACSCalibur flow cytometer (BD Pharmingen) and analyzed using FlowJo software (Treestar, Inc.). Protocols and antibodies used for FACS staining of T cells have been described previously[10]. Briefly, Th17 differentiation (percentage of IL-17+ IFNg− cells) was determined on day 4-cultured T cells following restimulation with phorbol myristate acetate (PMA; 10 nM) and ionomycin (1 mM), in the presence of brefeldin A (10 mg/ml) for 4–5 hours. Cytokine expression in restimulated cells was determined by intracellular cytokine staining as described[10]. In some experiments cytokine production by cells activated in non-polarizing conditions (ThN), Th1, or Th2 conditions was determined on day 5 following restimulation and intracellular cytokine staining as above. Inducible T regulatory (iTreg) differentiation was assessed by CD25 and Foxp3 upregulation on day 3-post activation using a commercially available Foxp3 intracellular staining kit (eBioscience).

Proline rescue of HF in Primary Fibroblasts

For assays of HF and proline effects on fibroblasts, MEFs were grown in 10% FCS/DMEM. For studies of TGFs signaling, ^{35}S methionine incorporation into total protein, and collagen production, cells were shifted into 0.2% FCS/DMEM 24 hours before the addition of HF or proline. For examination of protein expression and phosphorylation, cells were lysed in RIPA with 5 mM EDTA, 1x PhosStop (Roche), and 1x Complete protease inhibitor mix (Roche), and assayed by Western blot as described above. For analysis of gene expression, cells were lysed in Trizol (Invitrogen) and gene expression quantitated by quantitative RT-PCR using the Roche UPR Light-Cycler system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the reviewers for pointing out the need for tight binding inhibitor analysis, Dr. Robert Copeland (Epizyme) for advice on the execution of tight binding analysis, Winston Kuo (Harvard LITT) for assistance with the establishment of Q-PCR assays, and Drs. Chris Walsh (Harvard), Tom Roberts (DFCI), and Seddon Thomas (NIEHS) for their valuable comments on the manuscript. This work was supported by NIH GM089885 and a Harvard Technology Accelerator Award (to MW), PJ00812701 and PJ008196, The Next Generation BioGreen 21 Program, Rural Development Administration, Republic of Korea (to CYY and HKL); NIH AI40127 and JDRF 17-2010-421 (to A.R.).

Literature Cited

1. Clardy J, Walsh C. Lessons from natural molecules. Nature. 2004; 432:829–37. [PubMed: 15602548]
2. Howitz KT, Sinclair DA. Xenohormesis: sensing the chemical cues of other species. Cell. 2008; 133:387–91. [PubMed: 18455976]

3. Coatney GR, Cooper WC, Culwell WB, White WC, Imboden CA Jr. Studies in human malaria. XXV. Trial of febrifugine, an alkaloid obtained from Dichroa febrifuga lour., against the Chesson strain of Plasmodium vivax. J Natl Malar Soc. 1950; 9:183–6. [PubMed: 15422372]
4. Ryley JF, Betts MJ. Chemotherapy of chicken coccidiosis. AdvPharmacol Chemother. 1973; 11:221–93.
5. Pines M, Nagler A. Halofuginone: a novel antifibrotic therapy. Gen Pharmacol. 1998; 30:445–50. [PubMed: 9522159]
6. Elkin M, et al. Inhibition of bladder carcinoma angiogenesis, stromal support, and tumor growth by halofuginone. Cancer Res. 1999; 59:4111–8. [PubMed: 10463616]
7. McGaha T, et al. Effect of halofuginone on the development of tight skin (TSK) syndrome. Autoimmunity. 2002; 35:277–82. [PubMed: 12482197]
8. Pines M, Snyder D, Yarkoni S, Nagler A. Halofuginone to treat fibrosis in chronic graft-versus-host disease and scleroderma. Biol Blood Marrow Transplant. 2003; 9:417–25. [PubMed: 12869955]
9. Koon HB, et al. Phase II AIDS Malignancy Consortium trial of topical halofuginone in AIDS-related Kaposi sarcoma. J Acquir Immune Defic Syndr. 2010; 56:64–8. [PubMed: 21068672]
10. Sundrud MS, et al. Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. Science. 2009; 324:1334–8. [PubMed: 19498172]
11. Grohmann U, Bronte V. Control of immune response by amino acid metabolism. Immunol Rev. 2010; 236:243–64. [PubMed: 20636821]
12. Cobbold SP, et al. Infectious tolerance via the consumption of essential amino acids and mTOR signaling. Proc Natl Acad Sci U S A. 2009; 106:12055–60. [PubMed: 19567830]
13. Finlay D, Cantrell DA. Metabolism, migration and memory in cytotoxic T cells. Nat RevImmunol. 2011; 11:109–17.
14. Kilberg MS, Pan YX, Chen H, Leung-Pineda V. Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. Annu Rev Nutr. 2005; 25:59–85. [PubMed: 16011459]
15. Harding HP, et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell. 2003; 11:619–33. [PubMed: 12667446]
16. Harding HP, et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell. 2000; 6:1099–108. [PubMed: 11106749]
17. Peng T, Golub TR, Sabatini DM. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. Mol Cell Biol. 2002; 22:5575–84. [PubMed: 12101249]
18. Deval C, et al. Amino acid limitation regulates the expressionof genes involved in several specific biological processes through GCN2-dependent and GCN2-independent pathways. Febs J. 2009; 276:707–18. [PubMed: 19120448]
19. Palii SS, et al. Specificity of amino acid regulated gene expression: analysis of genes subjected to either complete or single amino acid deprivation. Amino Acids. 2008
20. Sancak Y, et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008; 320:1496–501. [PubMed: 18497260]
21. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011; 12:21–35. [PubMed: 21157483]
22. Powell JD, Delgoffe GM. The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism. Immunity. 2010; 33:301–11. [PubMed: 20870173]
23. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol. 2008; 8:923–34. [PubMed: 19029988]
24. Esposito M, et al. Rapamycin inhibits relapsing experimental autoimmune encephalomyelitis by both effector and regulatory T cells modulation. J Neuroimmunol. 2010; 220:52–63. [PubMed: 20149931]
25. Nath N, et al. Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis. J Immunol. 2009; 182:8005–14. [PubMed: 19494326]

26. Kamberov YG, Kim J, Mazitschek R, Kuo WP, Whitman M. Microarray profiling reveals the integrated stress response is activated by halofuginone in mammary epithelial cells. BMC Res Notes. 4:381. [PubMed: 21974968]
27. Kobayashi S, et al. Catalytic Asymmetric Synthesis of Antimalarial Alkaloids Febrifugine and Isofebrifugine and Their Biological Activity. J Org Chem. 1999; 64:6833–6841. [PubMed: 11674693]
28. Ruan B, et al. A unique hydrophobic cluster near the active site contributes to differences in borrelidin inhibition among threonyl-tRNA synthetases. J Biol Chem. 2005; 280:571–7. [PubMed: 15507440]
29. Copeland RA. Evaluation of enzyme inhibitors in drug discovery. A guide for medicinal chemists and pharmacologists. Methods Biochem Anal. 2005; 46:1–265. [PubMed: 16350889]
30. Kikuchi H, et al. Potent antimalarial febrifugine analogues against the plasmodium malaria parasite. J Med Chem. 2002; 45:2563–70. [PubMed: 12036365]
31. Heacock D, Forsyth C, Shiba K, Musier-Forsyth K. Synthesis and Aminoacyl-tRNA Synthetase Inhibitory Activity of Prolyl Adenylate Analogs. Bioorganic Chemistry. 1996; 24
32. Splan KE, Ignatov ME, Musier-Forsyth K. Transfer RNA modulates the editing mechanism used by class II prolyl-tRNA synthetase. J Biol Chem. 2008; 283:7128–34. [PubMed: 18180290]
33. Yaremchuk A, Tukalo M, Grotli M, Cusack S. A succession of substrate induced conformational changes ensures the amino acid specificity of Thermus thermophilus prolyl-tRNA synthetase: comparison with histidyl-tRNA synthetase. J Mol Biol. 2001; 309:989–1002. [PubMed: 11399074]
34. Ibba M, Soll D. Aminoacyl-tRNA synthesis. Annu Rev Biochem. 2000; 69:617–50. [PubMed: 10966471]
35. Boye K, Maelandsmo GM. S100A4 and metastasis: a small actor playing many roles. Am J Pathol. 2009; 176:528–35. [PubMed: 20019188]
36. Oslejskova L, et al. Metastasis-inducing S100A4 protein is associated with the disease activity of rheumatoid arthritis. Rheumatology (Oxford). 2009; 48:1590–4. [PubMed: 19828600]
37. Pleiss JA, Whitworth GB, Bergkessel M, Guthrie C. Rapid, transcript-specific changes in splicing in response to environmental stress. Mol Cell. 2007; 27:928–37. [PubMed: 17889666]
38. Mukhopadhyay R, Jia J, Arif A, Ray PS, Fox PL. The GAIT system: a gatekeeper of inflammatory gene expression. Trends Biochem Sci. 2009; 34:324–31. [PubMed: 19535251]
39. Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010; 328:321–6. [PubMed: 20395504]
40. Anderson RM, Weindruch R. Metabolic reprogramming, caloric restriction and aging. Trends Endocrinol Metab. 2009; 21:134–41. [PubMed: 20004110]
41. Haigis MC, Yankner BA. The aging stress response. Mol Cell. 2010; 40:333–44. [PubMed: 20965426]
42. Caro P, et al. Effect of 40% restriction of dietary amino acids(except methionine) on mitochondrial oxidative stress and biogenesis, AIF and SIRT1 in rat liver. Biogerontology. 2009; 10:579–92. [PubMed: 19039676]
43. Xiao F, et al. Leucine Deprivation Increases Hepatic Insulin Sensitivity Via GCN2/mTOR/S6K1 and AMPK Pathways. Diabetes. 2011
44. Zelante T, et al. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. Eur J Immunol. 2007; 37:2695–706. [PubMed: 17899546]
45. Huang L, Baban B, Johnson BA 3rd, Mellor AL. Dendritic cells, indoleamine 2,3 dioxygenase and acquired immune privilege. Int Rev Immunol. 2010; 29:133–55. [PubMed: 20367139]
46. von Bubnoff D, et al. Indoleamine 2,3-dioxygenase-expressing myeloid dendritic cells and macrophages in infectious and noninfectious cutaneous granulomas. J Am Acad Dermatol. 2011
47. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol. 2005; 5:641–54. [PubMed: 16056256]
48. Plouffe D, et al. In silico activity profiling reveals the mechanism of action of antimalarials discovered in a high-throughput screen. Proc Natl Acad Sci U S A. 2008; 105:9059–64. [PubMed: 18579783]

49. Ting SM, Bogner P, Dignam JD. Isolation of prolyl-tRNA synthetase as a free form and as a form associated with glutamyl-tRNA synthetase. J Biol Chem. 1992; 267:17701–9. [PubMed: 1517216]

50. Jahn MJ, Jahn D, Kumar AM, Soll D. Mono Q chromatography permits recycling of DNA template and purification of RNA transcripts after T7 RNA polymerase reaction. Nucleic Acids Res. 1991; 19:2786. [PubMed: 1710347]

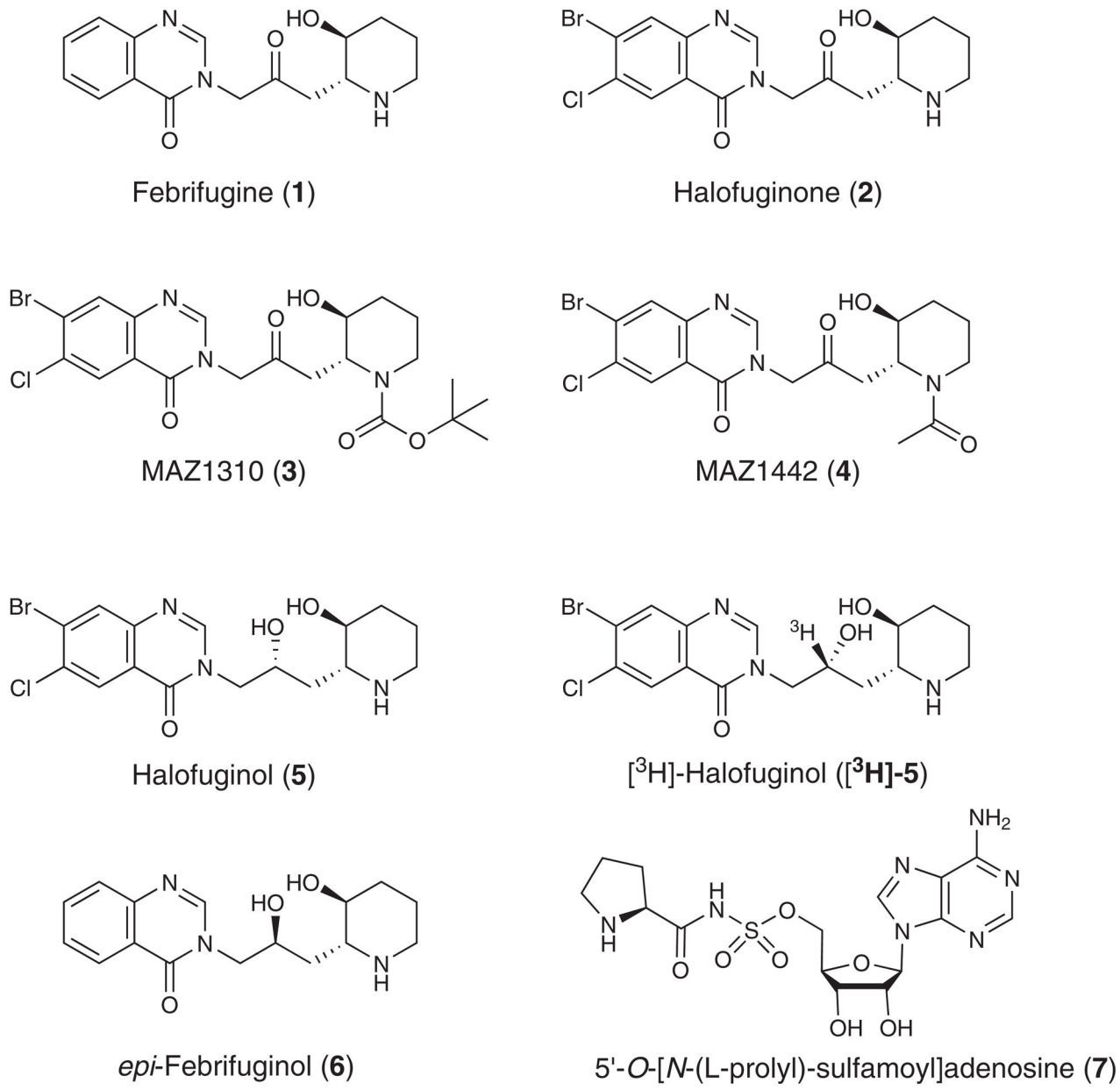

Fig. 1. Chemical structures of studied compounds

Except where otherwise specified halofuginone and derivatives were used as racemates.

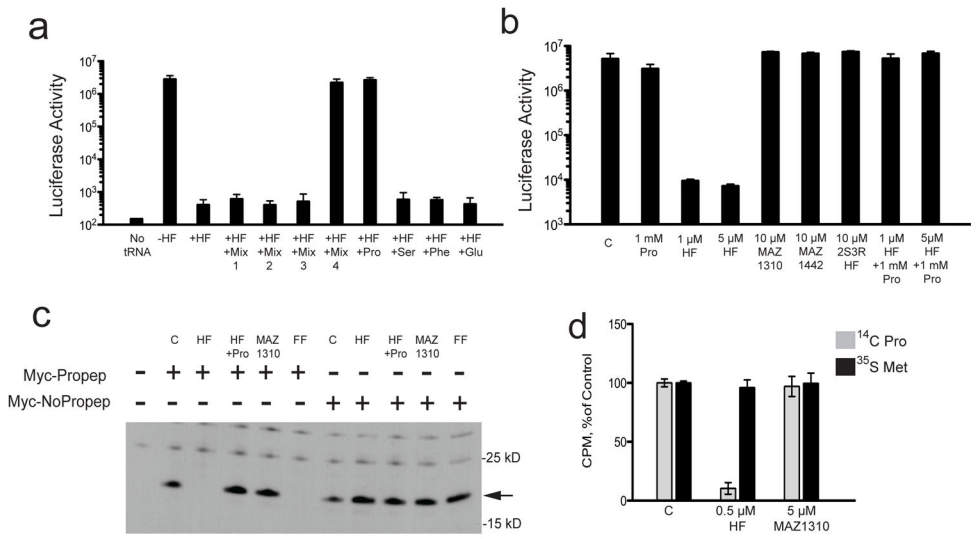

Fig. 2. HF and Febrifugine Inhibit Prolyl tRNA Synthetase Activity *In Vitro*

A) Rabbit reticulocyte lysate (RRL) was incubated with luciferase mRNA and translation quantitated in a luminescence assay. A solution of a mixture of amino acids or individual amino acids was added to give 1 mM of each in the reaction to rescue translational inhibition. Mix1: Asn, Arg, Val, Glu, Gly; Mix2: Lys, Ile, Tyr, Asp, Trp; Mix 3: His, Met, Leu, Ala, Thr; Mix 4: Ser, Phe, Pro, Gln. Note log scale of y-axis. **B)** Effect of HF and its derivatives in the presence or absence of proline supplementation on translation were assayed as in A. Error bars reflect standard deviation of triplicate determinations. **C)** Short myc-tagged polypeptides of identical sequence (see Supplementary Methods) with the exception that NoPropep lacks proline, while Propep contains a proline dipeptide, were translated in RRL in the presence of indicated inhibitors (each at a concentration of 1 µM, proline added at 1 mM). Translation was examined by anti-myc Western blot. **D)** ^{14}C Pro or ^{35}S Met (Perkin-Elmer) were incubated with RRL (Promega) and 1 µg/µl total bovine tRNA (Sigma) in the presence or absence of HF or MAZ1310 for 10′, tRNA was isolated using a MirVana tRNA isolation kit (Ambion), and radioactivity in tRNA was measured by liquid scintillation counting. Error bars reflect standard deviation of triplicate determinations.

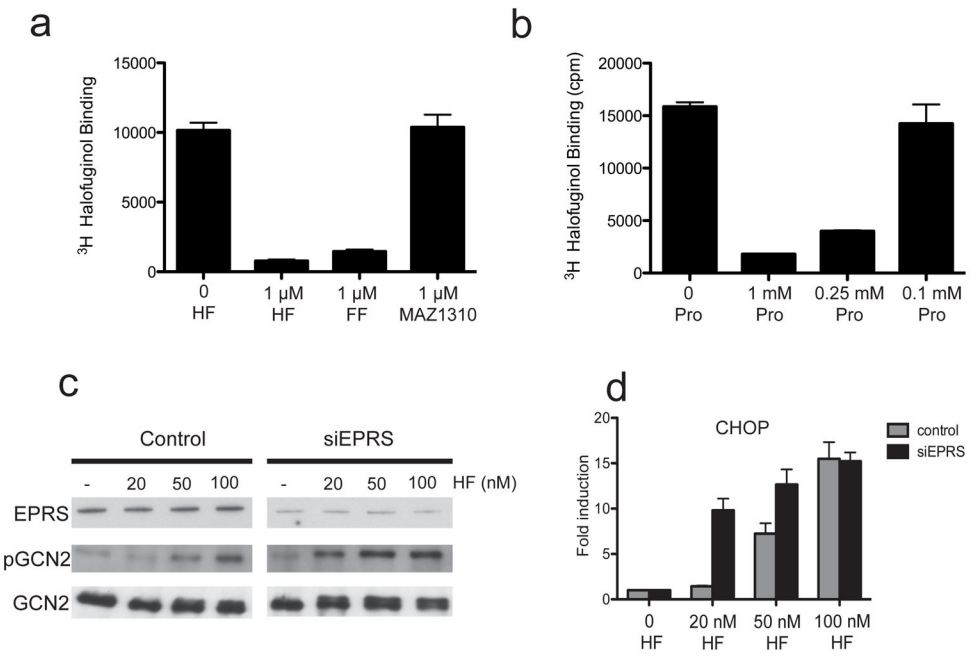

Fig. 3. EPRS binds to HF and determines sensitivity to HF in cells

A) [^{3}H] Halofuginol binds specifically to ProRS. Purified 6-his tagged ProRS (amino acids 998–1513 of human EPRS) was immobilized on N-NTA beads and incubated for 10′ at RT with 50 nM [^{3}H] Halofuginol (HFol) in the presence or absence of HF, FF, or the inactive HF derivative MAZ1310, 5 mM $MgCl_2$, and 2 mM ATP. Preliminary experiments established that binding was maximal by 10′, and that inclusion of tRNA had no effect on [^{3}H] HFol binding. **B)** [^{3}H] HFol binding was assayed as described above in the presence of indicated concentrations of proline. **C)** EPRS depletion sensitizes cells to HF. IMR90 lung fibroblasts were treated with a siRNAs directed against EPRS (Dharmacon) or a control siRNA mixture for 48 hours, and then treated with HF for 2 hours (pGCN2) and examined for EPRS protein levels, GCN2 total protein and phospho-GCN2 by Western blot. **D)** EPRS depleted IMR90 cells were compared to control cells with respect to HF induction of the AAR marker CHOP by Q-PCR. Equal concentrations of total cellular protein were loaded in each Western blot lane. CHOP expression was standardized to expression of phosphoglycerate kinase 1 (PGK1) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Data shown are representative of three separate experiments.

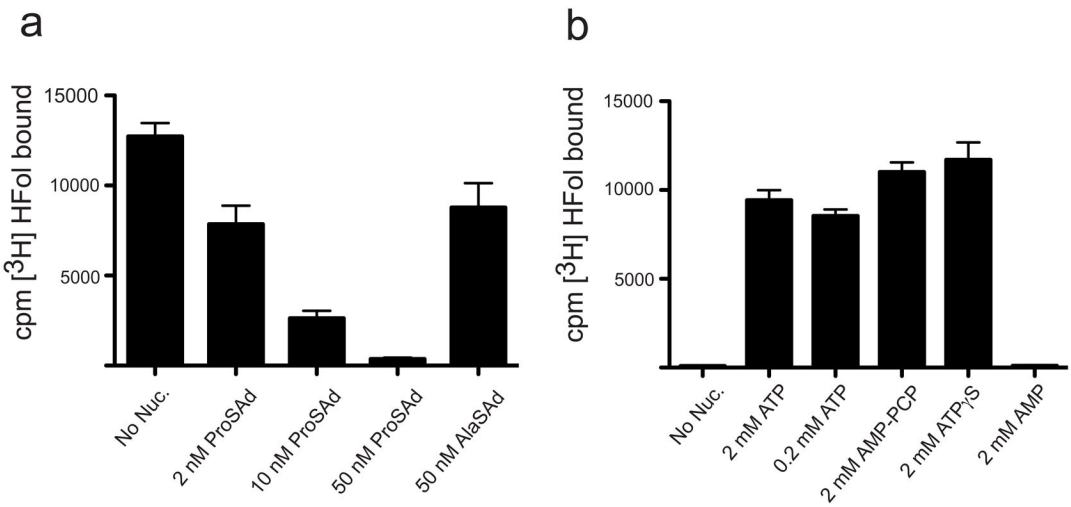

Fig. 4. HFol binds to the active site of EPRS in an ATP dependent manner

A) A prolyl adenylate analog potently inhibits HFol binding. Prolyl sulfamoyl adenosine (ProSAd) or alanyl sulfamoyl adenosine (AlaSAd), analogs of the corresponding aminoacyl adenylate, were coincubated with [^{3}H] HFol as in Fig. 2. **B)** HFol binding to EPRS requires ATP but not ATP hydrolysis. Binding of [^{3}H] HFol was assayed as in Fig. 3, except that the nucleotide type and concentration was varied as indicated.

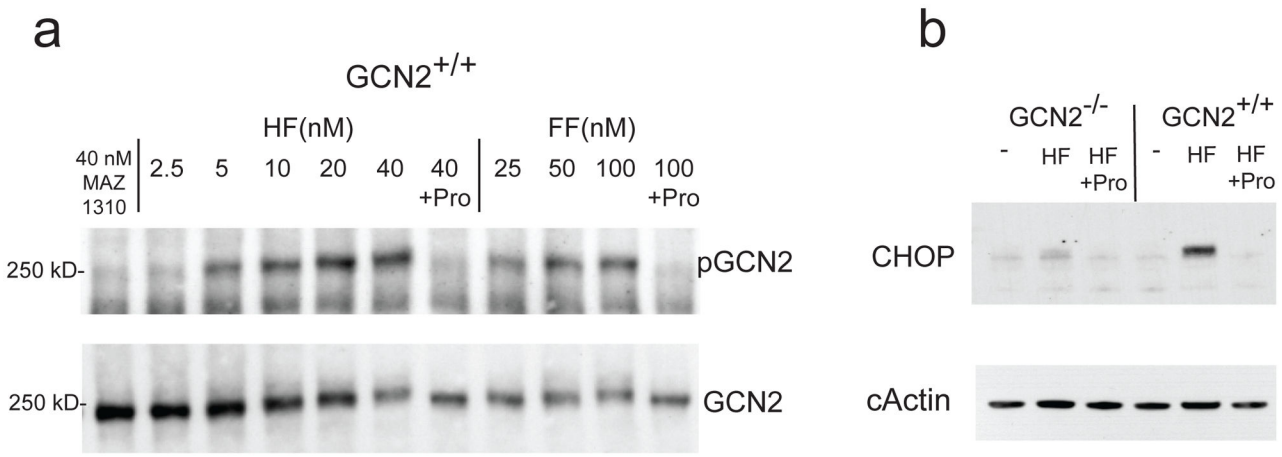

Fig. 5. Proline supplementation prevents activation of the AAR by HF

A) MEFs were treated with the indicated concentration of inhibitor in the presence or absence of 2 mM proline for 2 hours and assayed by Western blot for total GCN2 and GCN2 phosphorylated on Thr898 using a phospho-specific antibody (Cell Signaling). Data are representative of three separate experiments. **B)** MEFs were treated with 50 nM HF with or without 2 mM proline, lysed 6 hours later and analyzed by Western blot for expression of the AAR response marker CHOP. Cytoplasmic actin (cActin) is shown as a loading control.

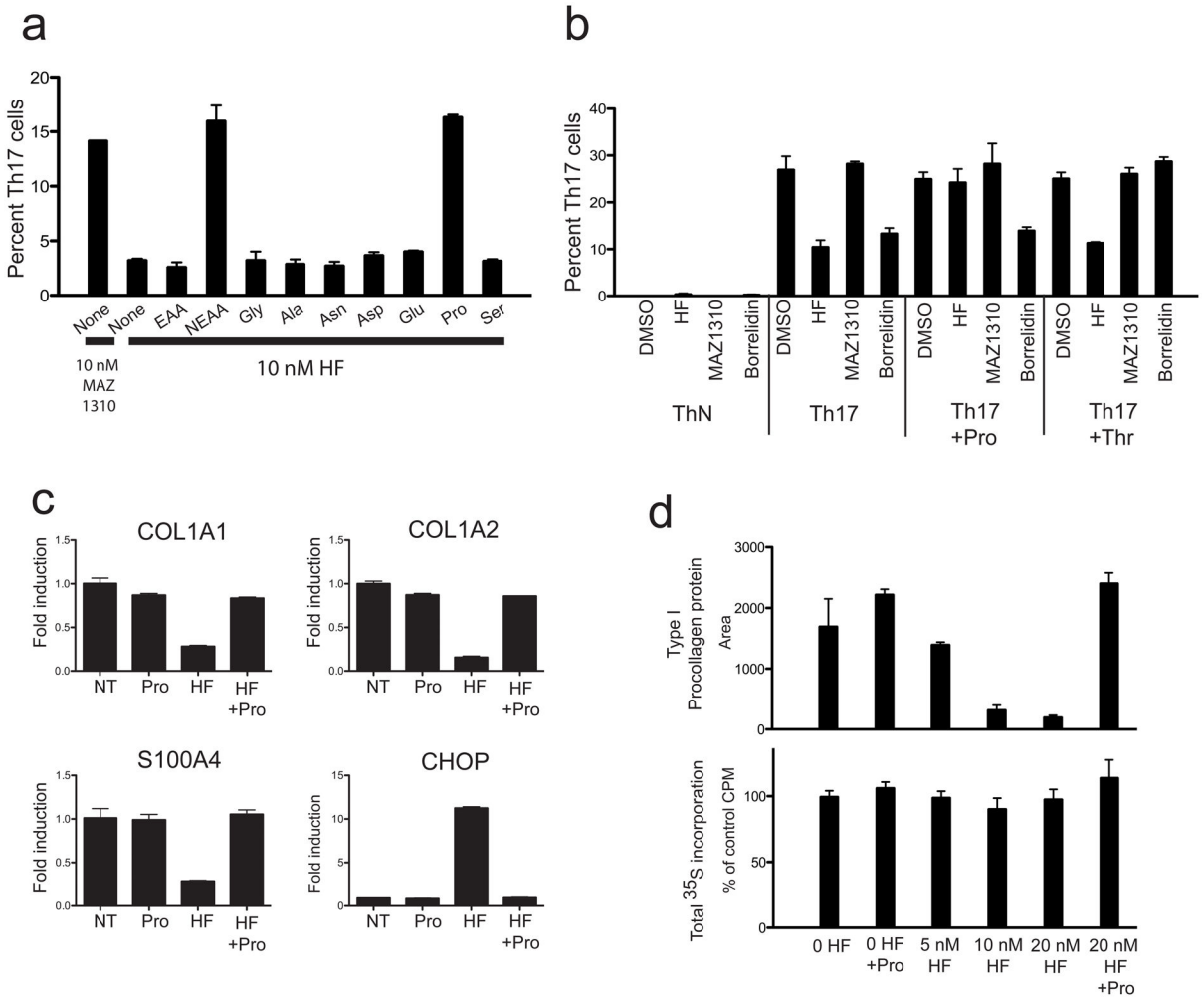

Fig. 6. Proline supplementation prevents the biological effects of HF

A) Primary murine $CD4^+$ $CD25^-$ T cells were activated through the TCR in Th17 polarizing conditions[10] in the presence of either 10nM MAZ1310 or HF and the following amino acid supplements: 10x concentration of essential (EAA) or non-essential (NEAA) amino acids mixtures (Biowhittaker or Invitrogen, respectively), or 10x concentrations (1 mM) of indicated individual amino acids. Data are presented as mean percentage of Th17 (IL-17+ IFNγ−) cells +/− SD from triplicate wells. **B)** Th17 differentiation was assayed as described above, in the absence or presence of HF or borrelidin, with or without 1 mM threonine or proline supplementation. **C)** MEFs were treated with or without HF (50 nM) and/or Proline (2 mM) for 4 hours (CHOP, S100A4) or 24 hours (ColIA1, Col1A2). mRNA expression was normalized to expression of TBP and is shown relative to untreated control. Error bars reflect standard deviation of triplicate determinations from triplicate plates of cells. Confidence intervals (p-value) for the effect of HF alone versus HF + Pro were determined using a two-tailed Student's t test. Data are representative of two separate experiments. **D)** Cells were pre-incubated for 24 hours with HF and proline as indicated, and secretion of Type I procollagen was measured after 24 hours in conditioned medium of cells plated by Western blot and quantitated using Image J software (top panel). Total protein synthesis was

measured as TCA-precipitable ^{35}S in a scintillation counter (Bottom panel). Error bars reflect standard deviation of triplicate determinations.