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A Fluoroacetamidine-Based Inactivator of Protein Arginine Deiminase 4: Design, Synthesis, and in Vitro and in Vivo Evaluation

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Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting ~1% of the worldwide population, and while a number of pharmaceuticals have been used to treat this disease, most only target its symptoms and not the underlying causes of the disease. Recent biochemical and genetic evidence has strongly suggested that a dys-regulated protein arginine deiminase (PAD) activity (Figure 1), in particular protein arginine deiminase 4 (PAD4), contributes to the onset and progression of RA.^{1–3} A role for PAD2 in the patho-physiology of multiple sclerosis has also been described.⁴ Additionally, PAD4 activity has been implicated in the control of gene transcription, primarily through its ability to catalyze the deimination of Arg residues present in histones H2A, H3, and H4, as well as p300, a transcriptional coactivator.^{5–7} However, the underlying mechanisms of this activity are only beginning to be understood.

Because of its potential as a therapeutic target for the treatment of RA, we initiated studies to characterize the molecular mechanism of PAD4 and to develop PAD4 inhibitors. Herein, we describe the *N*- α -benzoyl-*N*⁵-(2-fluoro-1-iminoethyl)-L-ornithine amide, **1**, hereafter referred to as F-amidine (Figure 2), the most potent PAD inhibitor described to date.

Although the catalytic mechanism of PAD4 is not fully established, it is clear from structural studies⁸ and precedents from related systems^{9,10} that PAD4 utilizes an active site Cys (Cys645) to catalyze the hydrolytic deimination of Arg residues through a proposed amidino-Cys intermediate.¹¹ Because the proposed mechanism is analogous to that employed by Cys proteases and because fluoromethyl ketones have long been used as covalent inactivators of Cys proteases, we hypothesized that such compounds designed to mimic the basic structure of small-molecule PAD substrates (i.e., benzoylated arginines)¹¹ would be reasonable inactivators of PAD4. However, these compounds have several less than desirable characteristics, including the fact that they would not be isosteric, they lack H-bond donors (vide infra), and they lack positive charge.¹² Therefore, we hypothesized that a fluoroacetamidine-containing compound based on the structure of a benzoylated Arg (Figure 2) would react with the active site Cys in PAD4 (Cys645) analogously to a fluoromethyl ketone (Figure 3), but would in fact provide superior inactivation kinetics because F-amidine is positively charged, it closely mimics the structure of Arg, and it possesses potential H-bond donors for both Asp350 and Asp473, two active-site residues that are important for substrate recognition and catalysis.

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Supporting Information Available: Additional references and complete author lists for refs 1, 5, and 7, materials, methods, and spectral characterization of F-amidine. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The synthesis of F-amidine, which is described in detail in the Supporting Information, utilized a solid-phase synthetic methodology that involved the on-resin coupling of an ethyl fluoroacetimidate hydrochloride to *N*- α -benzoyl ornithine (Scheme S1). The compound was obtained in >90% purity after cleavage from the resin and was further purified by reverse-phase HPLC.

To evaluate the potency of F-amidine, IC₅₀'s were determined by preincubating this compound with PAD4 in the absence or presence of Ca²⁺ for 15 min prior to assaying (Figure S1). The IC₅₀ of F-amidine is $21.6 \pm 2.1 \mu\text{M}$ when preincubated with Ca²⁺ versus an IC₅₀ of >200 μM when preincubated in the absence of Ca²⁺, thereby indicating that F-amidine inactivation, *vide infra*, of PAD4 is Ca²⁺-dependent. This observation is consistent with the hypothesis that F-amidine reacts with an active-site residue because PAD4 undergoes a conformational change upon Ca²⁺ binding that reorients active-site residues into positions that are competent for catalysis.^{8,11} In contrast to the potent inhibition observed for PAD4, F-amidine does not inhibit the methyltransferase activity of either PRMT1 or PRMT4, two Arg methyltransferases whose regiospecificity overlaps with that of PAD4 (not shown); thereby demonstrating that F-amidine selectively inhibits this Arg-modifying enzyme.

To determine whether F-amidine possesses time-dependent inhibition properties, time courses of product formation were monitored in either the absence or presence of this compound. The results of these experiments indicate that F-amidine does in fact display time-dependent inhibition properties (Figure 4A); that is, the progress curves are nonlinear and reach a plateau value, where $v_s = 0$. On the basis of these results, rapid dilution time course experiments (Figure 4B) were used to differentiate between reversible and irreversible inhibition. The results of these experiments indicate that there is no recovery¹³ of activity upon dilution of the preformed PAD4-F-amidine complex into assay buffer containing high concentrations of substrate ($\sim 7.5 \times K_m$), thereby indicating that F-amidine is an irreversible inactivator of PAD4. Consistent with this proposal is the fact that activity is not recovered even after dialyzing inactivated PAD4 for 20 h.

F-Amidine inactivates PAD4 in both a time- (as noted above) and concentration-dependent manner (Figure 4A). The K_I and k_{inact} values for this process are $330 \pm 90 \mu\text{M}$ and $1.0 \pm 0.1 \text{ min}^{-1}$, respectively, yielding a robust k_{inact}/K_I of $3000 \text{ M}^{-1} \text{ min}^{-1}$ (Figure 4C). Note that the rate of PAD4 inactivation by F-amidine can be decreased by increasing the concentration of substrate in the inactivation assays (Figure S2). Substrate protection of this type is consistent with our observation that inactivation is Ca²⁺-dependent and strongly suggests that F-amidine inactivates PAD4 by covalently modifying an active-site residue.

Although the site of modification in PAD4 has yet to be established, the fact that inactivation is substrate- and Ca²⁺-dependent is consistent with the modification of an active-site residue, and on the basis of similarities between the fluoroacetamidine warhead described herein and 2-chloroacetamidine, which covalently modifies the active-site Cys in a PAD4 paralogue, dimethylarginine dimethylamino hydrolase (DDAH),¹⁴ the most likely site of modification in PAD4 is Cys645, the active-site nucleophile.

The inhibitory properties of F-amidine were also evaluated *in vivo* using a mammalian two-hybrid assay that uses a luciferase reporter assay to monitor the interaction between the p300 GRIP1 binding domain (p300GBD) and GRIP1, an estrogen receptor transcriptional coactivator⁶—cotransfection of PAD4, which results in the deimination of the p300GBD and enhances its interaction with GRIP1.⁶ Treatment of the cells with F-amidine reduces the p300GBD and GRIP1 interaction as the concentration of this compound is increased, with maximal inhibition being noted at 200 μM (Figure 5). In contrast, control experiments in which the Cys645Ser mutant was transfected into cells in place of wild-type enzyme show minimal

inhibition, thereby indicating that the decrease in luciferase activity is not a nonspecific effect. And, while the mechanism of transport into these cells is not known, these results are particularly impressive because they indicate that F-amidine is bioavailable.

The development of inhibitors targeting PAD4 is critical for validating this enzyme as a drug target; therefore we designed and synthesized F-amidine, an irreversible inactivator of PAD4. In comparison to other known PAD inhibitors, for example, taxol ($IC_{50} \approx 5 \text{ mM}^{15}$) and 2-chloroacetamidine ($K_I = 20 \pm 5 \text{ mM}$; $k_{\text{inact}} = 0.7 \pm 0.1 \text{ min}^{-1}$; $k_{\text{inact}}/K_I = 35 \text{ M}^{-1} \text{ min}^{-1}$), F-amidine is the most potent PAD4 inhibitor described to date.¹⁴ The bioavailability of F-amidine suggests that this compound will be a powerful chemical probe that can be used to discern the role of PAD4 in the various signaling pathways (e.g., transcription control and differentiation) in which its activity has been implicated.

Additionally, the fact that F-amidine forms a covalent linkage to PAD4 indicates that, with the addition of appropriate functional groups, F-amidine derivatives will be robust activity-based protein-profiling and proteomic capture reagents. Such compounds will be useful for identifying the in vivo conditions under which this enzyme is activated and isolating activated PAD4, thereby enabling the identification of the numbers and types of post-translational modifications that occur to this enzyme during PAD4 activation in vivo. Finally, the fact that the synthesis of F-amidine relies on facile chemistry involving the coupling of an amine to an acetimidate hydrochloride indicates the ease of generating large, structurally diverse libraries containing this warhead, thereby leading to the identification of additional PAD4 inactivators with improved potency and selectivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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12. IC_{50} for N- α -benzoyl-N⁵-(2-fluoro-1-ketoethyl)-L-ornithine amide is $\gg 500 \mu M$.
13. Because the line representing the F-amidine rapid dilutions experiments fits well to a linear fit, the small amount of product formation observed after extended incubations is most likely due to the presence of a small amount of active PAD4 (<5%, based on comparing the slopes of the two lines) that was not inactivated during the preincubation of PAD4 with Ca^{2+} and F-amidine.
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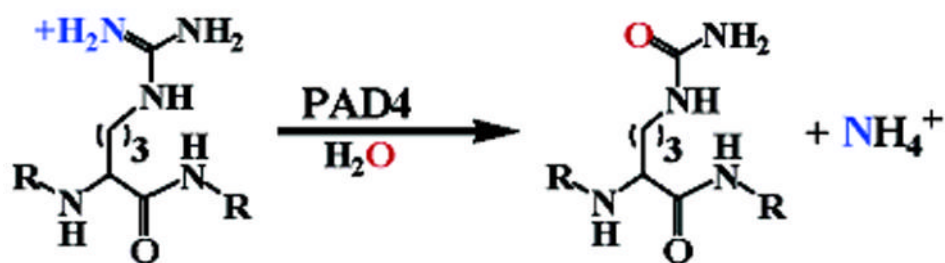


Figure 1.
Reaction catalyzed by PAD4. R = peptide backbone.

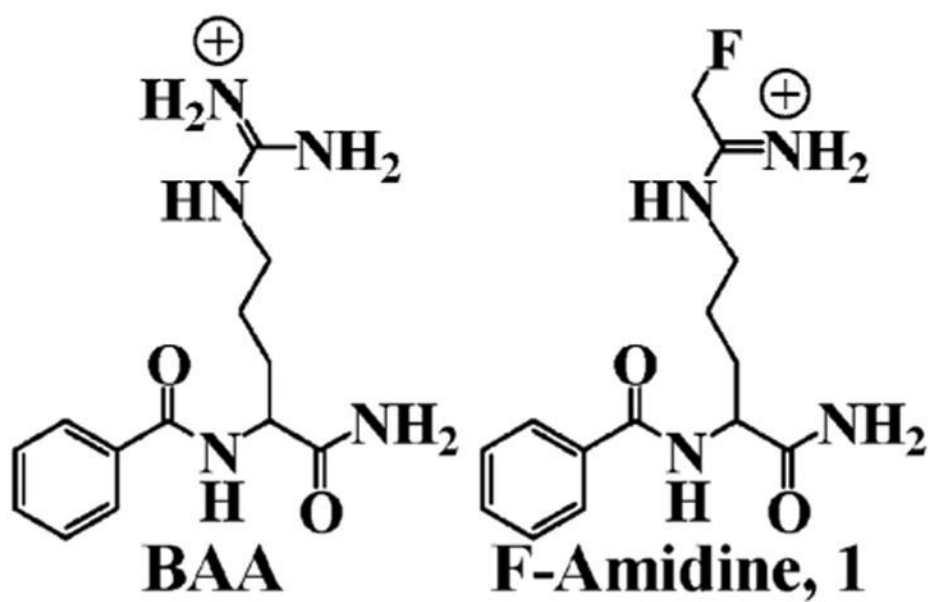


Figure 2.
Structure of F-amidine and benzoyl arginine amide (BAA), a nonphysiological PAD4 substrate.

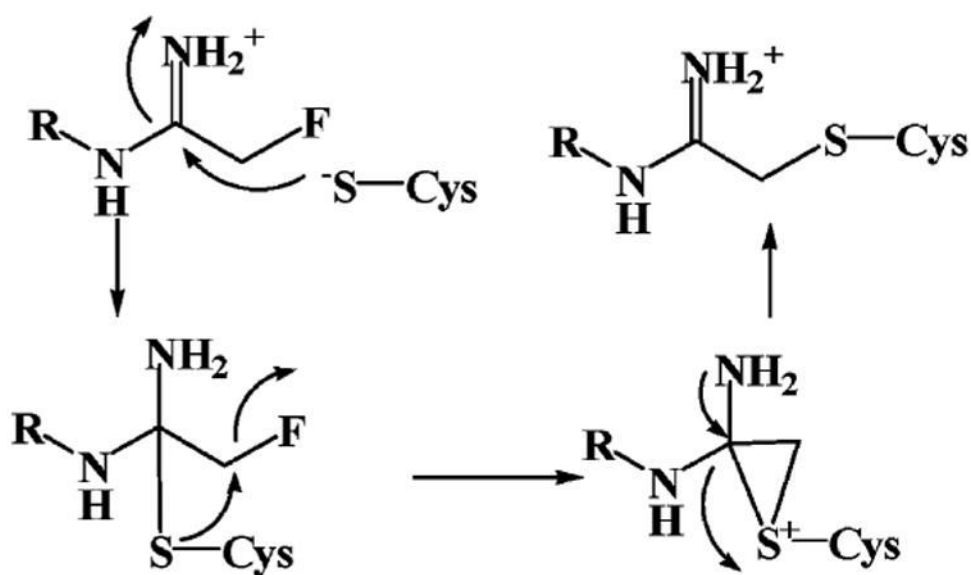


Figure 3.
Potential mechanism of PAD4 inactivation by F-amidine.

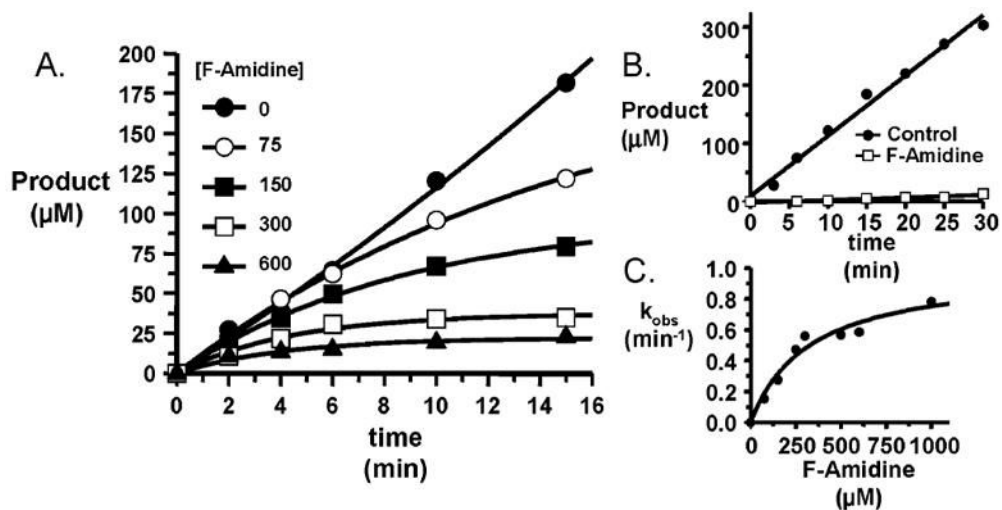


Figure 4.

F-Amidine is an irreversible inactivator of PAD4. (A) Plots of product formation versus time in the absence and presence of increasing concentrations of F-amidine. (B) Rapid dilution of preformed PAD4·F-amidine complexes into assay buffer containing excess substrate. (C) Concentration dependence of k_{obs} .

