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Activity Based Protein Profiling Reagents for Protein Arginine Deiminase 4 (PAD4): Synthesis and in vitro Evaluation of a Fluorescently-labeled Probe

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Abstract

Protein Arginine Deiminase 4 (PAD4), which catalyzes the post-translational conversion of peptidyl-arginine to peptidyl-citrulline, is widely regarded as one of the best new targets for the development of a novel Rheumatoid Arthritis therapeutic. In addition to its presumed role in this disease, PAD4 is also a calcium dependent histone deiminase that acts as a transcriptional corepressor. Herein we describe the design, synthesis and in vitro evaluation of two fluorescently labeled activity based protein profiling (ABPP) reagents that specifically and irreversibly modify the active, i.e. calcium bound, form PAD4 with equal affinity to previously described small molecule chemical probes of PAD4 function. These fluorescently tagged ABPPs will be useful for identifying the conditions under which this enzyme is activated *in vivo* and may prove to be useful RA diagnostics.

Protein Arginine Deiminase 4 (PAD4) has attracted significant attention in the past several years because of its presumed role as a causative factor in the pathophysiology of Rheumatoid Arthritis (RA), a disease affecting approximately 1% of the world's population. [1,2] Additionally, this enzyme has attracted the attention of molecular biologists interested in deciphering the factors controlling eukaryotic gene transcription because this enzyme downregulates the expression of genes under the control of the estrogen receptor, [3-5] presumably by catalyzing the post-translational modification of arginine residues in histones H2A, H3 and H4 to form peptidyl-citrulline and ammonia.

Because the activity of PAD4 appears to be upregulated in RA, we and others suggested that this calcium regulated enzyme (PAD4 is essentially inactive in the absence of mM amounts of calcium) [6] represents a valid therapeutic target for RA and initiated efforts to develop PAD4 inhibitors. [1,6-9] Those efforts have led to the successful identification of several bioavailable haloacetamidine based lead compounds, including F-amidine (**1**), Cl-amidine (**2**), and 2-chloro-acetamidine, that act as mechanism based inactivators of PAD4 (Figure 1). [7-9] These compounds inactivate PAD4 by covalent modification of Cys645, [7-9] a key catalytic residue, through one of two potential mechanisms (Figure 1) – Cys645 facilitates the deimination of arginine residues by forming a proposed thiouronium intermediate that is ultimately hydrolyzed to form citrulline.

F-amidine (k_{inact}/K_I of 3000 $M^{-1}min^{-1}$) [8] and Cl-amidine (k_{inact}/K_I of 13000 $M^{-1}min^{-1}$) [7] are the most potent PAD4 inhibitors to be described to date and are significantly more potent than 2-chloro-acetamidine (k_{inact}/K_I = 35 $M^{-1}min^{-1}$). [9] The increased potency is likely due to the binding energy gained by the addition of the benzoylated ornithine moiety that targets these inhibitors to the PAD4 active site.

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In addition to their high potency and ability to irreversibly modify PAD4, the fact that F- and Cl-amidine selectively modify the active, i.e. calcium bound, form of PAD4, [7,8] led us to consider that these compounds could be adapted for use as activity based protein profiling reagents (ABPPs; reviewed in refs. [10,11]). Herein, we describe the design, synthesis, and evaluation of two fluorescently tagged PAD4 ABPPs, rhodamine-tagged F-amidine (RFA; **3**) and rhodamine-tagged Cl-amidine (RCA; **4**) (Figure 1), that preferentially label the calcium bound, i.e. active, form of the enzyme.

During the design of these fluorescently-tagged ABPPs, we focused on using the copper(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction [12] to generate a triazole linker because this chemistry affords a level of versatility that is not provided by other linkers, i.e. the fluorescent tag can be added in a bio-orthogonal manner either before or after the inactivator has undergone reaction with the protein of interest. Note that while we only report our efforts to develop fluorescently tagged ABPPs in this communication, it will be trivial to adapt our methodologies to perform the couplings post-inactivation.

The synthesis of RFA and RCA, which is described in detail in the supplementary material, utilized a solid phase synthetic methodology that involved the on-resin coupling of an ethyl haloacetimidate hydrochloride to *N*-α-4-azidobenzoyl ornithine (Scheme S1). Subsequently, this compound was cleaved from the resin and coupled to a previously described rhodamine-alkyne construct [13] via the copper(I)-catalyzed azide-alkyne [3+2] cycloaddition reaction and then purified by reverse phase HPLC.

To evaluate the inhibitory properties of RFA and RCA, IC_{50}'s were determined using previously established methods. [8] Briefly, the compounds were pre-incubated with PAD4 in the absence or presence of saturating amounts of calcium (10 mM final) for 15 minutes prior to assaying (Figure 2). The IC_{50}'s of RFA and RCA are 23.7 ± 4.1 μM and 7.4 ± 0.8 μM, respectively, when preincubated with calcium, remarkably consistent with those obtained for F-amidine (21.6 ± 2.1 μM) and Cl-amidine (5.9 ± 0.3 μM); [7,8] thereby indicating that the reporter tag does not influence the interaction between these compounds and PAD4. Also noteworthy is the fact that the IC_{50}'s are significantly higher for both RFA (> 76 μM) and RCA (> 50 μM) when calcium is omitted from the preincubation step. This result is significant because it is consistent with the modification of an active site residue [7,8] – upon calcium binding, active site residues are moved into positions that are competent for catalysis. [14]

Having established that RFA and RCA inhibit PAD4 with comparable potency to their parent compounds, we then evaluated their ability to act as ABPPs by incubating them with purified recombinant PAD4 in the absence and presence of calcium. The reaction components were then separated on a 12% SDS-PAGE gel and fluorescently labeled proteins visualized (Figure 3). The results of these studies clearly demonstrate that RFA and RCA preferentially modify the active form of the enzyme, i.e. calcium bound PAD4; although at higher concentrations of RCA, PAD4 is modified in the absence of calcium by this compound. In contrast, a C645S mutant, which lacks the active site nucleophile and is essentially inactive, was not modified by RFA and only minimally modified by RCA (Figure S1), consistent with crystallographic and mass spectrometry experiments demonstrating that this is the only residue modified in PAD4 by F-amidine. [7,9] MALDI-MS experiments on full-length PAD4 treated with RFA and RCA showed respective mass shifts of 937 and 956 Da relative to control samples, within instrument error of the expected 940 Da mass shift, which is consistent with the preferential modification of a single site on the enzyme. Limit of detection assays demonstrated that ≥125 ng (or 1.7 pmol) of PAD4 can be detected with either RFA or RCA.

To evaluate the abilities of RFA and RCA to act as ABPPs in a complex protein mixture, we incubated *E. coli* cell extracts containing overexpressed GST (Glutathione *S*-transferase)-

tagged PAD4 or the GST-tagged C645S mutant with either RFA or RCA in the presence or absence of Ca^{2+}. The reaction components were separated on a 12% SDS-PAGE gel and fluorescently labeled proteins visualized (Figure 4). Remarkably, RFA and to a lesser extent RCA were highly selective for the calcium bound form of wild type but not mutant PAD4; thereby demonstrating that these ABPPs can readily label PAD4 even in the presence of highly complex cellular mixtures.

The ability of RCA, and in particular RFA, to preferentially label the active form of PAD4 indicates that these compounds are powerful small molecule chemical probes that will be useful for deciphering the normal and pathophysiological roles of this enzyme and how (or if) dysregulation of these pathways contributes to the onset and progression of RA. Specifically, these ABPPs will be useful for identifying the *in vivo* conditions under which this enzyme is activated, i.e. they will help to determine if post-translational modifications, e.g. proteolytic processing, occur to this enzyme during its *in vivo* activation; potentially offering an explanation for why non-physiological concentrations of calcium are required for *in vitro* enzyme activity. Furthermore, these probes may prove to be useful RA diagnostics and will undoubtedly aid the identification of non-specific targets of these compounds whose identities will aid the successful design and synthesis of PAD4-specific inhibitors. Finally, the fact that RFA is highly selective for the active form of PAD4 suggests that fluoroacetamidine containing compounds will have better pharmacological characteristics than chloroacetamidine containing compounds because they are likely to be more selective for PAD enzymes and would therefore be expected to have fewer off target effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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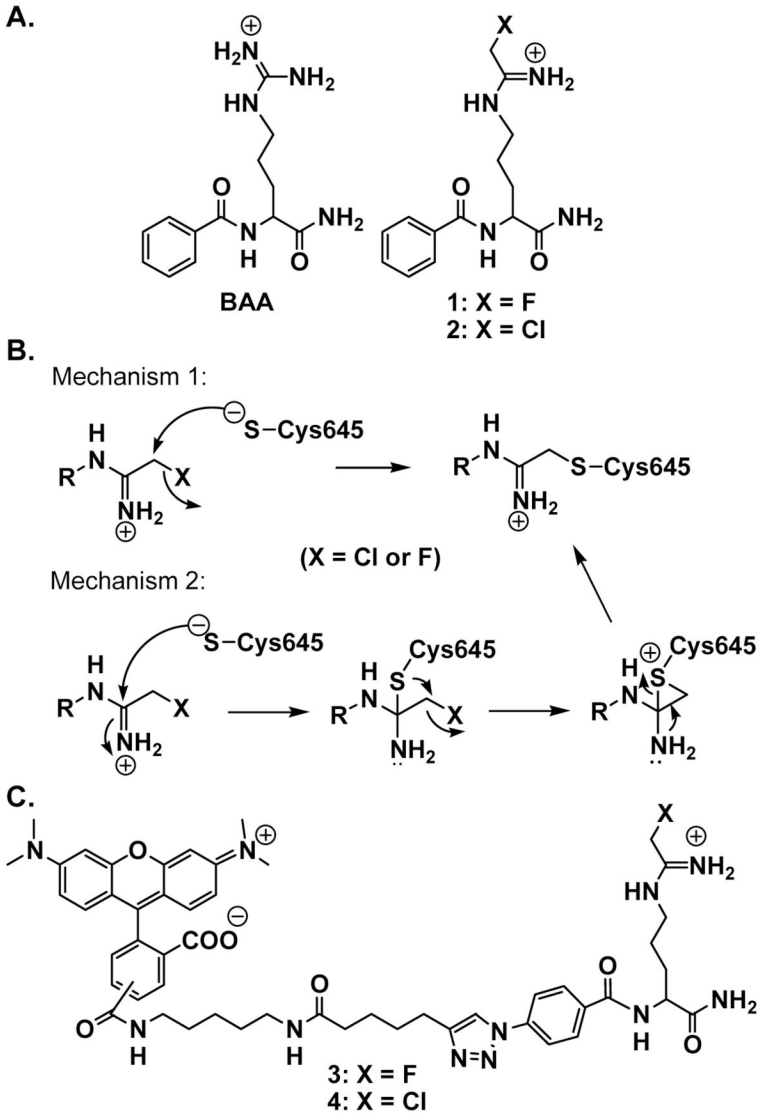

Figure 1.

Structures of inactivators and proposed mechanism of inactivation. (A) Structures of BAA, a PAD4 substrate, and the PAD4 inactivators F- and Cl-amidine. (B) Proposed mechanisms of inactivation. (C) Fluorescently labeled PAD4 inactivators.

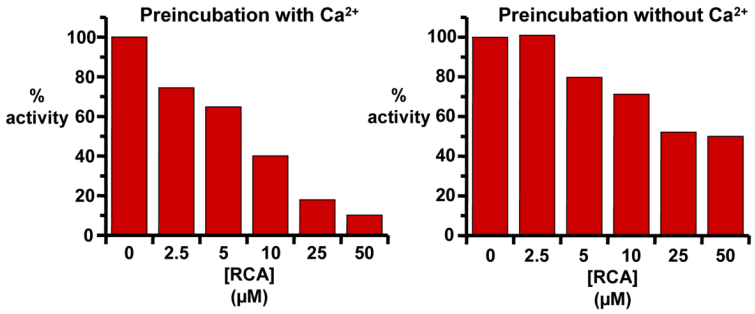

Figure 2.
Representative IC_{50} data for (**4**) determined with and without preincubation with calcium.

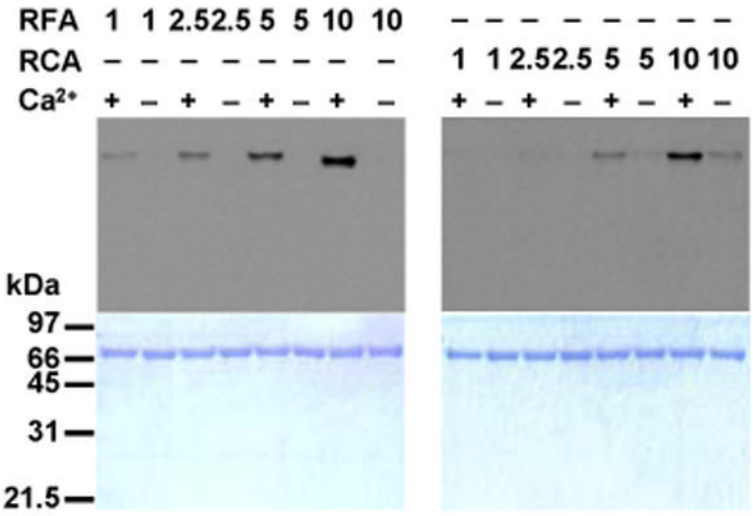

Figure 3.

In vitro labeling of PAD4 with RFA and RCA. The ratio of inactivator to protein is indicated at the top of the gel (*top*). The coomassie stained gels are shown to confirm equal loading of PAD4 in each lane. The numbers refer to the ratio [RXA]/[PAD4].

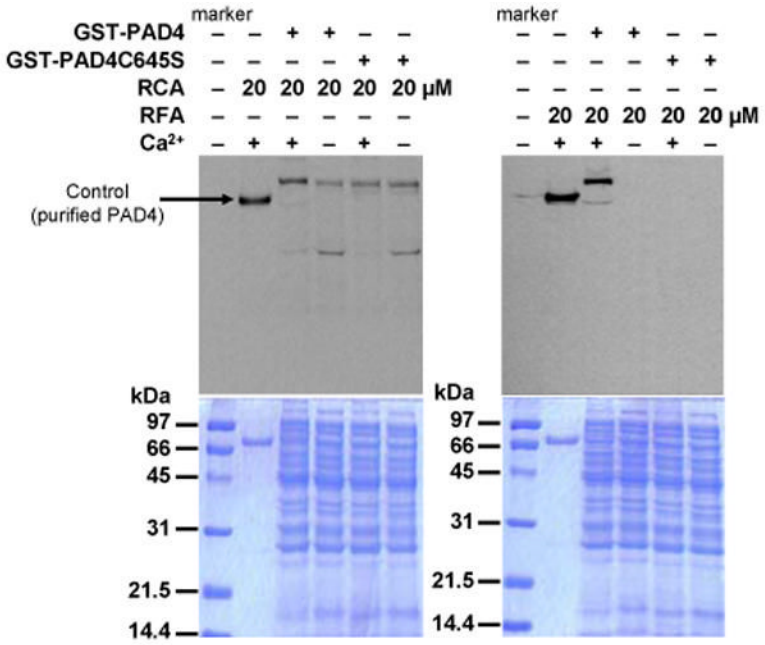

Figure 4.
In vitro labeling of *E. coli* cell lysates with RFA and RCA. The lysates were prepared from bacteria overexpressing either wild type or the C645S mutant. The coomassie stained gels (bottom) are shown to confirm equal loading of protein in each lane.