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Interrogation of the Active Sites of Protein Arginine Deiminases (PAD1, -2, and -4) Using Designer Probes

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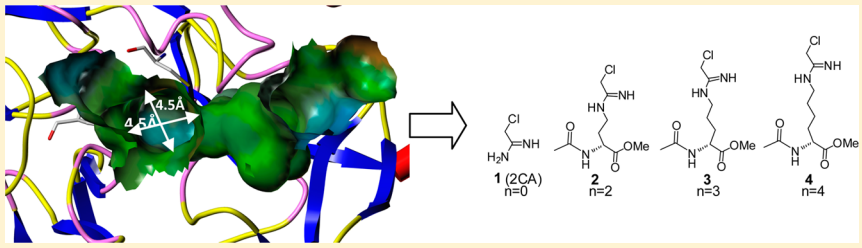
Supporting Information



ABSTRACT: Protein arginine deiminases (PADs) are involved in a number of cellular pathways, and they catalyze the transformation of peptidyl arginine residue into a citrulline as part of post-translational modifications. To understand ligand preferences, a group of probe molecules were investigated against PAD1, PAD2, and PAD4. These probe molecules carried a well-known covalent modifier of the catalytic cysteine residue, 2-chloroacetamidine moiety, which was tethered to an α-amino acid via a carbon linker. The chain length for the linker varied from 0 to 4. Time-dependent assays indicated that 2-chloroacetamidine (2CA) with no linker inhibited all PAD enzymes with a similar trend in the second-order rate constants, although with poor affinity. Among the other three probe molecules, compound **3** with a three-carbon linker exhibited the best second-order rate constants for optimal ligand reactivity with the binding site. These analyses provide insights into the relative patterns of covalent inactivation of PAD isozymes and the design of novel inhibitors targeting PAD enzymes as potential therapeutic targets.

KEYWORDS: *PAD, protein arginine deiminase, chemical probes, inhibitor design*

Protein arginine deiminases (PAD) are a group of five enzymes, PAD1−PAD4 and PAD6, involved in the post-translational modification of peptidyl arginine residues and are distributed in various tissues. PAD enzymes belong to the superfamily of amidinotransferases and are essential for a variety of cellular processes. In recent years, arginine-related biochemical pathways have been reported to have an important role in several major human diseases including rheumatoid arthritis, Alzheimer's disease, scrapie, Creutzfeld−Jacob disease, and multiple sclerosis.[1−5] Recently, it was also shown that PAD4 may be involved in cancer via the tumor suppressor genes; thus, PAD inhibitors may also have potential therapeutic benefits as anticancer agents.[6] Modulation of the activities of these enzymes, which are up-regulated in the above-mentioned diseases, may lead to novel therapeutic strategies against these diseases.

PAD hydrolyses peptidyl arginine residues to peptidyl citrulline and ammonia (Figure 1A). In the catalytic site of PAD, four residues appear to be critical for the deimination reaction: one cysteine, one histidine, and two aspartate residues, forming a closely interacting tetrad.[7] Although the mechanism is not fully established, there is sufficient evidence that PAD4, one of the five known human PAD isozymes, utilizes the active site cysteine, Cys645, to catalyze the hydrolytic deimination of arginine (Arg) residues involving a covalent mechanism.[7−11] Through a series of PAD4 mutant studies, it was shown that the active site Cys645 is indeed deprotonated and exists in the thiolate form prior to the substrate binding in the catalytic site.[7−11] Furthermore, it was suggested that a properly oriented His471 side chain is required for carrying out the acid−base chemistry for the deimination process.

A series of PAD4 inhibitors that utilize a covalent inhibition mechanism have been reported over the past 7 years,[12,13] including *N*-α-benzoyl-*N*^{5}-(2-chloro-1-iminoethyl)-L-ornithine

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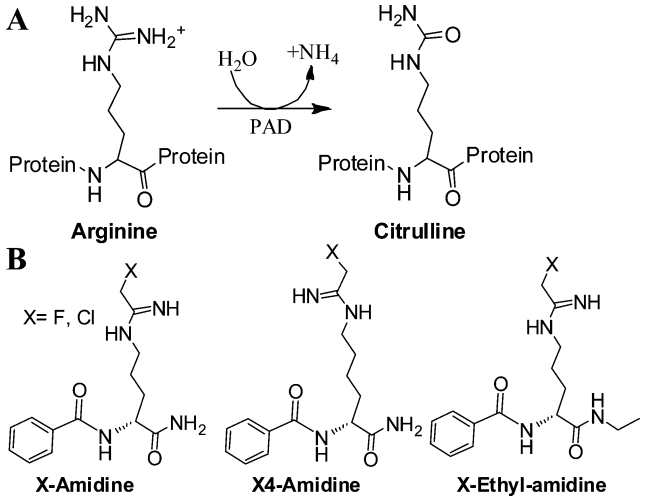

Figure 1. (A) Hydrolysis of peptidyl Arg residue into peptidyl citrulline by PAD enzyme. (B) Structures of haloamidines.

or Cl-amidine, with an IC_{50} of 5.9 ± 0.3 μM (Figure 1B).[6,14−16] Similarly, various other haloamidines (Figure 1B) were reported as potent inhibitors and as probe molecules to investigate PAD enzymes.[13] In the above studies, PAD4 enzyme was investigated using amidine molecular probes.[12] For comparative analysis with PAD1, -3, and -4, only one probe molecule with two different halogens (X-amidine, X = Cl, F; Figure 1B) was used to compare the relative inactivations of the PAD enzymes, primarily through inhibitor concentrations to inhibit 50% of enzyme activity (IC_{50}).[9]

As a part our medicinal chemistry program, we were interested in comparing the effectiveness of various inhibitors against PAD1, PAD2, and PAD4 enzymes, due to their therapeutic importance in neurological diseases. Here, we report comparative reactivities of PAD1, -2, and -4 isozymes using probe molecules designed with a comprehensive structural analysis and provide a strong link toward medicinal chemistry for selective inhibitors of PAD isozymes. Additionally, enzyme kinetics analyses with second-order rate constants provide a comparative tool across various isozymes and inhibitors classes for the study of enzymes and inhibitors.

Here, we present a systematic approach, using strategically designed chloroamidine derivatives carrying between zero and four side chain C−C bonds, to examine the depth of the catalytic site pocket and the varying reactivities with the PAD enzymes. Probe molecules in the current study include 2-chloroacetamidine (2CA) (**1**, Figure 2A) through chloroamidine structures **2**−**4**. The shortest of these, 2CA, is a known inhibitor of PAD enzymes, with very interesting pharmacological activities in multiple sclerosis.[17,18] The chloroamidine group is used as the reactive center because the simplest compound **1** has already been shown to be an active covalent inhibitor of PAD enzyme, and other derivatives with varied chain lengths could be synthetically accessed. Thus, compounds **1**−**4** were designed with varied lengths of the side chains connected to the chloroamidine moiety (Figure 2A). Three of the four compounds carry an acyl moiety on the amino terminus and a methyl ester on the carboxyl terminus. Because of the differences in the length of the side chains, these compounds are expected to enter the catalytic site of PAD with varying degrees of access to the reactive center for covalent bond formation. A detailed investigation in this direction would

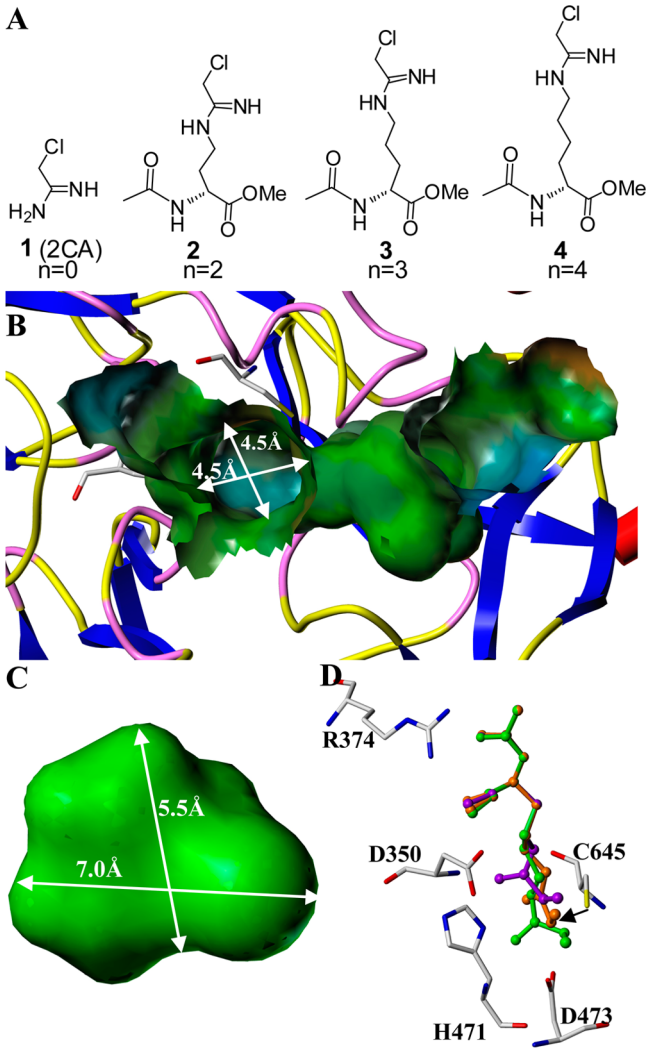

Figure 2. (A) Chemical probe molecules targeting PAD enzymes. (B) peptidyl Arg binding site in a tunnel shape in PAD4 (1WDA) and the entry of the substrate indicated by the arrow in white. (C) Connolly surface depicting the water accessible surface on **1** (2CA). (D) Compounds **1**−**4** docked into the active site of PAD4 (1WDA). Inhibitor molecules are shown as a ball-and-stick model with the following: compound **1**, red; **2**, purple; **3**, orange; and **4**, green. PAD4 residues are shown as capped-stick models, color-coded according to atom type.

help design inhibitors with improved precision to modulate the activities of PAD enzymes.

Compound **1** is a simple acetamidine derivative carrying the chloromethyl group as the reactive center but does not carry any other structural elements such as a linker moiety or the amino acidlike structural components (Figure 2A). Compound **1** is approximately 5.5−7.0 Å in length when one measures on the Connolly accessible surface on this molecule (Figure 2C). When one compares the size of **1** and the opening of the binding site in PAD4, they are very close to conclude that **1** could easily enter the binding site channel (Figures 2B). The remaining three probe molecules carry 2, 3, or 4 −C−C− bond spacers between the amidine moiety and the C_α of the amino acid. The amino acid backbone, that is, the −NH−CH−CO− moiety, keeps the side chain anchored at the opening of the binding pocket, in contrast to 2CA (**1**), which does not carry such an anchoring moiety. Because of this anchor and their

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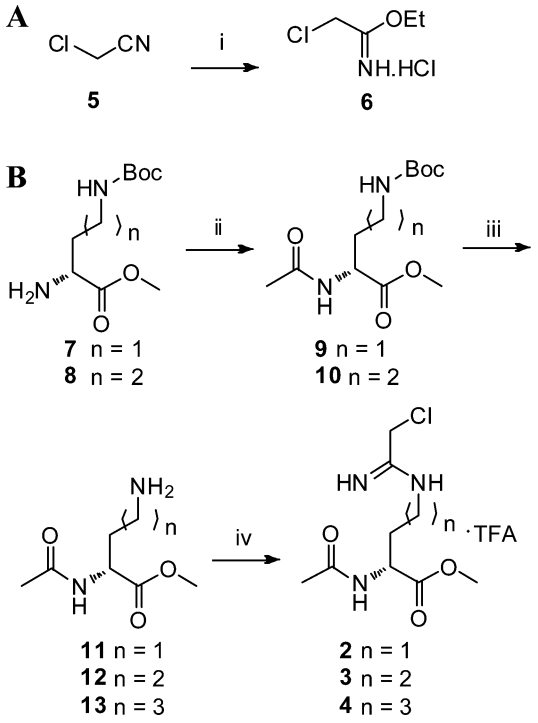
varying side chain lengths, compounds **2**–**4** reach into different depths of the binding pocket (Figure 2D). Hence, the reactive carbon for each compound is located at different depths in the binding pocket.

Compound **1** was used as a hydrochloride salt in enzymatic assays. Compounds **2**–**4** were synthesized according to Scheme 1.[15,19] Briefly, acylation of the commercially available starting

Scheme 1. Synthesis of Compounds 2–4[a]



[a]Reagents and conditions: (i) 2 M HCl/ether, EtOH. (ii) AcCl, Et_3N, DCM, rt, 5 h. (iii) TFA, DCM, rt. (iv) Compound **6**, Et_3N, EtOH, rt, 16 h.

materials **7** and **8** afforded the derivatives **9** and **10**, respectively (Scheme 1B). These two served as precursors for the final target compounds carrying the side chains with $n = 1$ and $n = 2$, respectively. The carbamate moiety was removed by treatment with TFA, yielding **11** and **12**, respectively. Compound **13**, a lysine analogue purchased from commercial sources, along with **11** and **12** were transformed into corresponding chloroamidine derivatives **2**–**4** by treatment with the imidoate derivative **6**. Compound **6** was synthesized from chloroacetonitrile **5** (Scheme 1A).[15,20] Compounds **2**–**4** were obtained as the corresponding TFA salts, and their purity was confirmed using rigorous analytical protocols (see the Supporting Information). Altogether, compounds **1**–**4** provided the probe molecules to interrogate the PAD enzymes' activities.

We evaluated compounds **1**–**4** against PAD1, PAD2, and PAD4 probing the ligand interactions in the context of binding site of PAD enzymes (Table 1). Compound **1** with no specific interactions at the binding site, other than carrying the reactive chloroimidine moiety, inhibited all three PAD enzymes in a time-dependent and concentration-dependent manner, with almost similar second-order rate constants with k_{inact}/K_I in the range of 9–60 M^{-1} min^{-1}.

There is a clear loss of PAD activity upon incubation with **1** over time, with a complete loss of activity at 5 mM concentration against PAD4 within 30 min. Poor affinity of **1** against PAD enzymes as demonstrated by the high concentrations required to achieve any inhibition, as well as the lowest second-order rate constants in comparison to those with inhibitors **2**–**4**. This is not surprising due to the absence of any structural features attributed to high affinity. Because of the small size of compound **1**, and possibly for its entry into the catalytic sites of PAD enzymes with little barrier, compound **1** exhibited similar affinities and kinetics of inactivation of the PAD enzymes (Table 1 and Figure 1B,C). Compound **2**, with a 2-carbon linker, exhibited a little higher affinity than that seen with **1** against PAD4 but failed to provide complete loss of activity in a time-dependent fashion against PAD4. Compound **3**, on the other hand, showed higher potency as well as faster inactivation of PAD4 than **1** and **2**. In fact, PAD4 lost all its activity within 3 min when incubated with **3** at 2 mM concentration and within 30 min at 100 μM concentration of **3** (k_{inact}/K_I = 865 ± 55 M^{-1} min^{-1}). As expected, compound **3** exhibited higher second-order rates of inactivation against PAD1 and PAD2 as well (k_{inact}/K_I = 497 ± 21 and 167 μM^{-1} min^{-1}, respectively).

Compound **4**, with the longest linker—a four-carbon linker between the chloroamidine moiety and the amino acid backbone—exhibited a decreased rate of inactivation of PAD1 and PAD4 (k_{inact}/K_I = 62 ± 2 and 50 ± 3 M^{-1} min^{-1}, respectively) when compared to those with **3**. Interestingly, when the inhibition profiles by **2** and **3** against PAD enzymes are compared, the general trend indicated that a 3-carbon chain as in **3** is optimal for the chloroamidine moiety to inactivate the enzyme in a time-dependent fashion, presumably through the participation of the catalytic Cys moiety. Compounds **2**–**4** carry simple groups on the carboxyl and amino ends, viz. a methyl ester and an acyl moiety, respectively. These substitutions do not present strong interactions at the binding site but do anchor the ligands to the opening of the active site channel. When compounds **2**–**4** were docked into the binding site of PAD4, compound **3** showed the optimal orientation and positioning for a potential reaction with the Cys645 residue, in comparison to that with **2** or **4** (indicated by an arrow, Figure 2D).[21]

This trend is also reflected when the efficiencies of inactivation are compared for each inhibitor, against each

Table 1. Enzyme Inhibition Kinetics for Compounds 1–4 Using Human PAD1, Mouse PAD2, and Human PAD4[a]

	k_{inact}/K_I (M^{-1} min^{-1})			
	1	**2**	**3**	**4**
Hs PAD1	32 ± 1 (1)	111 ± 9 (3.5)	497 ± 21 (15.5)	62 ± 2 (1.9)
Mm PAD2	9 ± 0.2 (1)	50 ± 3 (5.5)	167 ± 17 (18.5)	120 ± 14 (13.3)
Hs PAD4	60 ± 4 (1.2)	183 ± 23 (3.6)	865 ± 55 (17.3)	50 ± 3 (1)

[a]*Hs*, *Homo sapiens*; *Mm*, *Mus musculus*. Numbers in parentheses indicate relative rates of efficiency of inhibition of each PAD isozyme due to inhibitors **1**–**4**. Compound **3** with an optimal side chain length exhibited the best second-order rate constant for all three PAD enzymes.

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isozyme (Table 1, numbers in parentheses). Compound **3** inactivates each isozyme, specifically PAD4, 15−18 times faster than the corresponding poorest inhibitor, **1**. In fact, compound **1** is almost the least reactive compound against all three isozymes tested, and there appears to be no selectivity either. The Connolly surface for compound **1** (Figure 2C) indicates that this compound is small enough to enter the active sites of the PAD enzymes (Figure 2B) and equally be accessible to the thiolate in the active site for covalent bond formation. If there were no reactive moieties such as a chloroamidine group present on the inhibitors, high-affinity inhibitors could be designed to take advantage of the interactions inside the PAD active site, and the linker may not be a concern for optimal reactivity.

In summary, this report highlights the architecture of the active site of PAD enzyme through the reactive ligands and their inactivation rates of these enzymes for direct comparison with other inhibitors and enzymes families. Further systematic interrogation will provide tools to design nonreactive chemical probes as potential inhibitors of PAD enzymes with therapeutic potential.

■ ASSOCIATED CONTENT

Supporting Information

Compounds purity data, mass spectral data, and enzyme kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PAD, protein arginine deiminase; 2CA, 2-chloroacetamidine; Arg, arginine

■ REFERENCES

(1) Vossenaur, E. R.; Zendman, A. J.; van Venrooji, W. J.; Pruijin, G. J.; PAD, A. Growing Family of Citrullinating Enzymes: Genes, Features and Involvement in Disease. *Bioessays* **2003**, *25*, 1106−1115.

(2) Ishigami, A.; Oshawa, T.; Hiratsuka, M.; Taguchi, H.; Kobayashi, S.; Saito, Y.; Murayama, S.; Asaga, H.; Kimura, N.; Maruyama, N. Abnormal Accumulation of Citrullinated Proteins Catalyzed by Peptidylarginine Deiminase in Hippocampal Extracts from Patients with Alzheimer's Disease. *J. Neurosci. Res.* **2005**, *80*, 120−128.

(3) Jang, B.; Kim, E.; Choi, J. K.; Jin, J. K.; Kim, J. I.; Ishigami, A.; Maruyama, N.; Carp, R. I.; Kim, Y. S.; Choi, E. K. Accumulation of Citrullinated Proteins by Up-Regulated Peptidylarginine Deiminase 2 in Brains of Scrapie Infected Mice. A Possible Role in Pathogenesis. *Am. J. Pathol.* **2008**, *173*, 1129−1142.

(4) Jang, B.; Jin, J. K.; Jeong, Y. C.; Cho, H. J.; Ishigami, A.; Choi, K. C.; Carp, R. I.; Maruyama, N.; Kim, Y. S.; Choi, E. K. Involvement of Peptidylarginine Deiminase-Mediated Post-translational Citrullination in Pathogenesis of Sporadic Creutzfeld-Jacob Disease. *Acta Neuropathol.* **2009**, *119*, 199−210.

(5) Moscarello, M. A.; Wood, D. D.; Ackerley, C.; Boulias, C. Meylin in Multiple Sclerosis is Developmentally Immature. *J. Clin. Invest.* **1994**, *94*, 146−154.

(6) Wang, Y.; Li, P.; Wang, S.; Hu, J.; Chen, X. A.; Wu, J.; Fisher, M.; Oshaben, K.; Zhao, N.; Gu, Y.; Wang, D.; Chen, G.; Wang, Y. Anticancer Peptidylarginine Deiminase (PAD) Inhibitors Regulate the Autophagy Flux and the Mammalian Target of Rapamycin Complex 1 Activity. *J. Biol. Chem.* **2012**, *287*, 25941−25953.

(7) Arita, K.; Hashimoto, H.; Shimizu, T.; Nakashima, K.; Yamada, M.; Sato, M. Structural basis for Ca(2+)-induced Activation of Human PAD4. *Nature Struct. Mol. Biol.* **2004**, *11*, 777−783.

(8) Kearney, P. L.; Bathia, M.; Luo, Y.; Glascock, M. C.; Catchings, K. L.; Yamada, M.; Thompson, P. R. Kinetic Characterization of Protein Arginine Deiminase 4: A Transcriptional Corepressor Implicated in the Onset and Progression of Rheumatoid Arthritis. *Biochemistry* **2005**, *44*, 10570−10582.

(9) Knuckley, B.; Bhatia, M.; Thompson, P. R. Protein Arginine Deiminase 4: Evidence for a Reverse Protonation Mechanism. *Biochemistry* **2007**, *46*, 6578−6587.

(10) Ke, Z.; Zhou, Y.; Hu, P.; Wang, S.; Xie, D.; Zhang, Y. Active site cysteine is protonated in the PAD4Michaelis complex: evidence from Born-Oppenheimer ab initio QM/MM molecular dynamics simulations. *J. Phys. Chem. B* **2009**, *113*, 12750−12758.

(11) Ke, Z.; Wang, S.; Xie, D.; Zhang, Y. Born-Oppenheimer ab initio AM/MM molecular dynamics simulations of the hydrolysis reaction catalyzed by protein arginine deiminase 4. *J. Phys. Chem. B* **2009**, *113*, 16705−16710.

(12) Luo, Y.; Knuckley, B.; Lee, Y. H.; Stallcuo, M. R.; Thompson, P. R. A Fluoroacetamidine-Based Inactivator of Protein Arginine Deiminase 4: Design, Synthesis, and in Vitro and in Vivo Evaluation. *J. Am. Chem. Soc.* **2006**, *128*, 1092−1093.

(13) Luo, Y.; Arita, K.; Bhatia, M.; Knucley, B.; Lee, Y. H.; Stallcup, M. R.; Sato, M.; Thompson, P. Inhibitors and Inactivators of Protein Arginine Deiminase 4: Functional and Structural Characterization. *Biochemistry* **2006**, *45*, 11727−11736.

(14) Knuckley, B.; Causey, C. P.; Jones, J. E.; Bhatia, M.; Dreyton, C. J.; Osborne, T. C.; Takahara, H.; Thompson, P. R. Substrate Specificity and Kinetic Studies of PADs 1, 3 and 4 Identify Potent and Selective Inhibitors of Protein Arginine Deiminiase 3. *Biochemistry* **2010**, *49*, 4852−4863.

(15) Causey, C. P.; Jones, J. E.; Slack, J. L.; Kamei, D.; Jones, L. E., Jr.; Subramanian, V.; Knucley, B.; Ebrahimi, P.; Chumanevic, A. A.; Luo, Y.; Hashimoto, H.; Sato, M.; Hofseth, L. J.; Thompson, P. R. The Development of N-α-(2-Carboxyl)benzoyl-N5-(2-fluoro-1-iminoethyl)-L-ornithine Amide (o-F-amidine) and N-α-(2-Carboxyl)benzoyl-N5-(2-chloro-1-iminoethyl)-L-ornithine Amide (o-Cl-amidine) As Second Generation Protein Arginine Deiminase (PAD) Inhibitors. *J. Med. Chem.* **2011**, *54*, 6919−6935.

(16) Jones, J. E.; Slack, J. L.; Fang, P.; Zhang, X.; Subramanian, V.; Causey, C. P.; Coonrod, S. A.; Guo, M.; Thompson, P. Synthesis and Screening of a Haloacetamidine Containing Library to Identify PAD4 Selective Inhibitors. *ACS Chem. Biol.* **2012**, *7*, 160−165.

(17) Stone, E. M.; Schaller, T. H.; Bianchi, H.; Person, M. D.; Fast, W. Inactivation of two diverse enzymes in the amidinotransferase superfamily by 2-chloroacetamidine: dimethylarginase and peptidyl arginine deiminase. *Biochemistry* **2005**, *44*, 13744−13752.

(18) Moscarello, M. A.; Lei, H.; Mastronardi, F. G.; Winer, S.; Tsui, H.; Li, Z.; Ackerley, C.; Zhang, L.; Raijmakers, R.; Wood, D. D. Inhibition of peptidyl-arginine deiminase reverses protein-hypercitrullination and disease in mouse models of multiple sclerosis. *Dis. Models Mech.* **2013**, in press.

(19) Causey, C. P.; Thompson, P. R. An improved synthesis of haloacetamidine-based inactivators of protein-arginine deiminase-4 (PAD4). *Tetrahedron Lett.* **2008**, *49*, 4383−4385.

(20) Stillings, M. R.; et al. Substituted 1,3,4-thiadiazoles with anticonvulsant activity. 2. Aminoalkyl derivatives. *J. Med. Chem.* **1986**, *29*, 2280−2284.

(21) Models were generated from the X-ray crystal structure of PAD4 complexed with benzoyl-L-arginine amide, and the inhibitors **2**–**4** were overlapped with the ligand. The active site Cys645 was regenerated from Ala645 PAD4 inactive mutant used for crystallization.