Cellular Activity of New Small Molecule Protein Arginine Deiminase 3 (PAD3) Inhibitors

Haya Jamali, Hasan A. Khan, † Caroline C. Tjin, and Jonathan A. Ellman*

Department of Chemistry, Yale University, New Haven, Connecticut 06520, United States

Supporting Information

ABSTRACT: The protein arginine deiminases (PADs) catalyze the post-translational deiminination of arginine side chains. Multiple PAD isozymes have been characterized, and abnormal PAD activity has been associated with several human disease states. PAD3 has been characterized as a modulator of cell growth via apoptosis inducing factor and has been implicated in the neurodegenerative response to spinal cord injury. Here, we describe the design, synthesis, and evaluation of conformationally constrained versions of the potent and selective PAD3 inhibitor 2. The cell activity of representative inhibitors in this series was also demonstrated for the first time by rescue of thapsigargin-induced cell death in PAD3-expressing HEK293T cells.

KEYWORDS: Protein arginine deiminases, small molecule inhibitor, apoptosis inducing factor, spinal cord injury

P rotein arginine deminiases (PADs) catalyze the calcium-dependent hydrolytic conversion of arginine residues to citrulline side chains (Figure 1). Several PAD isozymes have been identified and characterized.1−4 In particular, PADs 1, 2, 3, and 4 have been shown to be catalytically active. PAD substrate side chains contain potential hydrogen bond donors and are also protonated at physiological pH, priming them for interactions with negatively charged groups such as nucleic acids.5,6 Due to the net loss of charge inherent in deimination of arginine side chains, the post-translational modification catalyzed by PADs may have dramatic effects on cell signaling. Though the isozymes collectively possess a high degree of sequence identity (50−55%),1,6 tissue-specific localization of each isozyme in humans has been observed.3,7 Significantly, abnormal activity of PADs has been demonstrated to play a role in multiple human disease states.3,8

PAD3 in particular has been characterized as a modulator of cell growth via AIF (apoptosis inducing factor) mediated apoptosis. Citrullination by PAD3 of AIF in hNSCs is required for its translocation to the nucleus to induce cell death, identifying PAD3 as an upstream regulator of Ca2+ dependent cell death.9 Notably, PAD3 activity has also been implicated in the neurodegenerative response to spinal cord injury10 as well as the citrullination of proteins during lactation.11 Cl-amidine 1, which irreversibly alkylates the active site cysteine of PADs as confirmed by X-ray structure, was developed by Thompson and co-workers and is the most extensively evaluated small molecule PAD inhibitor in cells and animal models12 and has furthered understanding of the role of PADs in different diseases (Figure 2).13 However, Cl-amidine is only moderately selective for PAD1, with significantly lower potency against PAD2 and PAD3 isozymes.14 While Thompson has subsequently developed significantly more potent cell permeable analogs,14,15 these inhibitors uniformly show high inhibitory activity against PAD1 and, depending on the

![Figure 1](https://example.com/figure1.png)

Figure 1. Conversion of arginine side chains by PADs.

![Figure 2](https://example.com/figure2.png)

Figure 2. Previously described PAD inhibitors.

Scheme 1. Urea and Carbamate Inhibitor Synthesis

![Scheme 1](https://example.com/scheme1.png)

*Reagents and conditions: (a) triphosgene, Et3N, CH2Cl2,0 °C, 30 min followed by NH2(CH2)2NHBoc for 4 and HO(CH2)2NHBOc for 5, 0 °C, 1 h; (b) 20% CF3CO2H in CH2Cl2, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et3N, MeOH, rt, 1 h.

Received: May 22, 2016
Accepted: July 11, 2016
Published: July 20, 2016

DOI: 10.1021/acsmedchemlett.6b00215
structure, strong inhibition of PAD2 or PAD4. In all cases, low inhibitory activity against PAD3 has been observed. A potent and isozyme-selective inhibitor of PAD3 would be extremely useful for deciphering the biological roles of this isozyme.

We have recently reported on the use of a fragment-based substrate screening approach for the discovery of potent PAD3-selective inhibitors, the best of which are >10-fold selective for PAD3 over the other isozymes. These low molecular weight and nonpeptidic inhibitors represent the only potent, PAD3-selective inhibitors described in the literature. Herein, we report on the further optimization of inhibitor 2 (Figure 2) to provide more potent inhibitors where the amide has been replaced by a heterocyclic functionality. Moreover, we have established that these inhibitors are active in cell culture by their protection of thapsigargin-induced cell death of HEK293T cells expressing PAD3.

Inhibitor 2, which was one of the most potent and selective PAD3 inhibitors that we had previously identified, was an appealing starting point for optimization. The flexible alkyl chain connecting the chloroacetamidine mechanism-based pharmacophore to the remainder of the inhibitor structure provides a key region for optimization with conformational constraints potentially benefiting inhibitor potency and/or selectivity. These types of conformational constraints have

---

**Table 1. Inhibition of PAD3 by Second-Generation Benzamide Analogs**

<table>
<thead>
<tr>
<th>Cmpd</th>
<th>Structure</th>
<th>IC50 (μM)</th>
<th>(k_{inact}/K_I (min^{-1}M^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td><img src="image1" alt="Structure" /></td>
<td>5.8 ± 1.2 (11000 ± 2000)</td>
<td>80 ± 10 46 ± 8 39 ± 5</td>
</tr>
<tr>
<td>6</td>
<td><img src="image2" alt="Structure" /></td>
<td>19 ± 1</td>
<td>&gt; 500 151 ± 1 36 ± 4</td>
</tr>
<tr>
<td>7</td>
<td><img src="image3" alt="Structure" /></td>
<td>12 ± 1</td>
<td>52 ± 4 140 ± 2 129 ± 23</td>
</tr>
<tr>
<td>10</td>
<td><img src="image4" alt="Structure" /></td>
<td>5.6 ± 1.0 (12400 ± 1800)</td>
<td>125 ± 18 18 ± 2 53 ± 2</td>
</tr>
<tr>
<td>13</td>
<td><img src="image5" alt="Structure" /></td>
<td>65 ± 6</td>
<td>408 ± 13 153 ± 46 64 ± 12</td>
</tr>
<tr>
<td>18</td>
<td><img src="image6" alt="Structure" /></td>
<td>5.4 ± 0.9 (21000 ± 2000)</td>
<td>34 ± 1 22 ± 5 28 ± 0.1</td>
</tr>
<tr>
<td>20</td>
<td><img src="image7" alt="Structure" /></td>
<td>7.3 ± 1.3 (15200 ± 600)</td>
<td>161 ± 23 13 ± 1 8.8 ± 0.2</td>
</tr>
<tr>
<td>23</td>
<td><img src="image8" alt="Structure" /></td>
<td>10.7 ± 0.1 (7500 ± 1900)</td>
<td>294 ± 70 110 ± 9 22 ± 1</td>
</tr>
</tbody>
</table>

---

**Scheme 2. Alkene Inhibitor Synthesis**

```
Reagents and conditions: (a) 2 M LiOH, MeOH, rt, 1 h; (b) 3, EDCI, Oxyma, Et₃N, CH₂Cl₂, rt, 1 h; (c) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (d) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.
```

**Scheme 3. Alkyne Inhibitor Synthesis**

```
Reagents and conditions: (a) 2 M LiOH, MeOH, rt, 1 h; (b) 3, EDCI, Oxyma, Et₃N, CH₂Cl₂, rt, 1 h; (c) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (d) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.
```
Reagents and conditions: (a) CuBr, EtOAc/CHCl₃, 65 °C, 5 h; (b) hexamine, CH₂Cl₂, rt, 1 h; (c) MeOH, HCl, rt, 1 h; (d) Boc-GABA-OH, TBTU, Et₃N, CH₂Cl₂, rt, 1 h; (e) NH₄OAc, AcOH, 140 °C, 2 h; (f) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (g) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) NCS, CH₃CN, rt, 12 h; (b) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) 1-azido-3-phenylbenzene, Na ascorbate, CuSO₄·H₂O/EtOH/PhMe, rt, 12 h; (b) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) CuBr, EtOAc/CHCl₃, 65 °C, 5 h; (b) hexamine, CH₂Cl₂, rt, 1 h; (c) MeOH, HCl, rt, 1 h; (d) Boc-GABA-OH, TBTU, Et₃N, CH₂Cl₂, rt, 1 h; (e) NH₄OAc, AcOH, 140 °C, 2 h; (f) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) NCS, CH₃CN, rt, 12 h; (b) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) 1-azido-3-phenylbenzene, Na ascorbate, CuSO₄·H₂O/EtOH/PhMe, rt, 12 h; (b) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) CuBr, EtOAc/CHCl₃, 65 °C, 5 h; (b) hexamine, CH₂Cl₂, rt, 1 h; (c) MeOH, HCl, rt, 1 h; (d) Boc-GABA-OH, TBTU, Et₃N, CH₂Cl₂, rt, 1 h; (e) NH₄OAc, AcOH, 140 °C, 2 h; (f) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) NCS, CH₃CN, rt, 12 h; (b) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) 1-azido-3-phenylbenzene, Na ascorbate, CuSO₄·H₂O/EtOH/PhMe, rt, 12 h; (b) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) CuBr, EtOAc/CHCl₃, 65 °C, 5 h; (b) hexamine, CH₂Cl₂, rt, 1 h; (c) MeOH, HCl, rt, 1 h; (d) Boc-GABA-OH, TBTU, Et₃N, CH₂Cl₂, rt, 1 h; (e) NH₄OAc, AcOH, 140 °C, 2 h; (f) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) NCS, CH₃CN, rt, 12 h; (b) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) 1-azido-3-phenylbenzene, Na ascorbate, CuSO₄·H₂O/EtOH/PhMe, rt, 12 h; (b) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) CuBr, EtOAc/CHCl₃, 65 °C, 5 h; (b) hexamine, CH₂Cl₂, rt, 1 h; (c) MeOH, HCl, rt, 1 h; (d) Boc-GABA-OH, TBTU, Et₃N, CH₂Cl₂, rt, 1 h; (e) NH₄OAc, AcOH, 140 °C, 2 h; (f) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) NCS, CH₃CN, rt, 12 h; (b) 20% CF₃CO₂H in CH₂Cl₂, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.

Reagents and conditions: (a) 1-azido-3-phenylbenzene, Na ascorbate, CuSO₄·H₂O/EtOH/PhMe, rt, 12 h; (b) ethyl-2-chloroacetimidate hydrochloride, Et₃N, MeOH, rt, 1 h.
inhibitor 18 according to the methods described previously. Inhibitor 18, though somewhat less selective than parent inhibitor 2, was 2-fold more potent as determined by $k_{\text{inact}}/K_i$. This is an exciting result because inhibitor 18 introduces an imidazole in place of the amide that has the potential to be susceptible to hydrolases.

The chloroimidazole inhibitor 20 (Scheme 5) was prepared due to the altered steric and modulation of the pK$_a$ relative to an unsubstituted imidazole as well as because of the reported success of this type of imidazole halogenation in protease inhibitors. Chlorination of the imidazole in intermediate 17 to give 19 was followed by replacement of the Boc protecting group with the chloroacetamidine functionality to give inhibitor 20. Chloroimidazole inhibitor 20 proved to be less potent than the corresponding imidazole inhibitor 18 and also was less selective.

Finally, inhibitor 23 was prepared and evaluated because the 1,2,3-triazole functionality has also been used as an amide surrogate. Amine 21, prepared by literature methods, was coupled with 3-biphenyl azide using Cu-catalyzed Click chemistry to give 22. Installation of the chloroacetamidine pharmacophore then provided inhibitor 23. Although replacement of the amide by the 1,2,3-triazole was reasonably well tolerated, inhibitor 23 proved to be less potent than both parent inhibitor 2 and imidazole inhibitor 18, as determined by IC$_{50}$ and by $k_{\text{inact}}/K_i$.

At this stage we chose to evaluate the most potent PAD3 inhibitor 18 as well as the parent inhibitor 2 for activity in cells. Feretti and co-workers have developed a cellular assay for the assessment of PAD3 inhibition. In this assay, HEK293T cells transfected with PAD3-containing vectors are treated with thapsigargin, a natural product used to rapidly increase the concentration of intracellular Ca$^{2+}$. The authors showed that thapsigargin, a natural product used to rapidly increase the concentration of intracellular Ca$^{2+}$, was the consequence of thapsigargin treatment. Significantly, the authors also observed that PAD3 inhibition mediated by the nonselective PAD inhibitor Cl-amidine with a $k_{\text{inact}}/K_i$ of $\sim$2000–2300 min$^{-1}$ M$^{-1}$14,16 against PAD3 rescued cell death at most concentrations of thapsigargin in hNSC cells.

We first recapitulated Cl-amidine mediated rescue of thapsigargin-induced cell death in HEK293T cells expressing PAD3, with concentrations of Cl-amidine as low as 10 $\mu$M being sufficient to induce cellular survival (Figure 3). The PAD3-selective inhibitors were next evaluated in the cellular assay (Figure 3). Both inhibitors 2 and 18 at 10 $\mu$M also rescued thapsigargin-induced cell death in HEK293T cells, thus demonstrating the cellular activity of this PAD3 inhibitor series for the first time.

In conclusion, a series of inhibitors was synthesized and evaluated that introduced conformational constraints and amide replacements within the chain connecting the mechanism-based pharmacophore to the remainder of the inhibitor structure. These types of modifications have not previously been explored with mechanism-based PAD inhibitors. The second-generation PAD3 inhibitor 18 with a potentially susceptible amide group replaced with a stable imidazole core lead to a 2-fold increase in inhibitor potency. Moreover, inhibitors 2 and 18 were for the first time evaluated for efficacy in cells and clearly demonstrated cellular rescue of HEK293T cells expressing PAD3 from thapsigargin-induced cell death. Further application of these inhibitors in PAD3 relevant models is in progress.


