ACS Medicinal Chemistry Letters

Letter

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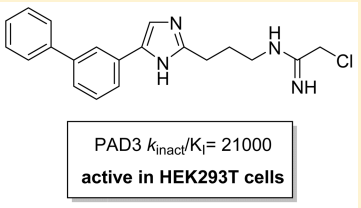
Cellular Activity of New Small Molecule Protein Arginine Deiminase 3 (PAD3) Inhibitors

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 deimination 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*

Protein arginine deiminases (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 Ca^{2+} dependent cell death.[9] Notably, PAD3 activity has also been implicated in the neurodegenerative response to spinal cord injury[10] 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 models[12] 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

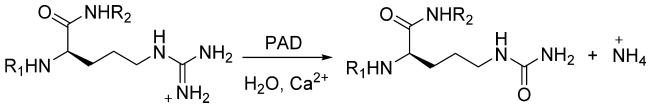


Figure 1. Conversion of arginine side chains by PADs.

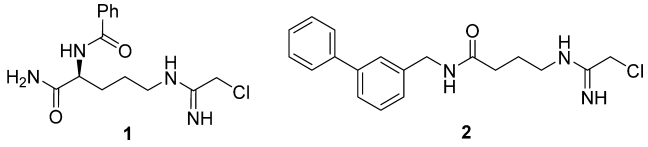
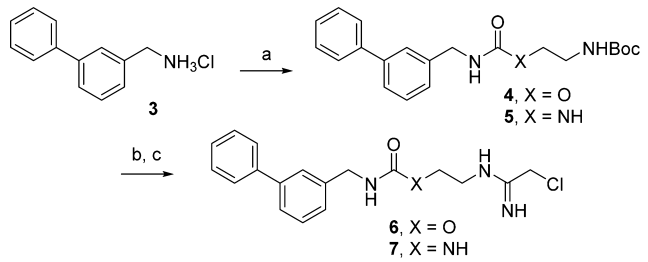


Figure 2. Previously described PAD inhibitors.

Scheme 1. Urea and Carbamate Inhibitor Synthesis[a]

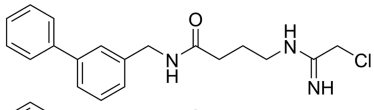
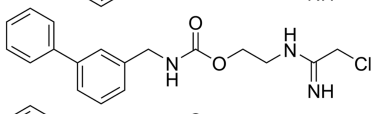
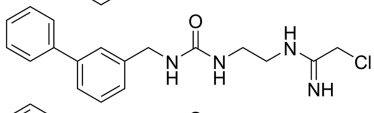
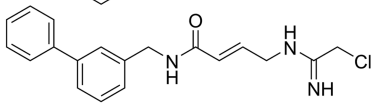
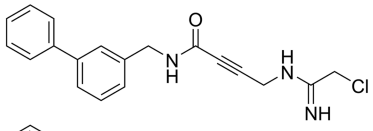
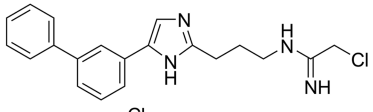
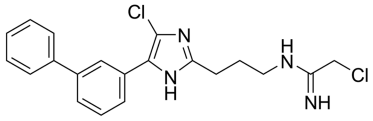
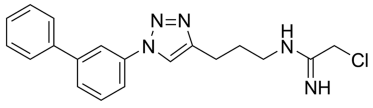


[a]Reagents and conditions: (a) triphosgene, Et_3N, CH_2Cl_2, 0 °C, 30 min followed by $NH_2(CH_2)_2NHBoc$ for **4** and $HO(CH_2)_2NHBoc$ for **5**, 0 °C, 1 h; (b) 20% CF_3CO_2H in CH_2Cl_2, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et_3N, MeOH, rt, 1 h.

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

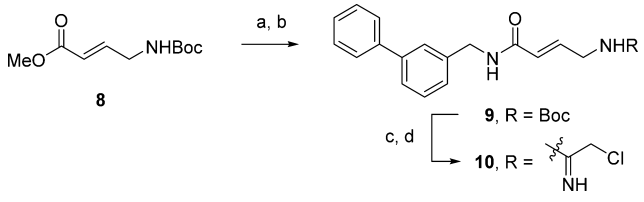
DOI: 10.1021/acsmedchemlett.6b00215
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

Table 1. Inhibition of PAD3 by Second-Generation Benzamide Analogs[a,b]

Cmpd	Structure	IC50 (μM) (k_{inact}/K_I ($min^{-1}M^{-1}$))			
		PAD3	PAD1	PAD2	PAD4
2		5.8 ± 1.2 (11000 ± 2000)	80 ± 10	46 ± 8	39 ± 5
6		19 ± 1	> 500	151 ± 1	36 ± 4
7		12 ± 1	52 ± 4	140 ± 6	129 ± 23
10		5.6 ± 1.0 (12400 ± 1800)	125 ± 18	18 ± 2	53 ± 2
13		65 ± 6	408 ± 13	153 ± 46	64 ± 12
18		5.4 ± 0.9 (21000 ± 2000)	34 ± 1	22 ± 5	28 ± 0.1
20		7.3 ± 1.3 (15200 ± 600)	161 ± 23	13 ± 1	8.8 ± 0.2
23		10.7 ± 0.1 (7500 ± 1900)	294 ± 70	110 ± 9	22 ± 1

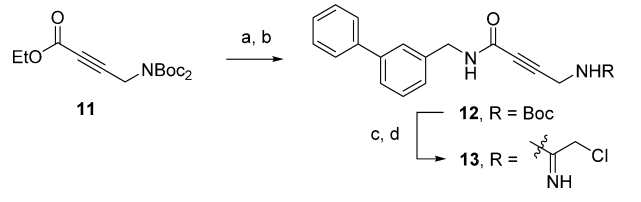
[a]IC_{50} values are reported as mean ± SD and were run in duplicate. [b]k_{inact}/K_I was determined using six concentrations of inhibitor at five time points. $K_{obs} = k_{inact}/K_I$ because $[I] \ll K_I$. The assays were run in quadruplicate. See Supporting Information for further assay details.

Scheme 2. Alkene Inhibitor Synthesis[a]



[a]Reagents and conditions: (a) 2 M LiOH, MeOH, rt, 1 h; (b) **3**, EDCI, Oxyma, Et_3N, CH_2Cl_2, rt, 1 h; (c) 20% CF_3CO_2H in CH_2Cl_2, rt, 1 h; (d) ethyl-2-chloroacetimidate hydrochloride, Et_3N, MeOH, rt, 1 h.

Scheme 3. Alkyne Inhibitor Synthesis[a]



[a]Reagents and conditions: (a) 2 M LiOH, MeOH, rt, 1 h; (b) **3**, EDCI, Oxyma, Et_3N, CH_2Cl_2, rt, 1 h; (c) 20% CF_3CO_2H in CH_2Cl_2, rt, 1 h; (d) ethyl-2-chloroacetimidate hydrochloride, Et_3N, MeOH, rt, 1 h.

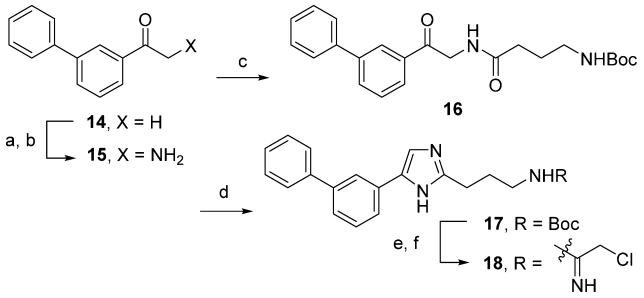
structure, strong inhibition of PAD2 or PAD4. In all cases, low inhibitory activity against PAD3 has been observed. A potent and isozyme-selective inhibitor of PAD3 would be extremely useful for deciphering the biological roles of this isozyme.

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.[16] 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

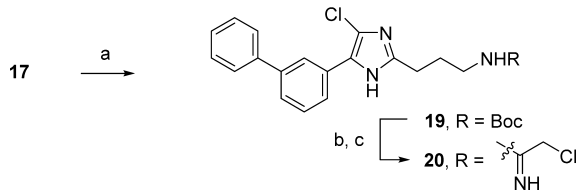
DOI: 10.1021/acsmedchemlett.6b00215
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

Scheme 4. Synthesis of Imidazole Inhibitor[a]



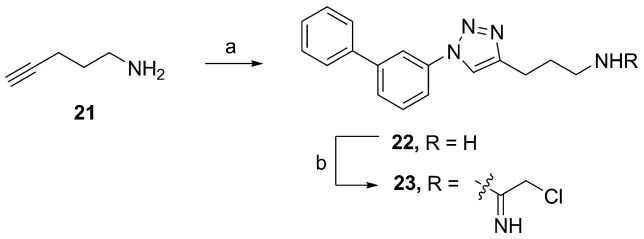
[a]Reagents and conditions: (a) CuBr, EtOAc/$CHCl_3$, 65 0 °C, 5 h; (b) (i) hexamine, CH_2Cl_2, rt, 1 h; (ii) MeOH, HCl, rt, 1 h; (c) Boc-GABA-OH, TBTU, Et_3N, CH_2Cl_2, rt, 1 h; (d) NH_4OAc, AcOH, 140 °C, 2 h; (e) 20% CF_3CO_2H in CH_2Cl_2, rt, 1 h; (f) ethyl-2-chloroacetimidate hydrochloride, Et_3N, MeOH, rt, 1 h.

Scheme 5. Synthesis of Chloroimidazole Inhibitor[a]



[a]Reagents and conditions: (a) NCS, CH_3CN, rt, 12 h; (b) 20% CF_3CO_2H in CH_2Cl_2, rt, 1 h; (c) ethyl-2-chloroacetimidate hydrochloride, Et_3N, MeOH, rt, 1 h.

Scheme 6. Synthesis of Triazole Inhibitor[a]



[a]Reagents and conditions: (a) 1-azido-3-phenylbenzene, Na ascorbate, $CuSO_4$, H_2O/EtOH/PhMe, rt, 12 h; (b) ethyl-2-chloroacetimidate hydrochloride, Et_3N, MeOH, rt, 1 h.

contributed to greatly enhanced selectivity in histone deactylase (HDAC) inhibitors,[17] but they have not previously been explored for PAD inhibitors. Replacement of the amide in **2** with heterocycle isosteres is also of value because it would eliminate susceptibility to hydrolases.

Replacement of the amide in **2** with carbamate and urea functionality reduces rotational flexibility while also modulating hydrogen bonding properties (Scheme 1). These modifications could readily be introduced using amine salt **3** as a common starting material, which was treated with triphosgene followed by coupling with either *N*-Boc ethylene diamine or *N*-Boc ethanolamine to give **4** and **5**, respectively. Cleavage of the Boc group followed by reaction with ethyl 2-chloroacetimidate then provided inhibitors **6** and **7**.

Replacement of the amide in **2** with carbamate (**6**) or urea (**7**) functionality was detrimental to PAD3 inhibitory potency with pronounced effects on the potency against other PAD isozymes also observed (Table 1). Interestingly, while both replacements resulted in 3-fold weaker PAD2 inhibition, urea

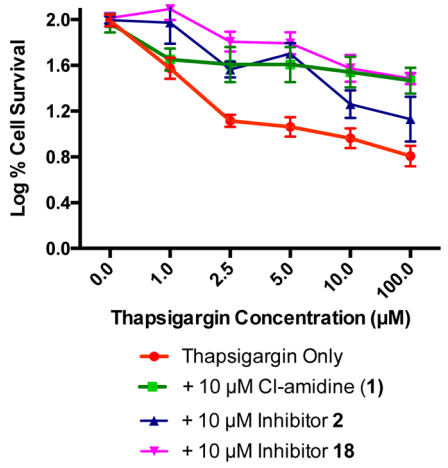


Figure 3. Rescue of thapsigargin-induced cell death in PAD3-expressing HEK293T cells by inhibitors (a) **1**, (b) **2**, and (c) **18**. Log % cell survival is reported as an average of four values ± SD.

and carbamate substitution had orthogonal effects on PAD1 and 4 inhibition. The carbamate in **6** resulted in a considerably weaker PAD1 inhibitor with little effect on PAD4, while the urea in **7** showed little effect on PAD1 inhibition but gave 3-fold weaker PAD4 inhibition.

Conformational constraints were also introduced in the center of the hydrocarbon chain by incorporation of an E alkene (Scheme 2). Saponification of the literature compound *N*-Boc γ-amino ester **8**[18] was followed by coupling the resulting acid with 3-phenylbenzylamine to provide amide **9**. Removal of the Boc group with trifluoroacetic acid followed by coupling with ethyl-2-chloroacetimidate then gave alkene inhibitor **10**. This conformationally constrained inhibitor showed comparable potency against PAD3 to parent inhibitor **2** though it did provide reduced selectivity over PAD2 (Table 1). More rigorous determinatination of k_{inact}/K_I for alkene inhibitor **10** confirmed that its potency was comparable to that of **2**.

An alkyne conformational constraint was also introduced (Scheme 3). As opposed to the alkene constraint in inhibitor **10**, the alkyne results in a much more significant displacement of the chloroacetamidine mechanism-based pharmacophore from the remainder of the inhibitor structure (Scheme 3). The *N,N*-bis-Boc γ-amino ester **11**, which was prepared according to literature procedures,[19] was treated with excess NaOH to remove one of the Boc groups along with concomitant saponification of the ester. Coupling with 3-phenylbenzylamine then provided amide **12**. Removal of the Boc group and coupling with ethyl-2-chloroacetimidate then gave alkyne inhibitor **13**.

Given the greater perturbation of the structure relative to the alkene isostere **10**, it is perhaps not surprising that inhibitor **13** incorporating the alkyne resulted in >10-fold reduction in PAD3 inhibition as well as weaker inhibition of all of the other PAD isozymes (Table 1).

Next, we explored heterocycle replacements of the amide present in inhibitor **2**. Imidazoles are well-known isosteres for amide bonds.[20,21] Therefore, the introduction of this functionality in place of the amide backbone was first explored (Scheme 4). Bromination of 3-phenyl acetophenone **14**[22] was followed by amine displacement to provide aminoketone **15**, which was then coupled with *N*-Boc γ-aminobutyric acid to give **16**. Heating **16** at elevated temperatures with NH_4OAc then provided imidazole **17**, which was converted to the imidazole

DOI: 10.1021/acsmedchemlett.6b00215
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

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_{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 sterics 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.[23] 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[24,25] (Scheme 6). Amine **21**, prepared by literature methods,[26] 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_{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.[9] 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 rapid, dose-dependent cell death as a result of PAD3-activation 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_{inact}/K_I of ~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 μM 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 μM also rescued thapsigargin-induced cell death in HEK293T cells, thus demonstrating the cell 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.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00215.

Complete experimental procedures and characterization data for all compounds described as well as IC_{50} data and k_{inact}/K_I data for inhibitors (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: jonathan.ellman@yale.edu.

Present Address

†(H.A.K.) AbbVie, 1 North Waukegan Road, North Chicago, Illinois 60064, United States.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Support has been provided by the NIH (R01-GM054051). H.J. also acknowledges support from the NIH Chemical Biology training grant (5T32 GM7499-34). Paul Thompson is gratefully acknowledged for providing constructs enabling the recombinant expression of PADs. Corey E. Perez, for his assistance with cloning, and Erin E. Duffy, for her help with cell culture experiments, are also acknowledged.

■ ABBREVIATIONS

AIF, apoptosis-inducing factor; Boc-GABA−OH, γ-(*tert*-butoxycarbonylamino)butyric acid; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HDAC, human deacetylase; HEK, human embryonic kidney; hNSC, human neural stem cells; PAD, protein arginine deiminase; TBTU, O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate

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DOI: 10.1021/acsmedchemlett.6b00215
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX