MedChemComm

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CONCISE ARTICLE

View Article Online
View Journal | View Issue

Cite this: *Med. Chem. Commun.*, 2013, **4**, 1109

Discovery of a structurally novel, drug-like and potent inhibitor of peptidylarginine deiminase

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Received 26th March 2013
Accepted 29th May 2013

DOI: 10.1039/c3md00091e

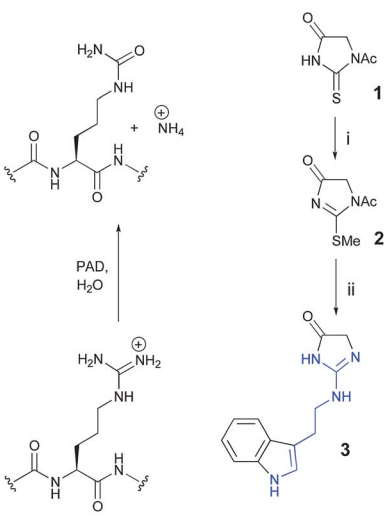
www.rsc.org/medchemcomm

The synthesis and biological properties of a structurally novel, potent and non-peptidic inhibitor of peptidylarginine deiminase are described. The novel drug-like PAD inhibitor contains a 3,5-dihydroimidazol-4-one ring that replaces the acyclic guanidine-binding unit present in arginine residues. This new drug-like PAD inhibitor was effective at 100 nM or below and could have relevance to diseases in which PAD expression is up-regulated, including rheumatoid arthritis, cancer, multiple sclerosis, and neural injury.

The family of calcium ion-dependent peptidylarginine deiminases (PADs, EC 3.5.3.15),[1] including the five known mammalian isoforms, PAD1–4 and PAD6, catalyse the formation of citrulline residues in proteins from arginine residues.[1–3] Such deimination (Scheme 1), also referred to as citrullination, results in loss of positive charge that can induce conformational changes in PAD protein structure,[2] and hence alters its interactions with other proteins. Elevated levels of citrullination, owing to up-regulation of PAD enzymes, have been associated with several major types of disease, including rheumatoid arthritis (mainly PAD4 involvement), in which antibodies to citrullinated proteins and anti-PAD autoantibody levels can act as important diagnostic aids.[4,5] Recently, *in vivo* efficacy of a PAD inhibitor against rheumatoid arthritis has been demonstrated.[6] Up-regulation of PAD2 has been implicated in the development of glaucoma[7] and autoimmune diseases such as multiple sclerosis, for which PAD inhibition in an animal model prevented onset of the disease.[8–10] Increased protein deimination has also been reported in patients with Alzheimer's disease[11] and in other neurodegenerative diseases.[12]

A role for PADs has also been demonstrated in traumatic injury. Tissue loss following spinal cord injury is associated with up-regulation of PAD3, but has been ameliorated using a non-selective PAD inhibitor. Cl-amidine,[13–15] an alkylating agent, decreases deimination, apoptosis and consequently tissue loss in injured chick spinal cords,[16] and ameliorates tissue damage following perinatal ischaemia.[1] Those effects can be modeled in cultures of human neural cells in which Cl-amidine inhibits apoptosis induced by increased cytoplasmic calcium ion concentration;[1,16,17] additionally, reduced deimination of PAD targets, including histone H3, is observed following treatment of injured chick spinal cords with Cl-amidine.[16] The putative role of PAD3 in spinal cord tissue loss following injury makes novel compounds that can effectively inhibit the activity of this isozyme of particular interest for neuroprotection.

Given the rapidly emerging importance of PADs in several physiological and pathological processes, targeting these enzymes could provide an attractive therapeutic strategy for several chronic diseases and also for traumatic neural injury. New PAD inhibitors are needed, given that PAD inhibitors are being increasingly investigated against a wide range of



Scheme 1 (a) PAD-catalysed citrullination of a peptidylarginine residue. (b) The structure and synthesis of a novel potent synthetic inhibitor of PAD. *Reagents and conditions*: (i) MeI, K_2CO_3, acetonitrile, 20 °C, 3 h, 54%; (ii) tryptamine, ethanol, reflux 4 days, 55%.

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biological targets,[10,16,18–20] and that PAD inhibitors are limited in number and chemical structure, being mainly halogenated amidine derivatives[13,14] and some guanidine derivatives.[21] Another general current limitation is that PAD isoform selectivity remains to be achieved. Mindful of those limitations, we sought to develop non-peptidic, potent PAD inhibitors with drug-like properties that cannot alkylate protein residues, and that bind to PAD only through non-covalent interactions. Herein is described the first potent reversible PAD inhibitor that, to our knowledge, fulfils those criteria.

Our hypothesis was that a potent and drug-like PAD inhibitor could be designed that lacked both a peptide unit and a chiral centre; those are both present in almost all previously reported PAD inhibitors including benzoyl L-arginine amide which has appreciable potency but is not CNS-active. Additionally, we considered that inhibitors containing a guanidine terminus, although likely to induce PAD inhibition by analogy with the natural substrate, would ultimately prove unsatisfactory, in part because of susceptibility to citrullination. All of those disadvantageous structural features were absent in the imidazolin-4-one **3** (clogP = 1.0, polar surface area = 69 Å^2, compared with Cl-amidine: clogP = 0.2, polar surface area = 108 Å^2) that was proposed: formal annulation of the guanidine terminus to give an imidazolin-4-one was expected to hinder deimination, and yet still permit extensive hydrogen bonding, in which two Asp residues near the active site of PAD enzymes have been invoked;[22] the peptide linkage and side-chain was replaced by a (1*H*-indol-3-yl)ethyl unit, isosteric in regard to its partial framework (as depicted in blue in Scheme 1).

N-Acetyl thiohydantoin (**1**), prepared by heating a mixture of glycine, ammonium thiocyanate and acetic anhydride at 100 °C,[23] was reacted with methyl iodide in the presence of potassium carbonate in acetonitrile to give 1-acetyl-2-methylthiohydantoin (**2**). Heating 1-acetyl-2-methylthiohydantoin (**2**) with tryptamine in ethanol initially gave the 1-acetyl derivative of **3** but on prolonged heating, deacetylation occurred to give imidazolin-4-one **3**.

The effect of imidazolin-4-one **3** on PAD activity in protein extracts from HEK293T cells over-expressing human recombinant PAD3 was measured using the BAEE assay. In this *in vitro* assay, imidazolin-4-one **3** showed comparable potency to Cl-amidine up to 1 mM, and markedly superior potency at 100 μM (Fig. 1). These results show that imidazolin-4-one **3** is a PAD inhibitor, can reduce human PAD3 activity, and appears to be more potent than Cl-amidine in the BAEE assay.

In a kinetic study using BAEE as the substrate for HEK293T cell extracts overexpressing human recombinant PAD3, the initial rates at various concentrations of imidazolin-4-one **3** (0, 10 and 100 μM) were determined. In the presence of compound **3**, the observed K_m increased with increasing concentration of **3**, whereas V_{max} remained constant, thereby displaying Michaelis–Menten kinetics consistent with competitive reversible inhibition (Fig. 2).

The effect of the novel PAD inhibitor 2-[2-(1*H*-indol-3-yl)-ethylamino]-3,5-dihydroimidazol-4-one (**3**) on human neural cells (methylene blue assay, Fig. 3) showed that compound **3** did not decrease the percentage of viable cells (Fig. 3) but rather increased it, indicating as little toxicity towards those cells as that of Cl-amidine.

We then further examined the effect of imidazolin-4-one **3** on human neural stem cells in culture in order to establish whether it was also more potent than Cl-amidine in live cells. Cell death was induced in human neural cells by treatment with 5 μM thapsigargin, and their pre-treatment with different concentrations of imidazolin-4-one **3** and Cl-amidine was examined for their ability to antagonise cell death (Fig. 4). Thapsigargin-induced cell death was not altered by Cl-amidine at or below concentrations of 1 μM, and a significant, though partial, increase in cell survival was observed only at concentrations above 10 μM (Fig. 4). In marked contrast, imidazolin-4-one **3**

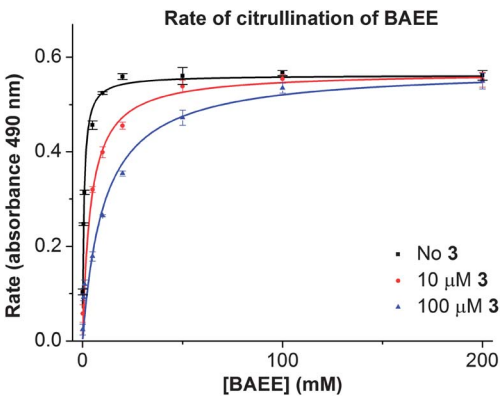

Fig. 2 Effect of imidazolin-4-one (**3**) on the rate of citrullination of BAEE by extracts from human HEK293T cells overexpressing recombinant PAD3.

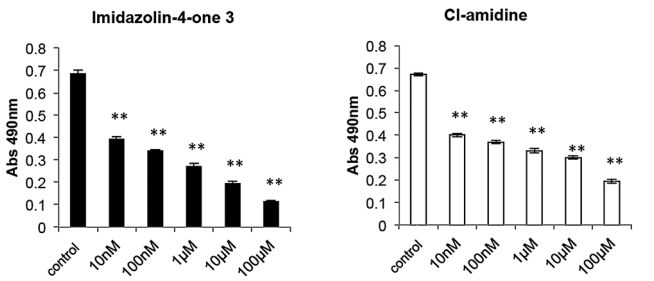

Fig. 1 Inhibition of PAD activity by Cl-amidine and imidazolin-4-one **3** assessed by the BAEE assay using extracts of HEK293T cells overexpressing human recombinant PAD3. Both compounds potently inhibit PAD activity *in vitro* (** $p \leq 0.01$).

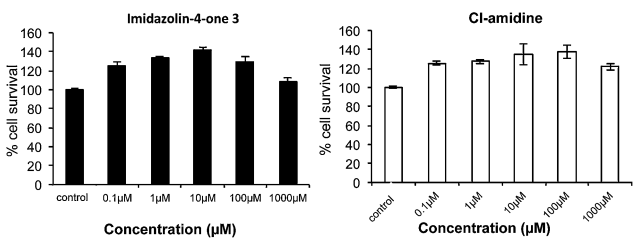

Fig. 3 Percentage of human neural stem cells surviving (detected by methylene blue assay) after treatment with either imidazolin-4-one **3** or Cl-amidine (Cl-am). No reduction in the percentage of live cells after 24 h treatment was observed at any of the concentrations tested.

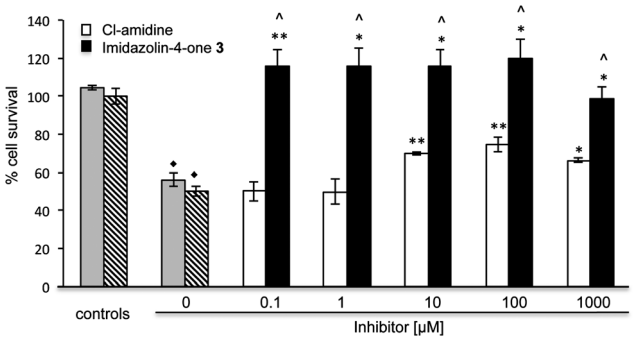

Fig. 4 Inhibition of thapsigargin-induced cell death by imidazolin-4-one **3** as compared to Cl-amidine. Controls and thapsigargin (5 μM) alone (no inhibitor) contain either ethanol (controls for Cl-amidine, grey bar) or both ethanol and DMSO (controls for imidazolin-4-one **3**, striped bar). Imidazolin-4-one **3** is more powerful than Cl-amidine in rescuing cells from thapsigargin-induced cell death as assessed by the methylene blue assay. $*p \leq 0.05$, $**p \leq 0.01$ (as compared to thapsigargin treated cells), $\diamond$ $p \leq 0.01$ (as compared to corresponding control) and ^ $p \leq 0.01$ (as compared to Cl-amidine at the same concentration).

maximally restored cell survival at 100 nM, and was highly effective at all the concentrations tested (up to 1 mM). The greater difference in potency between imidazolin-4-one **3** and Cl-amidine in the cell assay as compared to the enzymatic assay in protein extracts may be at least in part due to a higher concentration of the imidazolin-4-one **3** in the cell, given its greater lipophilicity.

Finally, we assessed whether the extent to which imidazolin-4-one **3** inhibited cell death in the HEK293T cells over-expressing the human recombinant PAD3. In PAD3-expressing HEK293T cells 1 μM thapsigargin was sufficient to induce cell death. A significant increase in cell survival, as assessed by the methylene blue assay, was achieved by treatment with both imidazolin-4-one **3** and Cl-amidine (Fig. 5). However, whereas imidazolin-4-one **3** was effective at 10 μM, the same extent of inhibition required Cl-amidine at a concentration of 100 μM. This further supports the view that imidazolin-4-one **3** is more potent than Cl-amidine in human cell-based assays and can inhibit human

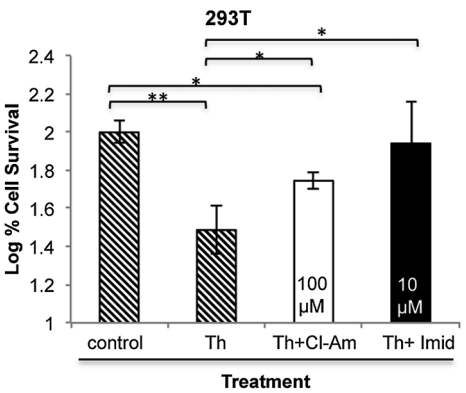

Fig. 5 Inhibition of cell death induced by thapsigargin (Th) in HEK293T cells expressing human recombinant PAD3 by Cl-amidine (Cl-am, 100 μM) and imidazolin-4-one **3** (Imid, 10 μM). Imidazolin-4-one **3** is effective at a 10 fold lower concentration ($*p < 0.51$; $**p < 0.01$). All samples including control and 1 μM thapsigargin alone (striped bars) contain both ethanol and DMSO.

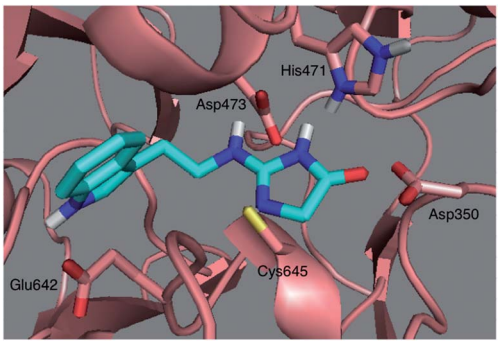

Fig. 6 Molecular modeling of the binding of imidazolin-4-one **3** (turquoise) to PAD4 using AutoDock3.0 (ref. 24) and viewed in PyMOL, with depiction of residues in and near the catalytic site.

PAD3 activity in live cells. This is of particular interest since PAD3 has been found to be up-regulated following spinal cord injury in which extensive neural tissue loss has occurred, and such loss was significantly reduced by Cl-amidine treatment.[16]

Using AutoDock3.0,[24] modeling of the binding of imidazolin-4-one **3** in PAD4 (prepared from PDB code 3B1U) showed a strong preference for a pose (Fig. 6) of the imidazolin-4-one ring in proximity to PAD4 residues involved in hydrolytic deimination,[22] including His471, the nucleophilic Cys645, and especially to Asp350 and Asp473 participating in hydrogen bonding. Even an unsubstituted indole ring proves to be an acceptable replacement for the amide chain present in BAA. This modeling confirms the efficacy of a 2-amino-3,5-dihydroimidazol-4-one moiety as a surrogate for a guanidine terminus, and also suggests that even more potent, and perhaps selective, inhibition of PADs should result from the addition of appropriate polar substituents to the indole ring present in **3**.

Conclusions

The structure-based design and synthesis of a novel non-peptidic drug-like PAD inhibitor **3** has been achieved, in which a heterocyclic ring replaces the acyclic guanidine-binding unit present in arginine residues. In human cell-based assays, imidazolin-4-one **3** showed significantly more potency than Cl-amidine, a benchmark PAD inhibitor, and in some cases between 10 and 100 times more potency. At all concentrations tested, the novel PAD inhibitor **3** exhibited no toxicity towards human neural stem cells and appeared to increase the percentage of live cells as compared to controls. This proof-of-principle of a new and drug-like PAD inhibitor effective on human cells, and with potencies at a therapeutic level is timely, given that PADs have been suggested as novel therapeutic targets for several diseases, including cancer, multiple sclerosis, rheumatoid arthritis and neural injury.[10,16–19]

Materials and methods

Synthesis of 2-[2-(1*H*-indol-3-yl)-ethylamino]-3,5-dihydroimidazol-4-one (3)

A mixture of 1-acetyl-2-methylthiohydantoin (0.469 g, 2.73 mmol) and tryptamine (0.438 g, 2.74 mmol) in ethanol (4 mL)

was heated at reflux until t.l.c. showed that the initially formed 1-acetyl-2-[2-(1*H*-indol-3-yl)-ethylamino]-3,5-dihydroimidazol-4-one (about 4 days) had been consumed. After allowing to cool, the precipitate was filtered and washed with cold ethanol to give 2-[2-(1*H*-indol-3-yl)-ethylamino]-3,5-dihydroimidazol-4-one **3** (0.36 g, 55%) as a yellow solid, mp 222–223 °C; IR (ν_{max}) (thin film) 3292, 1693, 1633, 1564 cm^{-1}; ^1H NMR (2 : 1 mixture of tautomers) (DMSO-d_6, 293 K, 300 MHz) δ_H 10.85 (1H, s, indole NH), 8.19 (1H, br. s, imidazolin-4-one ring NH, minor tautomer), 8.00 (1H, br. s, imidazolin-4-one ring NH, minor tautomer), 7.55 (1H, d, *J* 6.9 Hz, 4-indolyl and imidazolin-4-one ring NH, major tautomer), 7.36 (1H, d, *J* 6.9 Hz, 7-indolyl and imidazolin-4-one ring NH, major tautomer), 7.32 (1H, br. s, CH_2NH), 7.16 (1H, s, 2-indolyl), 7.08 (1H, t, *J* 6.9 Hz, 6-indolyl), 6.98 (1H, t, *J* 6.9 Hz, 5-indolyl), 3.65 (2H, br. m, CH_2CO), 3.50 (2H, br. m, CH_2CH_2NH), 2.93 (2H, t, *J* 7.0 Hz, CH_2CH_2NH); ^{13}C NMR (DMSO-d_6, 293 K, 75 MHz) δ_C 187.4, 186.7, 172.4, 171.6, 136.2, 127.1, 122.9, 122.8, 121.0, 118.3, 111.4, 111.3, 49.7, 42.5, 41.8, 25.5, 24.7 (tautomers); LRMS *m*/*z* (EI^+, %) 242 (M^+, 5), 143 (100), 130 (46), calcd for $C_{13}H_{14}N_4O$ 242.1162; found: 242.1157.

Treatment of cells with compounds

Imidazolin-4-one **3** was dissolved in (DMSO) and used at 0.1–1000 μM final concentration. Cl-amidine (Cambridge Bioscience Ltd.) was dissolved in phosphate buffer saline (PBS) and used at 0.1–1000 μM final concentration. Thapsigargin (Sigma) was dissolved in ethanol and used at either 1 or 5 μM final concentration. Cells were treated with each compound, or the appropriate vehicle(s) as a control, alone or in combination. In combination experiments, imidazolin-4-one **3** or Cl-amidine were applied 15 min before addition of thapsigargin.

Cells and methylene blue assay

Human Embryonic Kidney 293T (HEK293T) cells were grown and transfected when at 60% confluency with a human recombinant PAD3 plasmid using lipofectamine LTX (Invitrogen) as previously described.[17,25] The human embryonic neural stem cell line was grown as previously described.[17,26] Analysis of cell survival was assessed in 96-well plates after 24 h treatment with the different compounds using the methylene blue assay as previously reported.[27]

PAD enzymatic assay

The benzoyl L-arginine ethyl ester (BAEE) enzymatic assay was used to compare imidazolin-4-one **3** and Cl-amidine inhibitory activity in protein extracts from HEK293T expressing human recombinant PAD3. BAEE is a non-natural PAD substrate converted by PAD activity to sodium benzoyl-L-citrulline. The BAEE assay detects colorimetrically the amount of citrulline from BAEE produced by PAD activity using carbidino detection reagents and was carried out with minor modifications from published protocols.[7] The amount of red pigment developed upon heating at 100 °C for 10 min was measured at 490 nm (Sigma Protocol, EC 3.5.3.15) using a microplate reader (Revelation v4.21 Dynex Technologies, inc).

Acknowledgements

We acknowledge support from the BBSRC and the Child Research Appeal Trust.

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