Application Note

Histone H3 citrullination as a measure of PAD4 activity/inhibition

Julie M. Rumble, Ph.D., Elli M. Fackelman, and James L. Mobley, Ph.D.
Cayman Chemical Company

Key Features

- Citrullinated histone H3 (CitH3) can be used as a readout for PAD4 activity
- PAD4 inhibition decreases CitH3 in both HL-60 cells and PMN
- Differentiated HL-60 cells behave differently than primary neutrophils in regard to CitH3 measurements
- Sample preparation is key to designing experiments to analyze histone H3 citrullination

Introduction

Protein arginine deiminase (PAD) enzymes and the arginine to citrulline reaction that they catalyze (**Figure 1**) have increased in prominence in recent years, as dysregulation is associated with multiple inflammatory and autoimmune diseases. Under normal circumstances, PAD4, which is expressed by differentiated neutrophils, is required for the generation of neutrophil extracellular traps (NETs) upon encounter with certain pathogens. PAD4 citrullinates histone H3, which reduces its binding affinity for DNA and promotes the decondensation of chromatin and ensuing extrusion of nuclear DNA into a web-like structure that is studded with histones, antimicrobial enzymes, and defensin peptides.[1-3] These NETs are important in innate immune responses to infection, enabling the capture, killing, and phagocytosis of bacteria and other pathogens. As such, a loss of PAD4 can lead to the inability to eradicate otherwise harmless pathogens.[4] NET clearance is an equally important process, as excessive circulating NET fragments could be toxic and could stimulate autoantibody production. This process is thought to be involved in the pathogenesis of systemic lupus erythematosus, which is characterized by accumulation of autoantibodies to dsDNA and histones.[5]

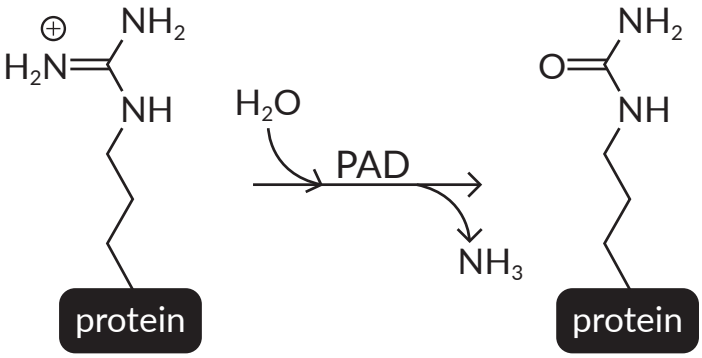

Figure 1. Citrullination is the process of deimination of charged arginine residues to neutral citrulline residues within proteins.

Antibodies against citrullinated proteins were first identified in rheumatoid arthritis (RA) patients in 1998,[6] and led to the creation of a diagnostic test for anti-citrullinated protein antibodies (ACPA) in patients suspected to have RA. Since then, an abundance of evidence has accumulated for a critical importance for PAD activity and NET formation and clearance in the pathogenesis of multiple inflammatory and autoimmune disorders.[7-10] PAD activity is largely restricted to specific tissues and is regulated transcriptionally, translationally, and by the availability of calcium.[11] Both PAD2 and PAD4 are expressed in immune cells, and overexpression and increased activity have been described not only in autoimmune diseases but also in cancer development.[7,8,12] The transient nature of NETs can make them challenging to study. Cayman Chemical has worked to develop a series of tools to enable researchers to study the processes involved in PAD activation and NETosis. Selective inhibition of PADs may prove to be a therapeutic target for some autoimmune disorders,[13] and development of simple methods for evaluating expression and activity in cells and *in vitro* will be useful in the pursuit of effective drugs. Here, we outline how these tools perform in a myelomonocytic cell line, HL-60, which can be differentiated into a neutrophil-like cell, and compare this to primary neutrophils.

Materials and Methods

Cells and treatments

HL-60 and THP-1 cells were differentiated for 72 hours at 37°C with either 25 nM PMA (Item No. 10008014), 1.25% DMSO, 100 mM DMF, 0.1 µM all-*trans* retinoic acid (ATRA; Item No. 11017), or 100 nM vitamin D_3 (vit D3; Item No. 11792). Polymorphonuclear leukocytes (PMN) were enriched using Cell-Based Assay Neutrophil Isolation Histopaque® (Item No. 600612) and red blood cells were lysed. For stimulation experiments, differentiated cells and PMN were stimulated with 25 µM A23187 (Item No. 11016) in complete IMDM (Iscove's Modified Dulbecco's Medium with 10% FBS, L-glutamine, and penicillin/streptomycin) for 2-3 hours. To test S7 nuclease treatment of conditioned media, 15 U/ml S7 nuclease was added to complete IMDM + 2.5 mM $CaCl_2$. For inhibition experiments, differentiated cells and PMN were pretreated with either 3 µM BB-Cl-amidine (Item No. 17079) or 10 µM GSK106 (hydrochloride) (Item No. 17490) or GSK199 (hydrochloride) (Item No. 17489) for 15 minutes before stimulation. Conditioned media were saved and cells were lysed as indicated by three freeze-thaw cycles in PBS or lysis buffer (1% Triton X-100 in 100 mM phosphate buffer, pH 7.0 or M-PER containing protease inhibitors) followed by treatment of the detergent-insoluble fraction with 0.4 M H_2SO_4 or 15 U/ml S7 nuclease in complete IMDM + 2.5 mM $CaCl_2$ for 30 minutes at 37°C, quenched by 10 mM EDTA (final).

Assays

A battery of assays was employed to better understand PAD4 expression and enzyme activity in commonly used cell types. First, inhibition of human recombinant PAD4 was tested using Cayman Chemical's PAD4 Inhibitor Screening Assay Kit (Ammonia) (Item No. 700560). Next, PAD2 and PAD4 were quantified in lysates using Cayman Chemical's PAD2 (human) ELISA Kit (Item No. 501450) and PAD4 (human) ELISA Kit (Item No. 501460), respectively. Then, citrullinated histone H3 was quantified by ELISA using Cayman Chemical's Citrullinated Histone H3 (Clone 11D3) ELISA Kit (Item No. 501620) and used as a functional cell-based readout for PAD4 activity.

Results and Discussion

To establish working concentrations for known inhibitors of PAD activity, Cayman Chemical's PAD4 Inhibitor Screening Assay Kit (Ammonia) was employed. This assay utilizes recombinant PAD4 to convert a substrate to ammonia, which reacts with a detector to form a fluorescent product. BB-Cl-amidine and GSK484 (hydrochloride) (Item No. 17488) inhibited PAD4 activity with pIC_{50} values of 4.99 ± 0.03 and 5.206 ± 0.07, respectively (**Figure 2**).

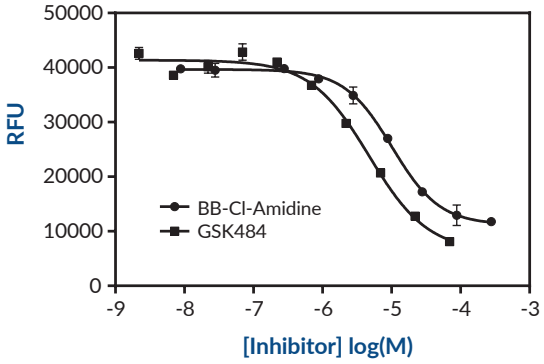

Figure 2. Inhibition of human recombinant PAD4 by BB-Cl-amidine and GSK484.

Results and Discussion *Continued*

To bring these inhibitors into a cell-based assay for PAD activity, we first needed to establish cell conditions for expression of PAD proteins. For this purpose, HL-60 and THP-1 cells were differentiated with either PMA, DMSO, DMF, ATRA, or vit D3 (**Figure 3**). Cells were lysed by three freeze-thaw cycles in PBS. PAD2 and PAD4 were quantified in clarified lysates by ELISA using Cayman Chemical's PAD2 (human) ELISA Kit and PAD4 (human) ELISA Kit. PAD2 expression was greatest in HL-60 cells after differentiation with ATRA, while PAD4 was induced in HL-60 cells by DMSO and DMF treatment, which are known to be strong inducers of neutrophilic differentiation.

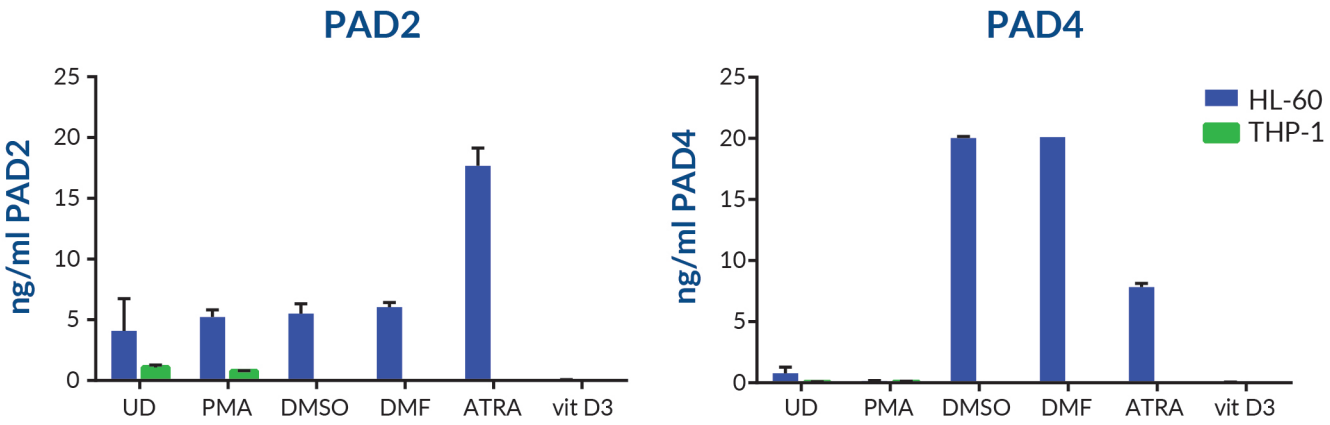

Figure 3. HL-60 cells can be induced to upregulate expression of PAD enzymes by specific differentiation stimuli.

Because citrullination of histone H3 in neutrophils is a well-known function of PAD4, we sought to use citrullinated histone H3 as a readout for PAD4 activity in differentiated HL-60 cells and primary human PMN. The conditions for detection were unknown, however, so several methods of cell lysis were tested for optimal detection of citrullinated histone H3. After HL-60 differentiation, cells were washed and stimulated with A23187 for 3 hours. Conditioned media were collected, and cells were lysed with either Triton X-100 or M-PER lysis buffer containing protease inhibitors followed by treatment of the detergent-insoluble fraction using S7 nuclease or H_2SO_4. Citrullinated histone H3 was quantified by ELISA in all lysates using Cayman Chemical's Citrullinated Histone H3 (Clone 11D3) ELISA Kit. Optimal specific detection of citrullinated histone H3 in stimulated cells was shown to be from conditioned media from HL-60 cells (**Figure 4A**). Under these conditions, A23187 induced a strong citrullination response in DMF- or DMSO-differentiated HL-60 cells, the same differentiation conditions that induced PAD4 expression. Notably, citrullination was detected in Triton X-100 and M-PER lysates, however M-PER lysis yielded substantially more "background", that is, citrullinated histone H3 in unstimulated cells, than Triton X-100 lysis (**Figure 4B and C**). Substantial citrullinated histone H3 was observed in insoluble lysates, but there was little to no increase upon differentiation or stimulation (**Figure 4D and E**).

Results and Discussion *Continued*

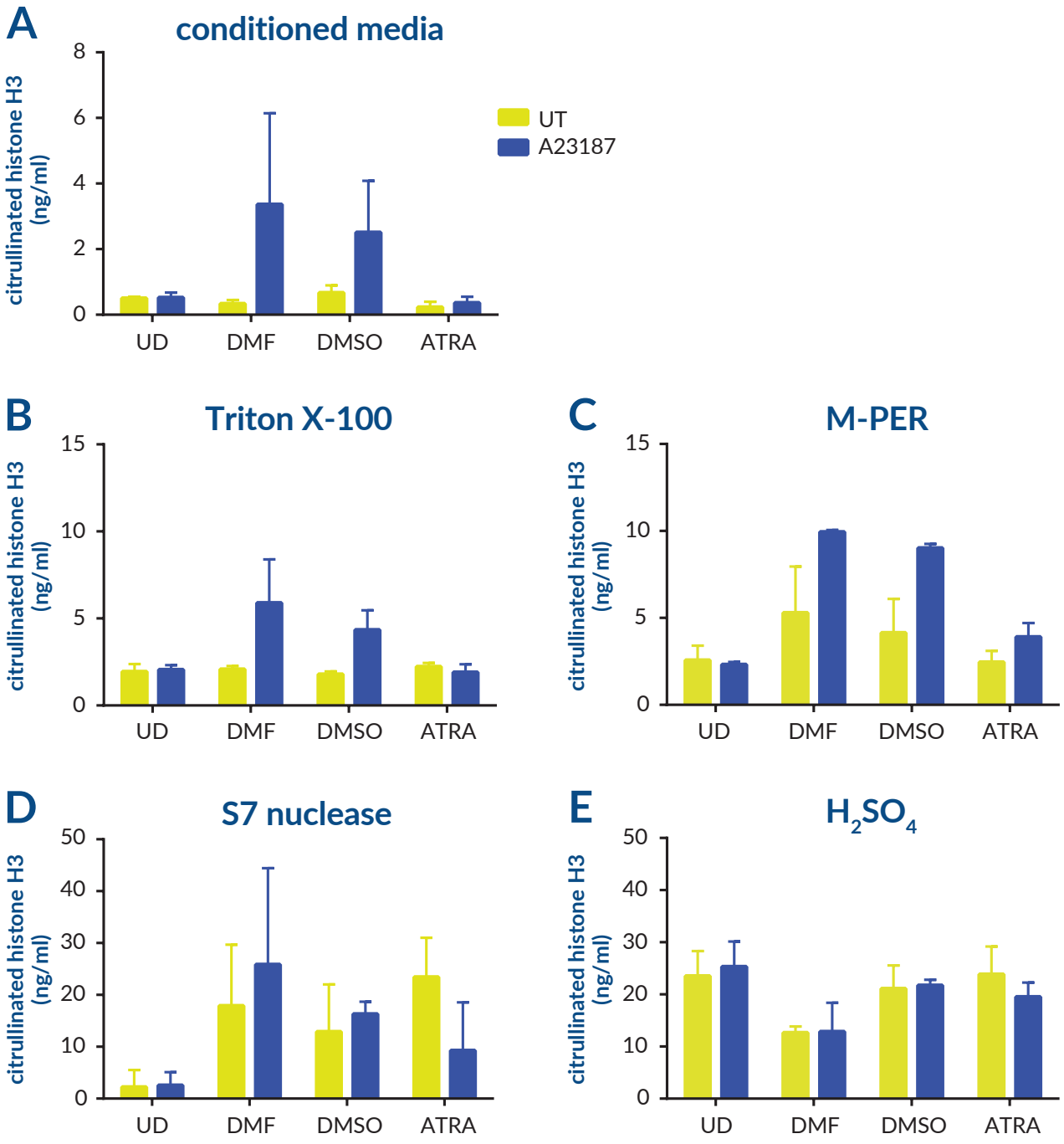

Figure 4. Citrullinated histone H3 is detectable in various fractions of differentiated HL-60 cells upon stimulation with A23187.

As differentiated HL-60 cells are designed to be a model of PMN, we tested the expression of PAD2 and PAD4 in the fractions described above from PMN cultured for 2 hours in the absence of stimulus. As shown in **Figure 5**, both PAD2 and PAD4 were expressed by unstimulated PMN, though PAD2 was mostly localized to the Triton X-100 soluble fraction, while PAD4 was found in the insoluble fraction, extracted by treatment with S7 nuclease. The expression pattern remained unchanged with A23187 stimulation (data not shown).

Results and Discussion *Continued*

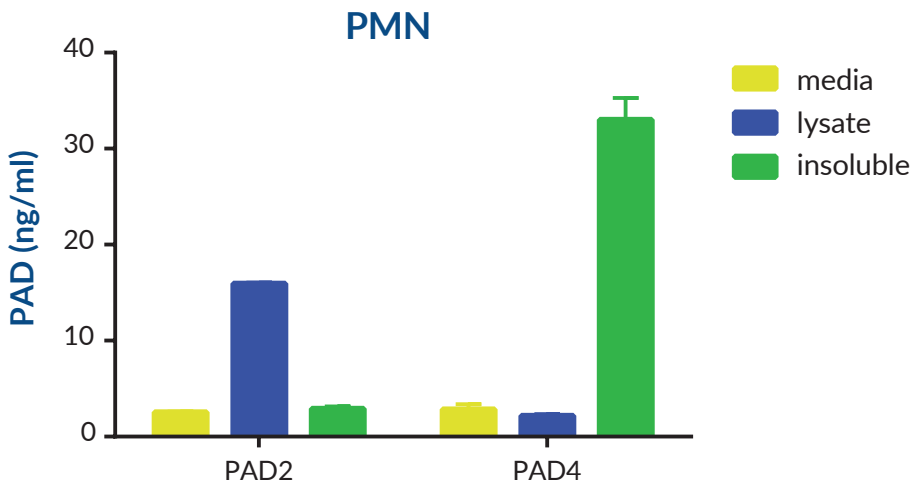

Figure 5. PAD2 and PAD4 are expressed by PMN.

PMN undergoing NETosis release complex NETs of DNA and protein, making detection of specific epitopes a concern within the conditioned media, so we tested the addition of S7 nuclease to the conditioned media of stimulated cells. Differentiated HL-60 and isolated PMN were left unstimulated or treated with A23187 in the presence or absence of S7 nuclease, and after 3 hours, conditioned media were collected and subjected to citrullinated histone H3 ELISA (**Figure 6**). Interestingly, S7 nuclease treatment dramatically increased specific detection in PMN cultures while decreasing detection in HL-60 cells. The increase in PMN was expected, as S7 nuclease should release histones from the accumulated DNA, but the decrease in HL-60 cultures was unexpected. It signals that differentiated HL-60 cells are not undergoing traditional NETosis, and citrullinated histone release in this cell line may have a different mechanism. Even so, differentiated, stimulated HL-60 cells produced significantly more citrullinated histone H3 than control cells, confirming the value of HL-60 conditioned media as a readout for citrullination of histone H3 and PAD activation.

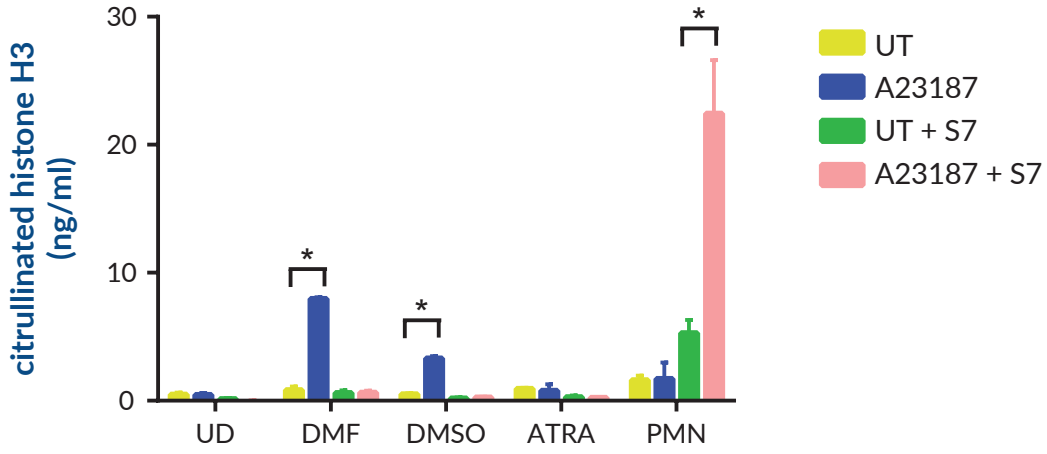

Figure 6. PMN require S7 nuclease for detection of citrullinated histone H3 in conditioned media, while S7 nuclease inhibits detection in HL-60 cells.

Results and Discussion *Continued*

Despite the finding that differentiated HL-60 may not utilize the same mechanism to citrullinate histone H3 and undergo NETosis, PAD4 was specifically upregulated in differentiated HL-60 cells, so we treated these cells and PMN with known PAD4 inhibitors to test the responsiveness of histone H3 citrullination to PAD4 inhibition. Differentiated HL-60 cells and enriched PMN were pretreated with GSK106 (negative control), GSK199, or BB-Cl-amidine followed by stimulation with A23187. In the PMN cultures, S7 nuclease was added to release histones from NETs. Conditioned media were collected 3 hours after stimulation, and citrullinated histone H3 was quantified by ELISA (**Figure 7**). In HL-60 cells, GSK199 trended towards decreasing, and BB-Cl-amidine significantly decreased citrullination of histone H3 as compared with GSK106. BB-Cl-amidine also significantly decreased histone H3 citrullination in PMN, while GSK199 did not have an effect.

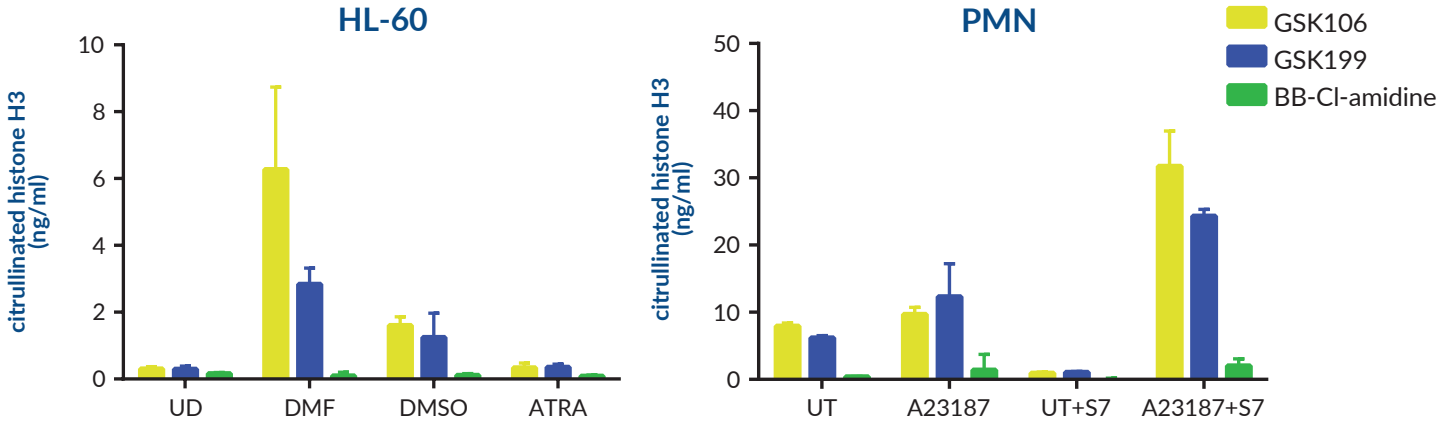

Figure 7. Known PAD inhibitor BB-Cl-amidine decreases citrullination of histone H3 detected in conditioned media from differentiated, stimulated HL-60 cells and PMN.

Methods for cell treatment and processing seem to be very important to the assays described in this note. Differentiation of HL-60 using DMSO or DMF upregulated PAD4 expression, while using ATRA upregulated PAD2 expression. The ability to focus on a single PAD may make this system an attractive one for better understanding PAD activity in cells, especially when trying to evaluate specific inhibitors. On the other hand, PMN express both PAD2 and PAD4, which seem to be associated with different compartments, as PAD2 is detergent-soluble, while PAD4 is localized to the detergent-insoluble fraction. The expression of PAD4 seems to be tightly associated with the citrullination of histone H3 in both HL-60 and PMN. Using Cayman's Citrullinated Histone H3 (Clone 11D3) ELISA Kit in HL-60 cells, citrullinated histone H3 was coincident with the conditions under which maximal PAD4 was expressed. In PMN, the presence of PAD4 in the detergent-insoluble fraction could also indicate association with histones, though more thorough localization studies would be required to strengthen this evidence. For preparation of samples for detection of citrullinated histone H3, conditioned media from stimulated HL-60 and PMN yielded the most specific citrullinated histone signal in HL-60 cells. Importantly, conditioned media from PMN needed to be treated with nuclease for optimal detection, while HL-60 conditioned media do not tolerate nuclease addition. We used these two cell systems to evaluate two different known PAD4 inhibitors, using citrullination of histone H3 as a readout for PAD4

Results and Discussion *Continued*

activity. Here, PMN citrullination was not affected by GSK199, while citrullination was significantly decreased by BB-Cl-amidine. It should be noted that PMN were shown to express both PAD2 and PAD4, and it is possible that PAD2 could compensate for inhibition of PAD4. *In vitro* studies have shown that PAD2 is capable of citrullinating histone H3.[14] In HL-60 cells, the GSK compound led to a non-significant decrease in citrullination of histone H3, while BB-Cl-amidine significantly decreased it. Again, these cells expressed much lower levels of PAD2, making the likelihood of compensation lower.

Contact contractresearch@caymanchem.com for full-service contract screening and profiling to identify particular modulators of PAD.

Cayman products used in this application

Assays

Item No.	Product Name
600612	Cell-Based Assay Neutrophil Isolation Histopaque®
501620	Citrullinated Histone H3 (Clone 11D3) ELISA Kit
501450	PAD2 (human) ELISA Kit
501460	PAD4 (human) ELISA Kit
700560	PAD4 Inhibitor Screening Assay Kit (Ammonia)

Biochemicals

Item No.	Product Name
11016	A23187
17079	BB-Cl-Amidine
17490	GSK106 (hydrochloride)
17489	GSK199 (hydrochloride)
17488	GSK484 (hydrochloride)
10008014	Phorbol 12-myristate 13-acetate
11017	Retinoic Acid
11792	Vitamin D_3

References

1. Lewis, H.D., Liddle, J., Coote, J.E., *et al. Nat. Chem. Biol.* **11(3)**, 189-191 (2015).
2. Li, Y., Smith, T., Grabski, S., *et al. J. Biol. Chem.* **273(45)**, 29830-29837 (1998).
3. Brinkmann, V., Reichard, U., Goosmann, C., *et al. Science* **303(5663)**, 1532-1535 (2004).
4. Ceccon, M., Mologni, L., Bisson, W., *et al. Mol. Cancer Res.* **11(2)**, 122-132 (2013).
5. Garcia-Romo, G.S., Caielli, S., Vega, B., *et al. Sci. Transl. Med.* **3(73)**, 73ra20 (2011).
6. Schellekens, G.A., de Jong, B.A.W., van den Hoogen, F.H.J., *et al. J. Clin. Invest.* **101(1)**, 273-281 (1998).
7. Slack, J.L., Causey, C.P., and Thompson, P.R. *Cell Mol. Life Sci.* **68(4)**, 709-720 (2011).
8. Umeda, N., Matsumoto, I., Kawaguchi, H., *et al. Clin. Rheumatol.* **35(5)**, 1181-1188 (2016).
9. Savchenko, A.S., Martinod, K., Seidman, M.A., *et al. J. Thromb. Haemost.* **12(6)**, 860-870 (2014).
10. Matsumoto, H., Ma, W.-g., Daikoku, T., *et al. J. Biol. Chem.* **277(32)**, 29260-29267 (2002).
11. Vossenaar, E.R., Radstake, T.R.D., van der Heijden, A., *et al. Ann. Rheum. Dis.* **63(4)**, 373-381 (2004).
12. Chang, X. and Han, J. *Mol. Carcinog.* **45(3)**, 183-196 (2006).
13. Jones, J.E., Causey, C.P., Knuckley, B., *et al. Curr. Opin. Drug Discov. Devel.* **12(5)**, 616-627 (2009).
14. Dreyton, C.J., Knuckley, B., Jones, J.E., *et al., Biochemistry* **53(27)**, 4426-4433 (2014).