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ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 739–745
Bioorganic & Medicinal Chemistry

Profiling Protein Arginine Deiminase 4 (PAD4): A novel screen to identify PAD4 inhibitors

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Received 24 August 2007; revised 26 September 2007; accepted 10 October 2007
Available online 13 October 2007

Abstract—Protein Arginine Deiminase 4 (PAD4) has emerged as a leading target for the development of a Rheumatoid Arthritis (RA) pharmaceutical. Herein, we describe the development of a novel screen for PAD4 inhibitors that is based on a PAD4-targeted Activity-Based Protein Profiling reagent, denoted Rhodamine-conjugated F-Amidine (RFA). This screen was validated by screening 10 Disease Modifying Anti-Rheumatic Drugs (DMARDs) and identified streptomycin, minocycline, and chlortetracycline as micromolar inhibitors of PAD4 activity.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic progressive autoimmune disorder that ultimately leads to the destruction of the cartilage surrounding the joint. It is the second most common type of arthritis with symptoms first appearing in patients between 40 and 60 years of age.[1,2] Current RA therapeutics can be classified into 3 groups: Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), corticosteroids, and Disease Modifying Anti-Rheumatic Drugs (DMARDs).[3] The NSAIDs (e.g., aspirin, ibuprofen, naproxen) and corticosteroids encompass a large group of clinically effective compounds whose mode of action is well established – these compounds relieve pain and reduce inflammation by preventing prostaglandin synthesis through inhibition of cyclooxygenase 2 and the production of arachidonic acid, respectively.[3] The DMARDs are an equally large group of therapeutics that includes both chemical (i.e., small molecules) and biological agents, for example, antibody-based therapies. Examples of biological DMARDs include drugs such as etanercept, infliximab, and tocilizumab, which are therapeutically effective because they reduce the levels of inflammatory cytokines.[3] Examples of chemical DMARDs include methotrexate, minocycline, and leflunomide.[3] Interestingly, and in contrast to the well established modes of action of the NSAIDs, corticosteroids, and biological DMARDs, the molecular mechanisms by which the chemical DMARDs function as RA therapeutics are incompletely understood in several cases, for example, minocycline.[3]

Protein Arginine Deiminase 4 (PAD4), which catalyzes the conversion of peptidyl-arginine to peptidyl-citrulline,[4] is widely believed to play a causative role in RA disease onset and progression because RA-associated mutations in the PAD4 gene have been identified in a variety of populations[5–7] and RA patients produce autoantibodies that recognize citrulline-containing proteins.[8,9] Interestingly, the anti-citrulline autoantibodies are considered to be the most specific diagnostic marker of this disease[8–10] and there is a direct correlation between the levels of citrullinated proteins and disease severity, especially in the formative stages of RA.[11] In total, the serological and genetic data suggest that PAD4 activity is dysregulated in RA; thereby suggesting this enzyme as a target for the development of a novel RA therapeutic.

While we have reported the development of the two most potent PAD4 inhibitors described to date,[12,13] we were curious to see if one or more of the aforementioned chemical DMARDs could inhibit this enzyme and thereby offer an explanation for their clinical efficacy. However, the standard PAD4 assay, which measures citrulline formation, is not readily amenable to high or even low-throughput screens because it suffers from

Keywords: Protein Arginine Deiminase 4; Minocycline; Streptomycin; Chlortetracycline; Tetracycline; Rheumatoid Arthritis; DMARD; arginine; Assay; Screen; Arginine deiminase; Inhibitor; Rhodamine; F-amidine; Fluoro; RFA; Activity-Based Protein Profiling; ABPP.
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doi:10.1016/j.bmc.2007.10.021

several limitations, including the fact that it requires the use of strong acids, toxic reagents, and high temperatures to convert the ureido group into a chromophore that absorbs light at 540 nm.[14] Additionally, a number of compounds interfere with this assay,[14] suggesting that potential inhibitors may be missed during the screening process. Therefore, we developed a novel inhibitor screen that takes advantage of a recently described PAD4-targeted Activity-Based Protein Profiling (ABPP) reagent that is denoted Rhodamine-conjugated F-amidine (RFA)[15] (Fig. 1)—RFA links a novel mechanism-based inactivator[13] to a fluorophore and has previously been used to label purified PAD4 as well as enzyme present in cell extracts.[15] The screen described herein is essentially a competition assay in which library components compete with RFA for binding and covalent modification of PAD4. While similar to competitive ABPP strategies for identifying inhibitors in complex proteomes,[16–18] this screening assay is, to our knowledge, the first reported use of competitive ABPP to overcome the limitations of current assays in a system with purified proteins. Herein we report the first description of this ABPP-based screen and demonstrate its utility in identifying PAD4 inhibitors. Significantly, streptomycin, chlortetracycline, and minocycline were all identified as PAD4 inhibitors; and while the potency of these compounds is relatively weak, their identification suggests several new chemical scaffolds that can be exploited in the design of future PAD4 inhibitors.

2. Results

The development of rapid and accurate detection methods of enzyme activity is vital for the discovery of enzyme inhibitors via high-throughput screening of compound libraries. However with regard to PAD4, current assays are not readily amenable to high-throughput screens. Therefore, a novel inhibitory screen was developed. This is a competition assay in which an individual member of a library of compounds competes with a recently described PAD4-targeted ABPP, that is, RFA, for binding to PAD4 (Fig. 1). Briefly, compounds are incubated individually with PAD4 in the presence of RFA for 30 min at 37 °C; at which point the reactions are quenched with SDS–PAGE loading dye and the mixtures are run on an SDS–PAGE gel. The amount of fluorescent PAD4 can then be quantified using a molecular imaging system. PAD4 inhibitors are readily identified by a visual decrease in fluorescence intensity. Using this simple but straightforward assay, we were able to rapidly screen 10 DMARDs (sulfamethoxazole, trimethoprim, 5-aminosalicylic acid, minocycline, leflunomide, methotrexate, sulfapyridine, azathioprine, clindamycin, azithromycin) as well as streptomycin for their ability to inhibit PAD4. Streptomycin was tested because this antibiotic contains two guanidinium groups that could potentially mimic the guanidinium group of an Arg residue and thereby act as either a substrate or inhibitor of PAD4. The results of these studies were quite interesting because they quickly identified minocycline and streptomycin as PAD4 inhibitors (Fig. 2), as can be seen by the strong decrease in fluorescence intensity in the lanes corresponding to these two compounds.

To help validate this novel ABPP-based inhibitory screen, we determined the IC_{50} values for all of the aforementioned compounds. The results of these studies, which are depicted in Table 1 and Figure 2b, are

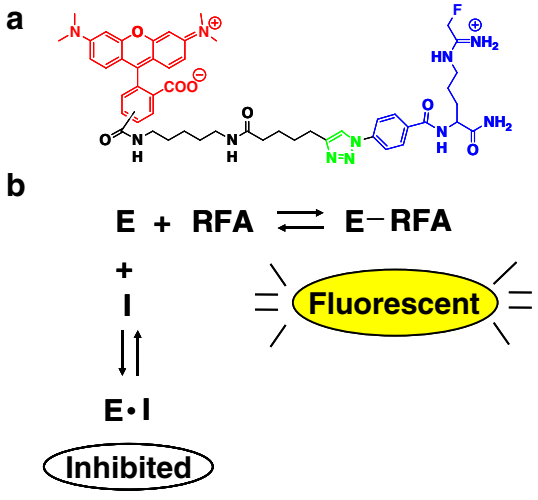


Figure 1. (a) The structure of Rhodamine-conjugated F-Amidine (RFA). (b) RFA can covalently modify the active site of the enzyme (E), rendering the protein fluorescent, or the inhibitor (I) can bind to the enzyme and inhibit this process.

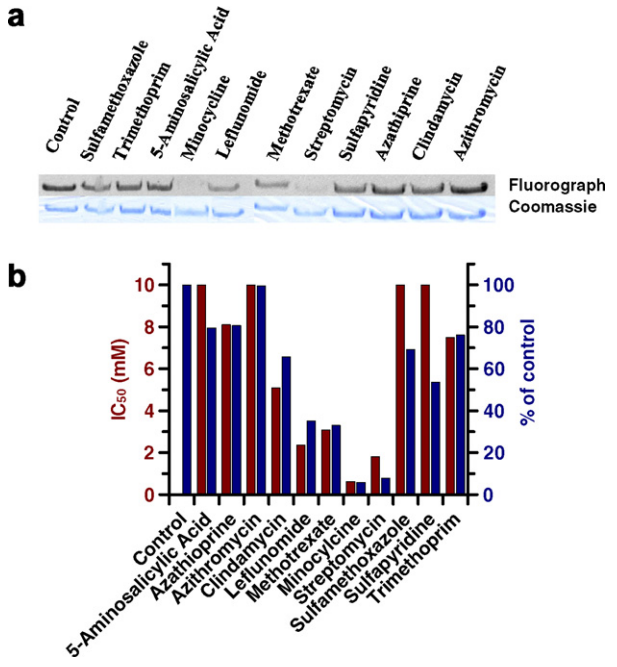
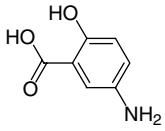
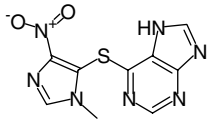
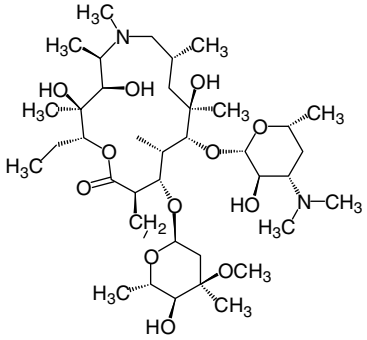
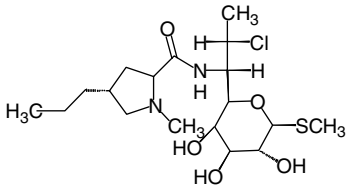
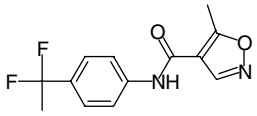
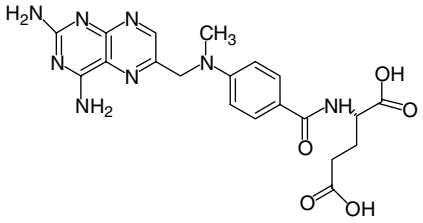
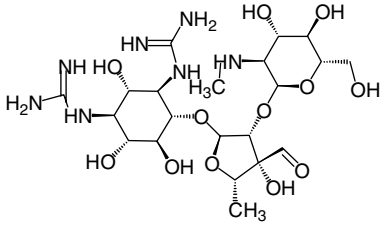
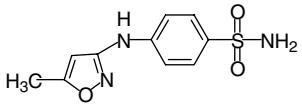


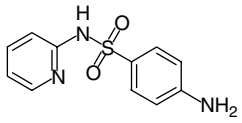
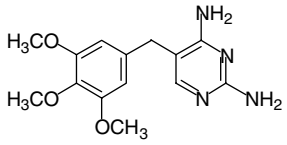
Figure 2. ABPP-based screen for PAD4 inhibitors. (a) Fluorescent image of SDS–PAGE gels (top) in which PAD4 was treated with RFA (10 μM final) in the absence and presence of the indicated DMARDs. The Coomassie stained SDS–PAGE gel is shown (bottom) to demonstrate equal protein loading. (b) A plot of the IC_{50} values for the various DMARDs compared to the fluorescence quantified as a percent of the control. Blue bars represent the fluorescence intensity as a percent of control. Red bars represent the IC_{50} of individual library components.

Table 1. Structures and IC_{50}s of DMARDs tested in this study

Compound	Structure	IC_{50}[a] (mM)
5-Aminosalicylic acid		>10
Azathioprine		8.1 ± 0.8
Azithromycin		>10
Clindamycin		5.1 ± 0.3
Leflunomide		2.4 ± 0.8
Methotrexate		>10
Streptomycin		1.8 ± 0.3
Sulfamethoxazole		>10

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	IC_{50}[a] (mM)
Sulfapyridine		>10
Trimethoprim		7.5 ± 0.2

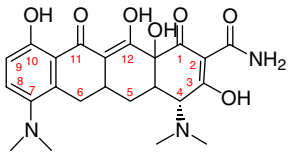
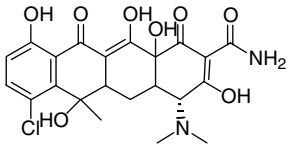
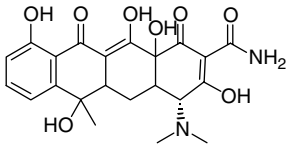
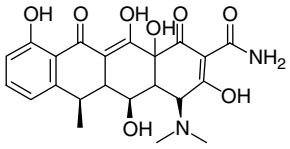
[a] IC_{50} values for the DMARDs screened determined using 1 mM BAEE.

consistent with our ABPP-based assay. For example, the IC_{50}s of 5-aminosalicylic acid, azathioprine, azithromycin, sulfamethoxazole, sulfapyridine, and trimethoprim were all above 7.5 mM in our standard assay and there was little to no decrease in the fluorescence of PAD4 when these compounds were incubated with the enzyme and RFA (Fig. 2). In contrast, the compounds that decreased the intensity of PAD4 fluorescence the most, that is, streptomycin and minocycline, were also the most potent compounds in our standard assay, with IC_{50} values of 1.8 and 0.62 mM, respectively. Intermediate effects on PAD4 fluorescence were observed for the remaining compounds (i.e., clindamycin, leflunomide, and methotrexate), consistent with their IC_{50}s. Please note that streptomycin was not deiminated by PAD4 despite the fact that this compound possesses two guanidinium groups.

The fact that minocycline was the most potent inhibitor identified in this screen suggested that other tetracycline derivatives might inhibit PAD4 with equal or greater potency. Therefore, we determined IC_{50} values for tetracycline, doxycycline, and chlortetracycline. The results of these studies (Table 2) indicate that chlortetracycline (IC_{50} = 100 μM) is significantly more potent (~5-fold) than minocycline (IC_{50} = 620 μM). Chlortetracycline differs from minocycline by the addition of a hydroxyl and methyl group at position 6 along with a chloro group replacing the dimethylamine moiety at position 7.

To further explore the significance of these findings, we evaluated the kinetic mechanism by which chlortetracycline, minocycline, and streptomycin inhibit PAD4 activity. For these studies, PAD4 activity was measured as a function of substrate concentration in the absence and presence of increasing concentrations of each inhibitor. Note that in all cases product formation was linear with respect to time, indicating that these compounds are reversible PAD4 inhibitors. Lineweaver–Burk plots ($1/v$ vs $1/[S]$) of the chlortetracycline inhibition data are consistent with mixed inhibition (Fig. 3a). The K_{ii} and K_{is} values are 0.11 ± 0.01 and 0.54 ± 0.53 mM, respectively. Minocycline was also a mixed type inhibitor with K_{ii} and K_{is} values of 0.63 ± 0.15 and 0.18 ± 0.06 mM, respectively (Fig. 3b). In contrast,

Table 2. Structures and IC_{50}s of tetracycline derivatives tested in this study

Compound	Structure	IC_{50}[a] (mM)
Minocycline		0.62 ± 0.01
Chlortetracycline		0.10 ± 0.01
Tetracycline		0.78 ± 0.14
Doxycycline		0.86 ± 0.08

[a] IC_{50} values for the tetracycline derivatives using 1 mM BAEE.

streptomycin is a competitive inhibitor of PAD4, as evidenced by the family of lines intersecting on the $1/v$-axis (Fig. 3c), with a K_{is} value equal to 0.56 ± 0.17 mM.

3. Discussion

While the molecular mechanisms linking a dysregulated PAD4 activity to RA are incompletely defined, an abundance of evidence strongly suggests that non-specific Arg deimination causes the production of citrulline-containing epitopes that the immune system recognizes as foreign, leading first to a break in tolerance, and then ultimately resulting in RA disease onset and progres-

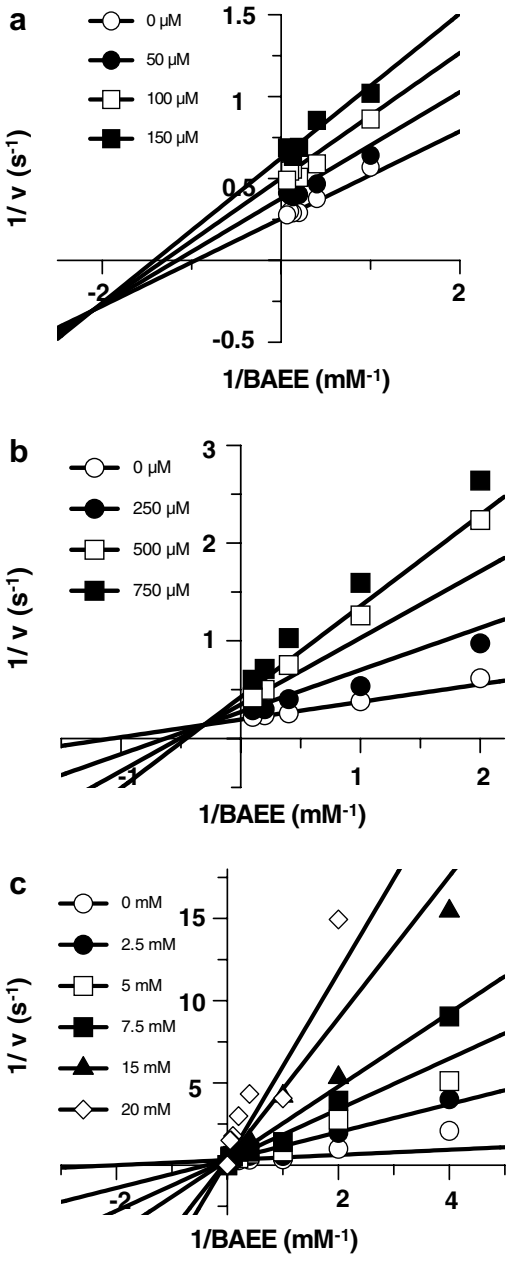


Figure 3. Lineweaver–Burk analyses of PAD4 inhibitors identified using the ABPP screen. (a) The mixed inhibition plot for chlortetracycline. (b) The mixed inhibition plot for minocycline. (c) The competitive inhibition plot for streptomycin. BAEE was used as the varied substrate.

sion. Because PAD4 inhibitors would decrease the levels of citrulline-containing proteins, this enzyme has recently emerged as an exciting new target for the development of a novel RA therapeutic. The need for new RA drugs is highlighted by the fact that current therapeutic regimens, for example, the NSAIDs and corticosteroids, are focused primarily on alleviating the pain and inflammation associated with this autoimmune disorder and not an underlying cause of the disease.

The well established utility of high-throughput screening efforts in drug discovery suggests that it may be possible to screen a large library of compounds and thereby identify novel PAD4 inhibitors and/or pharmacophores. However, the standard assay for PAD activity suffers from a host of limitations, including the need for strong acids (e.g., H_2SO_4 and H_3PO_4), high temperatures, and toxic compounds (e.g., diacetyl monoxime),[14] that preclude its widespread adoption as an assay for a high-throughput screen. Therefore, to overcome these limitations, we developed a novel method for screening large libraries of compounds for PAD4 inhibitors.

The methodology described herein is a simple and straightforward, but nonetheless elegant, assay that is based on a recently described ABPP that selectively modifies an active site Cys in PAD4, rendering the enzyme fluorescent. As proof of concept, we screened 10 DMARDs for their ability to inhibit PAD4. These FDA approved drugs were chosen for our initial screen because they have shown some efficacy in ameliorating RA disease progression (although side effects are common) and we felt that their value as RA therapeutics might be due in part to their ability to inhibit PAD4 activity.

Using this ABPP-based assay, we rapidly identified minocycline and streptomycin as PAD4 inhibitors. Their ability to act as PAD4 inhibitors was confirmed by comparing the decrease in fluorescence intensity to the IC_{50} values determined using the standard PAD4 assay. The ABBP-based assay was further validated by determining the IC_{50} values for the remaining compounds present in the screen and those values are consistent with the relative change in fluorescence intensity observed for all of the compounds tested. Because minocycline is a member of the tetracycline family of antibiotics, the inhibitory properties of chlortetracycline, tetracycline, and doxycycline were evaluated and this secondary screen identified chlortetracycline as a significantly more potent PAD4 inhibitor.

Kinetic studies with chlortetracycline, minocycline, and streptomycin—the three most potent inhibitors identified in our primary and secondary screens—revealed that chlortetracycline and minocycline are mixed inhibitors whereas streptomycin is a competitive inhibitor. These results are consistent with the notion that streptomycin binds within the active site of PAD4, or alternatively in close proximity to it—the two guanidinium groups on streptomycin are likely important for this interaction. In contrast, the fact that minocycline and chlortetracycline are mixed inhibitors suggests that these two compounds bind to a site distal from the active site. Unfortunately, we have been unable to obtain structures of PAD4 bound to these compounds using soaking methods (M. Sato, personal communication). Therefore, it is difficult to comment on how or where these compounds bind to PAD4. Studies to co-crystallize these compounds with PAD4 are ongoing in the lab. While it is also difficult to comment on the increased potency of chlortetracycline relative to both minocycline and tetracycline, it should be noted that the major distinguishing feature of these three compounds is the presence of a chloro group at position 7 (Table 2). While speculative, the increased affinity of chlortetracycline

for PAD4 may be due to the electron withdrawing effects of the chloro group altering the electronics of the ring system.

Although the fact that the majority of the DMARDs evaluated here are relatively weak PAD4 inhibitors is somewhat disappointing, these findings are significant and will nonetheless be appreciated by the RA community because the data suggest that these compounds do not derive their efficacy from inhibiting PAD4. Furthermore, the identification of the tetracycline derivatives as micromolar inhibitors of PAD4 is particularly interesting because they not only represent potential scaffolds that could be further elaborated to identify inhibitors with greater potency and selectivity but their identification as PAD4 inhibitors is also consistent with previously reported studies on their usefulness as RA therapeutics.[19] For example, we have shown that tetracycline is a relatively weak inhibitor of PAD4 activity and this compound has been shown to be ineffective as an RA treatment.[19,20] In contrast, minocycline shows modest efficacy as an RA therapeutic, consistent with its being a more potent PAD4 inhibitor than tetracycline.[19,20] While the concentrations of minocycline required to inhibit PAD4 in vitro are quite high, we note that minocycline is known to become concentrated in inflammatory cells.[21] Although it is tempting to speculate that the efficacy of minocycline and other tetracycline derivatives is due in part to their ability to inhibit PAD4, we note that tetracycline derivatives are known to inhibit a wide range of enzymes, including collagenase,[22–24] poly(ADP-ribose) polymerase-1 (PARP-1),[25] and several cysteine proteinases[26], that may also contribute to their efficacy as an RA therapeutic and their ability to impair neutrophil chemotaxis and act as anti-inflammatory agents.[27]

In summary, we have developed a novel ABPP-based assay that overcomes the limitations of the commonly used PAD4 assay by eliminating the need for strong acids, high temperatures, and toxic compounds. While additional PAD activity assays have recently been described in the literature (e.g., an ELISA based assay that monitors PAD4 activity through the use of an anti-citrulline antibody[28] and a coupled assay that couples the production of ammonia to the glutamate dehydrogenase catalyzed reductive amination of α-ketoglutarate[29]), these assays provide only an indirect measure of PAD4 activity. In contrast, the assay described herein allows for the direct visualization of PAD4 inhibition and is therefore superior. Furthermore, this report represents the first use of a competitive ABPP assay to overcome the limitations of previous assays in a system with purified proteins; thereby providing an additional application for ABPP reagents and suggesting a general application for ABPPs in drug discovery. It is also important to recognize that while this report describes the use of a gel-based detection screen, it should be trivial to adapt the methodology described herein to a solution-based system using a 96-well multiscreen filter plate; thereby facilitating the screening of large libraries of compounds for PAD4 inhibitors. Finally, the tetracycline and streptomycin structures identified with this screen represent important chemical scaffolds that can serve as a starting point for the synthesis of future PAD4 inhibitors.

4. Experimental

4.1. General

Azathioprine, azithromycin, clindamycin, minocycline, streptomycin, sulfamethoxazole, 5-aminosalicylic acid, sulfapyridine, trimethoprim, tetracycline, dithiothreitol (DTT), and benzoyl L-arginine ethyl ester (BAEE) were acquired from Sigma–Aldrich (St. Louis, MO). Methotrexate was purchased from Fluka. Leflunomide was purchased from Toronto Research Chemicals (Ontario, Canada). Chlortetracycline and doxycycline were purchased from Alexis Biochemicals (San Diego, CA). The synthesis of RFA has previously been described.[15] Recombinant human PAD4 was expressed and purified based on previously described methods.[30]

4.2. Inhibitor screen

The compounds tested in the screen and their chemical structures are listed in Table 1. RFA (10 μM final) and a particular compound from the library (2.5 mM final) were pre-incubated in screening buffer (500 μM TCEP, 10 mM $CaCl_2$, 100 mM HEPES 7.6, 50 mM NaCl) for 10 min at 37 °C. PAD4 (2 μM final) was then added to this mixture and incubated for 30 min at 37 °C. The reaction was quenched with 6× SDS–PAGE Dye, incubated at 95 °C for 10 min, and then loaded onto a 12% SDS–PAGE gel. After electrophoresis, the gel was placed in destaining buffer (10% glacial acetic acid, 45% methanol, 45% ddH_2O) until the fluorescence was measured using a fluorescence based molecular imaging station (Kodak Image Station 2000MM; excitation at 535 nm, emission at 600 nm). The protein gel was then stained and destained to ensure equal protein loading. A sample without a potential inhibitor was used as a control.

4.3. IC_{50} studies

IC_{50} values were determined by pre-incubating various amounts of the potential inhibitor in reaction buffer (10 mM $CaCl_2$, 2 mM DTT, 50 mM NaCl, 100 mM Tris HCl, pH 7.6) and 0.2 μM PAD4 at 37 °C. After 15 min, BAEE (1 mM final; $0.74 \times K_m$) was allowed to proceed for 15 min (60 μL total volume). Reactions were quenched by flash freezing in liquid N_2. The amount of Cit produced was then quantified according to previously described methods.[14,31] IC_{50} values were determined by fitting the data to the following equation:

$$\text{Fractional activity} = 1/(1 + [\text{I}]/\text{IC}_{50}) \qquad (1)$$

using GraFit version 5.0.11.[32]

4.4. Inhibition studies

Inhibition constants were determined for minocycline, chlortetracycline, and streptomycin using standard kinetic

analyses. Briefly, the compounds were incubated in reaction buffer containing various concentrations of BAEE (0–20 mM) for 10 min at 37 °C. PAD4 (0.2 μM) was then added to initiate the reaction and allowed to proceed for six additional minutes. Reactions were then quenched by flash freezing in liquid N_2 and the amount of Cit produced was quantified as previously described.[14,31] The data obtained for streptomycin, minocycline, and chlortetracycline were fit to the following equations:

$$\frac{1}{v_o} = \frac{\left(1 + \frac{[I]}{K_{ii}}\right)K_m}{V_{max}}\left(\frac{1}{[S]}\right) + \frac{\left(1 + \frac{[I]}{K_{is}}\right)}{V_{max}} \quad (2)$$

$$\frac{1}{v_o} = \frac{\left(1 + \frac{[I]}{K_{ii}}\right)K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (3)$$

using GraFit version 5.0.11.

Acknowledgments

We thank Monica Bhatia and Justin Jones for critical reading of the manuscript and Jessica Slack for technical assistance. We thank the NIH and the American Heart Association for financial support.

References and notes

1. Akil, M.; Amos, R. S. *Br. Med. J.* **1995**, *310*, 587–590.
2. Finesilver, A. G. *Wisconsin Med. J.* **2003**, *102*, 34–37.
3. Smolen, J. S.; Steiner, G. *Nat. Rev. Drug. Discov.* **2003**, *2*, 473–488.
4. Thompson, P. R.; Fast, W. *ACS Chem. Biol.* **2006**, *1*, 433–441.
5. Suzuki, A.; Yamada, R.; Chang, X.; Tokuhiro, S.; Sawada, T.; Suzuki, M.; Nagasaki, M.; Nakayama-Hamada, M.; Kawaida, R.; Ono, M.; Ohtsuki, M.; Furukawa, H.; Yoshino, S.; Yukioka, M.; Tohma, S.; Matsubara, T.; Wakitani, S.; Teshima, R.; Nishioka, Y.; Sekine, A.; Iida, A.; Takahashi, A.; Tsunoda, T.; Nakamura, Y.; Yamamoto, K. *Nat. Genet.* **2003**, *34*, 395–402.
6. Iwamoto, T.; Ikari, K.; Nakamura, T.; Kuwahara, M.; Toyama, Y.; Tomatsu, T.; Momohara, S.; Kamatani, N. *Rheumatology (Oxford)* **2006**, *45*, 804–807.
7. Lee, Y. H.; Rho, Y. H.; Choi, S. J.; Ji, J. D.; Song, G. G. *Rheumatol. Int.* **2007**, *27*, 827–833.
8. Schellekens, G. A.; de Jong, B. A.; van den Hoogen, F. H.; van de Putte, L. B.; van Venrooij, W. J. *J. Clin. Invest.* **1998**, *101*, 273–281.
9. Schellekens, G. A.; Visser, H.; de Jong, B. A.; van den Hoogen, F. H.; Hazes, J. M.; Breedveld, F. C.; van Venrooij, W. J. *Arthritis Rheum.* **2000**, *43*, 155–163.
10. Girbal-Neuhauser, E.; Durieux, J. J.; Arnaud, M.; Dalbon, P.; Sebbag, M.; Vincent, C.; Simon, M.; Senshu, T.; Masson-Bessiere, C.; Jolivet-Reynaud, C.; Jolivet, M.; Serre, G. *J. Immunol.* **1999**, *162*, 585–594.
11. Lundberg, K.; Nijenhuis, S.; Vossenaar, E. R.; Palmblad, K.; van Venrooij, W. J.; Klareskog, L.; Zendman, A. J.; Harris, H. E. *Arthritis Res. Ther.* **2005**, *7*, R458–R467.
12. Luo, Y.; Arita, K.; Bhatia, M.; Knuckley, B.; Lee, Y. H.; Stallcup, M. R.; Thompson, P. R. *Biochemistry* **2006**, *45*, 11727–11736.
13. Luo, Y.; Knuckley, B.; Lee, Y. H.; Stallcup, M. R.; Thompson, P. R. *J. Am. Chem. Soc.* **2006**, *128*, 1092–1093.
14. Knipp, M.; Vasak, M. *Anal. Biochem.* **2000**, *286*, 257–264.
15. Luo, Y.; Knuckley, B.; Bhatia, M.; Thompson, P. R. *J. Am. Chem. Soc.* **2006**, *128*, 14468–14469.
16. Li, W.; Blankman, J. L.; Cravatt, B. F. *J. Am. Chem. Soc.* **2007**, *129*, 9594–9595.
17. Leung, D.; Hardouin, C.; Boger, D. L.; Cravatt, B. F. *Nat. Biotechnol.* **2003**, *21*, 687–691.
18. Leung, D.; Du, W.; Hardouin, C.; Cheng, H.; Hwang, I.; Cravatt, B. F.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1423–1428.
19. Trentham, D. E.; Dynesius-Trentham, R. A. *Rheum. Dis. Clin. North Am.* **1995**, *21*, 817–834.
20. Gompels, L. L.; Smith, A.; Charles, P. J.; Rogers, W.; Soon-Shiong, J.; Mitchell, A.; Dore, C.; Taylor, P. W.; Mackworth-Young, C. G. *J. Rheumatol.* **2006**, *33*, 224–227.
21. Pruzanski, W.; Vadas, P. *J. Rheumatol.* **1992**, *19*, 1495–1497.
22. Golub, L. M.; McNamara, T. F.; D'Angelo, G.; Greenwald, R. A.; Ramamurthy, N. S. *J. Dent. Res.* **1987**, *66*, 1310–1314.
23. Greenwald, R. A.; Golub, L. M.; Lavietes, B.; Ramamurthy, N. S.; Gruber, B.; Laskin, R. S.; McNamara, T. F. *J. Rheumatol.* **1987**, *14*, 28–32.
24. Golub, L. M.; Lee, H. M.; Lehrer, G.; Nemiroff, A.; McNamara, T. F.; Kaplan, R.; Ramamurthy, N. S. *J. Periodontal Res.* **1983**, *18*, 516–526.
25. Alano, C. C.; Kauppinen, T. M.; Valls, A. V.; Swanson, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 9685–9690.
26. Imamura, T.; Matsushita, K.; Travis, J.; Potempa, J. *Antimicrob. Agents Chemother.* **2001**, *45*, 2871–2876.
27. Forsgren, A.; Schmeling, D.; Quie, P. G. *J. Infect. Dis.* **1974**, *130*, 412–415.
28. Zendman, A. J.; Raijmakers, R.; Nijenhuis, S.; Vossenaar, E. R.; Tillaart, M.; Chirivi, R. G.; Raats, J. M.; van Venrooij, W. J.; Drijfhout, J. W.; Pruijn, G. J. *Anal. Biochem.* **2007**, *369*, 232–240.
29. Liao, Y. F.; Hsieh, H. C.; Liu, G. Y.; Hung, H. C. *Anal. Biochem.* **2005**, *347*, 176–181.
30. Knuckley, B.; Bhatia, M.; Thompson, P. R. *Biochemistry* **2007**, *46*, 6578–6587.
31. Kearney, P. L.; Bhatia, M.; Jones, N. G.; Luo, Y.; Glascock, M. C.; Catchings, K. L.; Yamada, M.; Thompson, P. R. *Biochemistry* **2005**, *44*, 10570–10582.
32. Leatherbarrow, R. J.; Erathicus Software: Staines, UK, 2004.