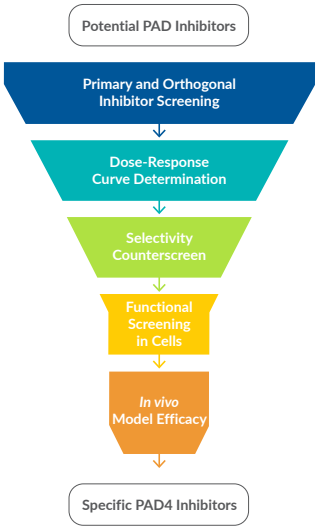
Screening Inhibitors of Citrullination

A Screening Funnel to Identify Selective PAD Inhibitors

The citrullination process is catalyzed by protein arginine deiminase (PAD) enzymes, whose activity is largely restricted to specific tissues and is regulated transcriptionally, translationally, and by the availability of calcium. Of the five known PAD isotypes, PAD2 and PAD4 are the most studied due to their impact in inflammation, histone modification, gene regulation, and autoimmune diseases. PAD2 is ubiquitously expressed in immune cells, skeletal muscle, spleen, brain, secretory glands, etc., and PAD4 is primarily restricted to neutrophils and eosinophils. Overexpression and increased activity of PAD2 and PAD4 have been described in several inflammatory and autoimmune diseases, as well as cancer.



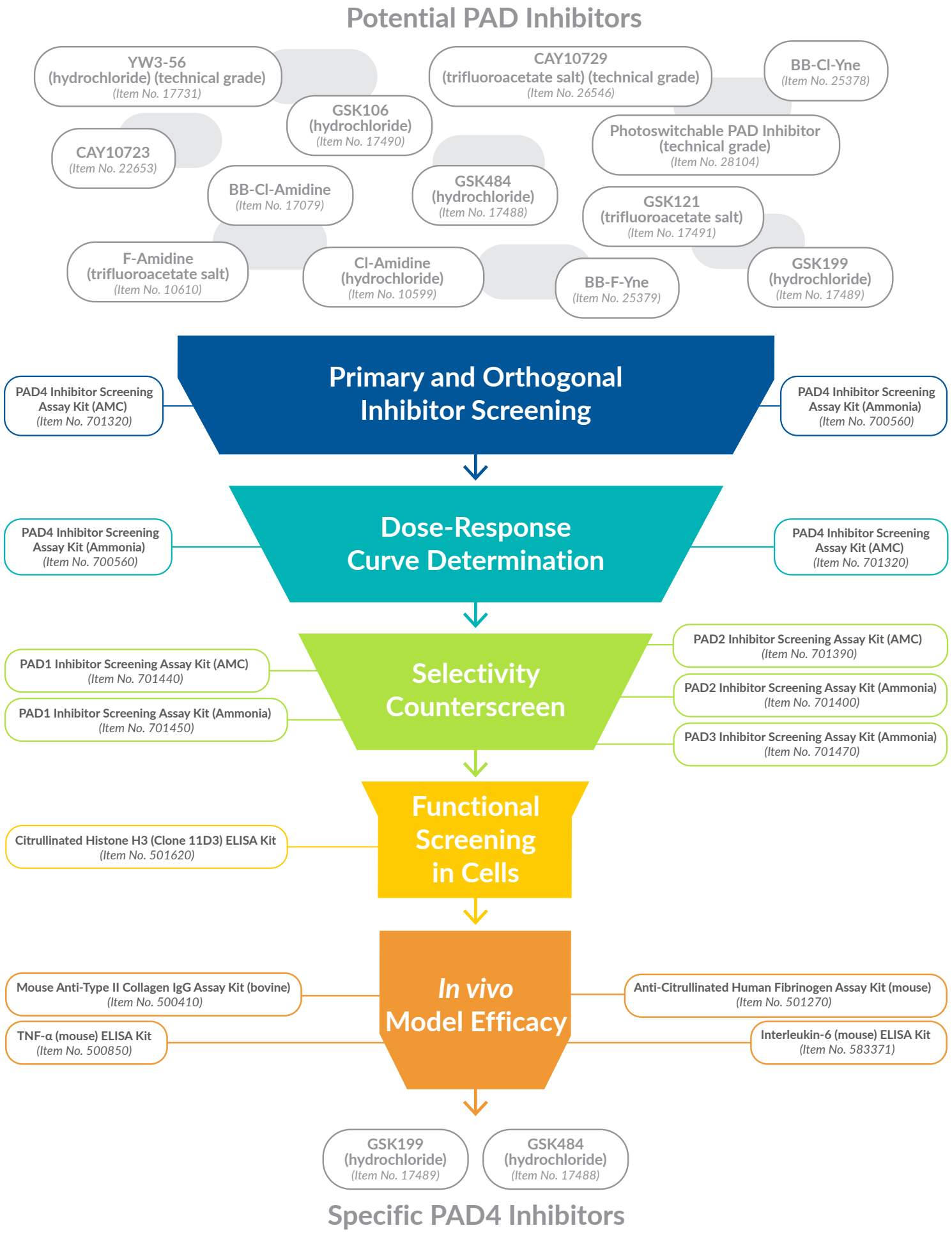
Because selective inhibition of PAD2 and PAD4 may prove to be a therapeutic target for certain human disorders, Cayman has developed a complete line of assays to enable the discovery of novel inhibitors of these enzymes. Antibodies, recombinant proteins, and a selection of inhibitors and probes have been developed to aid in evaluating the expression and activity of PAD in cells and *in vivo* efficacy models. Here we outline a logical screening workflow that exemplifies the use of Cayman's assays to narrow down specific PAD4 inhibitors from a large library of potential compounds in the pre-clinical pursuit of effective disease treatments (**see next page**). Though the example is specific for PAD4, a parallel set of screening assays is also available to specifically evaluate PAD1, PAD2, and PAD3 inhibitor activity.

Primary Inhibitor Screening

Cayman's PAD4 Inhibitor Screening Assay Kit (AMC) provides a convenient method to initially identify small molecules that inhibit human PAD4. This assay utilizes a fluorescent substrate consisting of a modified arginine residue coupled to a 7-amino-4-methylcoumarin (AMC) fluorophore. Acylation of AMC onto the arginine residue masks the fluorescence of the AMC. In the absence of PAD4, the substrate remains unaltered, allowing the developer to release free AMC. In the presence of PAD4, the arginine of the substrate is citrullinated, and when the reaction is quenched by the addition of the developer, free AMC cannot be released. In this reaction, the fluorescent signal is inversely proportional to the amount of citrullination by PAD4. Thus, a more intense signal indicates a greater degree of inhibition, allowing inhibitory compounds to be easily confirmed by visualizing a fluorescent signal.

Orthogonal Inhibitor Screening

Because false positives are inevitable in every high-throughput screening campaign, orthogonal assays are recommended to confirm hits and eliminate errors. Cayman's PAD4 Inhibitor Screening Assay Kit (Ammonia) provides an alternative method for screening human PAD4 inhibitors. In this assay, ammonia is produced when PAD4 deiminates N-α-benzoyl-L-arginine ethyl ester, a non-natural substrate with similar kinetic properties to PAD's natural substrates. Ammonia reacts with a detector, resulting in a fluorescent product. A negative result in this orthogonal assay would indicate that the primary hit was almost certainly an assay format-dependent artifact and not specific to the inhibition of PAD4. Compounds found to be active in both the AMC and ammonia formats are candidates for further analysis.



Pre-clinical screening funnel to identify selective PAD4 inhibitors.

Specificity and Selectivity Determination

Either the PAD4 Inhibitor Screening Assay Kit (AMC) or the PAD4 Inhibitor Screening Assay Kit (Ammonia) can once again be used to perform dose-response curve (DRC) experiments. Compounds must be tested over a wide range of concentrations to determine the concentration that results in half-maximal activity (IC_{50}). Subsequently, selectivity of the candidate small molecules for PAD4 over PAD1, PAD2, or PAD3 can be tested with Cayman's parallel set of screening assays for PAD1, PAD2, or PAD3. This will allow for determination of compounds that show minimal potency towards an alternative target.

Cell-Based, Functional Screening

Secondary screening of confirmed hits should next be tested in a functional cellular assay to determine efficacy. Activity in this secondary screen will confirm that compounds are able to function in a more complex biological system as opposed to the simple, isolated recombinant PAD4 protein used in the primary screen. HL-60 cells are one such model system that can be induced with DMSO to differentiate along the granulocyte lineage and express higher levels of PAD4. Upon calcium ionophore treatment, increased levels of PAD4-dependent histone citrullination are observed in these cells. Alternatively, histone citrullination can be observed in primary human neutrophils treated with a variety of stimuli. The measurement of citrullinated histones in this system can be a convenient cellular assay to test PAD4 inhibition. Cayman's Citrullinated Histone H3 (Clone 11D3) ELISA Kit measures citrullination at residues R2, R8, and R17 on histone H3 from cell lysates and, thus, can be used for the functional readout of pharmacological PAD inhibition in differentiated HL-60 cells or human primary neutrophils.

PAD-Dependent Efficacy Models

Prior to lead optimization, work must be performed to examine compound efficacy in an appropriate *in vivo* model. Recently, a novel PAD4-selective inhibitor, GSK199 (hydrochloride), was shown to be effective in the mouse collagen-induced arthritis (CIA) model of rheumatoid arthritis (RA). Cayman offers a series of products designed to support a PAD-dependent efficacy model of CIA. Cayman's Mouse Anti-Type II Collagen IgG Assay Kit (bovine) is an immunometric assay that can be used to measure anti-type II collagen (anti-CII) antibody in plasma or serum. This kit uses a bovine collagen-coated plate and an affinity-purified polyclonal antibody isolated from mice with CIA as a standard to provide a highly accurate measurement of anti-CII concentration in experimental plasma samples. A number of pro- and anti-inflammatory cytokines, including TNF-α and interleukin-6, are expressed in the joints of mice with CIA. These cytokines can be measured using Cayman's TNF-α (mouse) ELISA Kit and Interleukin-6 (mouse) ELISA Kit.

As an alternative to the CIA model, mice (especially those expressing the human HLA-DR4 transgene) can be immunized with citrullinated human fibrinogen to produce an arthritic response driven by the production of antibodies that recognize citrullinated epitopes. The polyclonal antibody response produces antibodies reactive with both citrullinated human fibrinogen and unmodified, non-citrullinated human fibrinogen. Cayman's Anti-Citrullinated Human Fibrinogen Assay Kit (mouse) is an immunometric assay that can be used to distinguish the antibody response to citrullinated human fibrinogen from the antibody response to unmodified human fibrinogen in mouse serum or plasma. A human fibrinogen affinity sorbent is provided with the kit so that any antibodies capable of reacting with non-citrullinated (unmodified) fibrinogen can be removed prior to analysis of the remaining anti-citrullinated fibrinogen antibodies for an accurate analysis of the anti-citrulline response.

Ready for Lead Optimization

By utilizing a systemic battery of tests as outlined above, select molecules can be swiftly sifted from a library of candidates for further optimization. Cayman has created this specific line of assays to give you the accuracy and efficiency needed to identify specific PAD inhibitors. We also offer full-service contract screening and profiling, including lead optimization and development, to help you identify particular modulators of PAD enzymes.

Primary Screening Assays for Identifying Inhibitors of PAD Activity

Item No.	Product Name	Sample Types	Readout
701440	PAD1 Inhibitor Screening Assay Kit (AMC)	Small molecules	Fluorescence plate reader (ex 355-365 nm, em 445-455 nm)
701450	PAD1 Inhibitor Screening Assay Kit (Ammonia)	Small molecules	Fluorescence plate reader (ex 405-415 nm, em 470-480 nm)
701390	PAD2 Inhibitor Screening Assay Kit (AMC)	Small molecules	Fluorescence plate reader (ex 355-365 nm, em 445-455 nm)
701400	PAD2 Inhibitor Screening Assay Kit (Ammonia)	Small molecules	Fluorescence plate reader (ex 405-415 nm, em 470-480 nm)
701470	PAD3 Inhibitor Screening Assay Kit (Ammonia)	Small molecules	Fluorescence plate reader (ex 405-415 nm, em 470-480 nm)
701320	PAD4 Inhibitor Screening Assay Kit (AMC)	Small molecules	Fluorescence plate reader (ex 355-365 nm, em 445-455 nm)
700560	PAD4 Inhibitor Screening Assay Kit (Ammonia)	Small molecules	Fluorescence plate reader (ex 405-415 nm, em 470-480 nm)

Secondary Functional Assay for Identifying PAD Activity in Cells

Item No.	Product Name	Sample Types	Readout
501620	Citrullinated Histone H3 (Clone 11D3) ELISA Kit	Cell culture supernatants, cell lysates, human plasma, and human serum	Colorimetric plate reader

Efficacy Assessment in PAD-Dependent Models of Arthritis

Item No.	Product Name	Sample Types	Readout
501270	Anti-Citrullinated Human Fibrinogen Assay Kit (mouse)	Mouse plasma or serum	Colorimetric plate reader
500410	Mouse Anti-Type II Collagen IgG Assay Kit (bovine)	Mouse plasma or serum	Colorimetric plate reader
583371	Interleukin-6 (mouse) ELISA Kit	Mouse plasma, serum, and other sample matrices	Colorimetric plate reader
500850	TNF-α (mouse) ELISA Kit	Mouse plasma, serum, and other sample matrices	Colorimetric plate reader

Assay to Detect Human PAD4 Autoantibodies

Item No.	Product Name	Sample Types	Readout
500930	PAD4 Autoantibody ELISA Kit	Human plasma or serum	Colorimetric plate reader

PAD Inhibitors and Probes to Detect Citrullination

Item No.	Product Name	Description
17079	BB-Cl-Amidine	Potent, stable pan-PAD inhibitor with increased cellular potency (EC_{50} = 8.8 µM in cells for PAD4)
25378	BB-Cl-Yne	Clickable inhibitor of PAD1-4 (K_{inact}/K_Is = 6,400, 3,600, 10,800, and 4,900 $M^{-1}min^{-1}$, respectively)
25379	BB-F-Yne	Clickable inhibitor of PAD1-4 (K_{inact}/K_Is = 900, 1,200, 3,400, and 3,750 $M^{-1}min^{-1}$, respectively)
10599	Cl-Amidine (hydrochloride)*	Irreversible inhibitor of PAD1, PAD3, and PAD4 (IC_{50}s = 0.8, 6.2, and 5.9 µM, respectively)
10610	F-Amidine (trifluoroacetate salt)*	Irreversible inhibitor of PAD1, PAD3, and PAD4 (IC_{50}s = 29.5, 350, and 21.6 µM, respectively)
17450	Citrulline-specific Probe-biotin	Fluorescent probe for citrulline-containing protein detection
16172	Citrulline-specific Probe-rhodamine	Affinity probe for citrullinated protein detection through interaction with the biotin ligand
17489	GSK199 (hydrochloride)	Selective inhibitor of PAD4 (IC_{50} = 200 nM)
17488	GSK484 (hydrochloride)	Selective inhibitor of PAD4 (IC_{50} = 50 nM)

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