FLUORESCENCE POLARIZATION ASSAY FOR DETERMINING HISTIDINE DECARBOXYLASE ACTIVITY

RELATED APPLICATIONS

This application claims the benefit of US provisional application No. 60/628,242 filed November 16, 2004 the contents of which are incorporated herein.

BACKGROUND OF THE INVENTION

1. TECHNICAL FIELD

The field of the invention relates to fluorescence polarization assays for detecting HDC activity that can be used in the diagnosis of disease and identification of HDC inhibitor agents.

2. BACKGROUND INFORMATION

Histamine is a potent bioamine with multiple activities in various pathological and physiological conditions (Jutel M, Watanabe T, Akdis M, Blaser K, Akdis CA: Immune regulation by histamine. *Curr. Opin. Immunol* 2002;14:735-740). In addition to its well-characterized effects on the acute inflammatory and allergic responses, histamine regulates several aspects of antigen-specific immune responses (Schneider E, Rolli-Derkinderen M, Arock M, Dy M: Trends in histamine research: new functions during immune responses and hematopoiesis. *Trends Immunol* 2002;23:255-263). Recent findings, such as the discovery of a novel histamine receptor (H4) on immunocompetent cells and the demonstration of a role for H1 and H2 receptors on T helper cell polarization, have generated much interest in the immune-regulatory mechanisms triggered by histamine (Schneider E, Rolli-Derkinderen M, Arock M, Dy M. ; Trends Immunol. 2002 May; 23(5):255-63).

Histidine decarboxylase (HDC) is the rate-limiting enzyme in the biosynthesis of histamine (Watanabe T, Yamatodani A, Maeyama K, Wada H: Pharmacology of α-fluoromethylhistidine, a specific inhibitor of histidine decarboxylase. *Trends Pharmaceutical Sci* 1990;11:363-367.) Mammalian HDC is a member of a large family of pyridoxal 5-phosphate (PLP)-dependent enzymes (Christen P, Mehta P: From Cofactor to

enzymes. The molecular evolution of pyridoxal-5'-phosphate-dependent enzymes. *Chemical Record* 2001;1:436-447.) HDC is expressed in most tissues but the highest levels are found in the skin, the GI track and the airways. HDC is a 74 Kd enzyme that is converted to a shorter 54 Kd form (Yatsunami K, Tsuchikawa M, Kamada M, Hori K, Higuchi T: Comparative studies of human recombinant 74- and 54- kDa L-histidine decarboxylase. J. Biol. Chem. 1995;270:30813-30817). Both forms are active in vitro but they are not found in the same subcellular compartments; the 74 Kd form being found predominantly in the endoplasmic reticulum (Tanaka S, Nemoto K, Yamamura E, Ohmura S, Ichikawa A: Degradation of the 74 kDa form of l-histidine decarboxylase via the ubiquitin-proteasome pathway in a rat basophilic/mast cell line (RBL-2H3). *FEBS* Letters 1997; 417:203-207).

The recent generation of HDC-deficient mice provided a good system to study the role of endogenous histamine in a broad range of normal and disease processes (Ohtsu H, Watanabe T: New functions of histamine found in histidine decarboxylase gene knockout mice. *Biochem Biophys Res Commun* 2003; 443-447). The $HDC^{-/-}$ mice have a reduced number of mast cells and reduced granular content such as mast cell proteases (Ohtsu H, Tanaka S, Terui T, Hori Y, Makabe-Kobayashi Y, Pejler G, Tchougounova E, Hellman L, Gertsenstein M, Hirasawa N, Sakurai E, Buzas E, Kovacs P, Csaba G, Kittel A, Okada M, Hara M, Mar L, Numayama-Tsuruta K, Ishigaki-Suzuki S, Ohuchi K, Ichikawa A, Falus A, Watanabe T, Nagy A: Mice lacking histidine decarboxylase exhibit abnormal mast cells. *FEBS* 2001; 502:53-56.) These mice show reduced airway hyperresponsiveness (Kozma GT, Losonczy G, Keszei M, Komlosi Z, Buzas E, Pallinger E, Appel J, Szabo T, Magyar P, Falus A, Szalai C: Histamine deficiency in gene-targeted mice strongly reduces antigen-induced airway hyper-responsiveness, eosinophilia and allergen-specific IgE. *International Immunol.* 2003;15:963-973, reduced vascular permeability (Ohtsu et al. Plasma extravasation induced by dietary supplemented histamine in histamine-free mice.Eur J Immunol. 2002; 32:1698-708), reduced skin inflammation (Ghosh AK, Hirasawa N, Ohtsu H, Watanabe T, Ohuchi K: Defective angiogenesis in the inflammatory granulation tissue in histidine decarboxylase-deficient mice but not in mast cell-deficient mice. *J. Exp. Med.* 2002;195:973-982.) and increased bone density (Fitzpatrick LA, Buzas

E, Gagne TJ, Nagy A, Horvath C, Ferencz V, Mester A, Kari B, Ruan M, Falus A, Barsony J. Targeted deletion of histidine decarboxylase gene in mice increases bone formation and protects against ovariectomy-induced bone loss. *Proc Natl Acad Sci U S A.* 2003; 100(10):6027-32). Thus, potent inhibitors of HDC activity might prove useful in allergic, inflammatory, immunological, bone and cardiovascular disorders. Histamine has also been shown to be a positive regulator of proliferation in some types of cancers (Hegyesi H, Somlai B, Varga VL, Toth G, Kovacs P, Molnar EL, Laszlo V, Karpati S, Rivera E, Falus A, Darvas Z. Suppression of melanoma cell proliferation by histidine decarboxylase specific antisense oligonucleotides. *J Invest Dermatol.* 2001 Jul;117(1):151-3).

The biological role of histamine has been extensively studied with pharmacological approaches using histamine receptor specific agonists or antagonists. Despite the important role of HDC in allergic and inflammatory responses, very few small molecule inhibitors of this enzyme are known. Most of these inhibitors were discovered by rational design strategies and are histidine analogues. A well characterized HDC inhibitor is the irreversible inhibitor alpha-fluoromethyl histidine (Watanabe T, Yamatodani A, Maeyama K, Wada H.Pharmacology of alpha-fluoromethylhistidine, a specific inhibitor of histidine decarboxylase. *Trends Pharmacol Sci.* 1990 11:363-7).

The ability to identify novel classes of HDC inhibitors is limited by the lack of assays that are suitable to HTS. The most commonly used assay to measure HDC activity is based on the o-phthalaldehyde (OPT) method (Roskoski R, Roskoski LM: A rapid histidine decarboxylase assay. *Analytical Biochem.* 1978;87:293-297.) This assay is not selective for histamine over histidine and involves a chromatographic separation of the enzyme product from the substrate. Another more sensitive HDC assay utilizes the conversion of [14C]-labeled histidine to [14C]-labeled-histamine. Thin layer chromatography is then used to resolve substrate and product. Histamine ELISA kits could potentially be adapted to measure HDC activity. However, these assays require an acetylation step (acetylated histamine) to reach any useful selectivity and sensitivity. Moreover, these procedures require many washing steps rendering them less amenable for HTS.

BRIEF SUMMARY OF THE INVENTION

A fluorescence polarization assay for determining the HDC modulating activity of a candidate compound comprising the steps of:

a) providing a reaction mixture comprising HDC, histidine, a fluorescently labeled histamine probe, a candidate compound and an anti histamine antibody having selectivity for histamine at least 10 fold greater than histidine;

b) incubating the reaction mixture;

c) determining whether inhibition of HDC has occurred in the presence of the test compound, wherein an increase in fluorescence polarization signal is an indication that the test compound inhibits the activity of the HDC.

In another embodiment of the invention the anti histamine antibody has a selectivity for histamine of at least 100 fold greater than histidine.

In another embodiment of the invention the reaction mixture is incubated for more than 15 minutes and more preferable for between 60 and 120 minutes and most preferably for between about 80 to 100 minutes.

In another embodiment of the invention uses a human HDC.

In another embodiment of the invention the HDC is a recombinant enzyme or alternatively partially purified.

In another embodiment of the invention the histamine probe has an affinity of greater than 1μm for the anti histamine antibody.

In another embodiment of the invention the antihistamine antibody used is generated by immunizing mice with a histamine bridged by a linker region to a carrier and where said linker is structurally homologous to the fluorescein probe.

In another embodiment of the invention the linker region is 1,4-benzoquinone and the carrier is albumin.

In another embodiment of the invention the fluorescently labeled histamine probe is chosen from FITC, rhodamine, TAMRA, or Cy5.

In another embodiment of the invention the histidine concentration is between 10μM to 5 mM and more preferably between 100μm and 1mM.

Another aspect of the invention provides a method for detection of HDC activity in a patient sample as a diagnostic tool for a disease, wherein said method comprises:

a) contacting said sample with a reaction mixture comprising a HDC, histidine, a fluorescently labeled histamine probe and an anti histamine antibody having selectivity for histamine at least 10 fold greater than histidine;

b) incubating the reaction mixture;

c) determining whether an increase in HDC activity has occurred in the patient sample compared to the level of HDC activity in control sample, wherein a decrease in fluorescence polarization signal relative to the control sample is an indication that the patient sample is at risk for the disease.

In another embodiment of the invention the antihistamine antibody used is generated by immunizing mice with histamine bridged by a linker region to a carrier wherein said linker is structurally homologous to the histamine - fluorescein probe.

In another embodiment of the invention the disease is chosen from cancer, asthma and mastocytoma, immunological disorders, and gastrointestinal disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the structure of histamine.

Figure 1B shows the structure of N-[3',6'-dihydroxy-3- oxospiro[isobenzofuran-1(3H),9'-[9H]xanthene-5(or 6)-yl]-N'-[2-(1H-imidazol-4-yl)ethyl]-2, 4-dimethylthiourea, disodium salt (FITC-histamine) the probe molecule.

Figure 2 shows the binding of FITC-histamine to the anti-histamine antibody. The assay was run in duplicate in a 96-well plate as described in the Methods, with FITC-histamine at 6nM. The data was fit in SAS and a Kd of 3.9nM was determined.

Figure 3 shows the displacement of FITC-histamine binding to the anti-histamine antibody by histamine (●) or histidine (□). Triplicate determinations in a 96-well plate were performed as described in the Methods, with 6nM FITC-histamine, 50nM anti-histamine antibody, and the indicated concentration of competitor ligand.

Figure 4A shows a time course of HDC at various enzyme concentrations. Triplicate reactions in a 384-well plate were initiated by the addition of the indicated concentration of HDC, and fluorescence polarization was determined at time points from 0 to 180 minutes of incubation. Probe, substrate, and antibody concentrations were as described in the standard assay.

Figure 4B. HDC titration at 90 minutes. Assays (384-well) were performed in quadruplicate using the indicated concentration of HDC for 90 minutes at 37°C on the Allegro™ system. The assay window is defined as the difference in mP between the blank (no enzyme) and reaction wells.

Figure 5 shows the inhibition of HDC by methyl ester histidine (A), α-fluoromethylhistidine (B), and the dipeptide histidine-phenylalanine (C). Reactions were run using the standard conditions as described in the Methods and the indicated concentration of inhibitor. IC50 values were obtained by fitting the data using XLFit4 (IDBS Software). Error bars show the mean ± S>D of triplicate determinations.

Figure 6 shows the scatter plot of blank (•) and positive control (□) values from a single day's screening run of 90 plates (384 wells).

DETAILED DESCRIPTION OF THE INVENTION

Florescent labeled histamine probe. The present invention provides for a fluorescent probe. The preferred probe is FITC-Histamine (Thiourea, N-[3',6'dihydroxy-3-oxospiro[isobenzofuran-1 (3H),9'-[9H]xanthene-5(or 6-yl)-N'-[2-(1H-imidazol-4-yl)ethyl]-2,4-dimethyl-, disodium salt) which can be obtained from Molecular Probes (Eugene, OR). Other suitable probes include histamine labeled with rhodamine, TAMRA, or Cy5.

Anti-histamine antibodies. The present invention provides an assay that uses anti histamine antibodies. One suitable monoclonal antibody that can be used is histamine antibody D22.12 which can be obtained from Argene (Varilhes, France). The D22.12 antibody was generated by immunizing mice with 2-histaminyl-1,4-benzoquinone coupled to albumin (Guesdon JL, Chevrier D, Mazie JC, David B, Avrameas S: Monoclonal anti-histamine antibody. Preparation, characterization and application to enzyme immunoassay of histamine. J. Immunol. Methods 1986; 87:69-78.) whereas all the other antibodies we tested were generated by immunization with histamine or acetylated histamine coupled to albumin. The high binding affinity of D22.12 for histamine-fluorescein could result from a structural homology between the immunogen used to obtain D22.12 (histaminyl benzoquinone) and the histamine-fluorescein probe. Other antihistamine antibodies suitable for use in the invention could be generated using immunogens with similar linkers that also have structural homology to the histamine probe. Different anti-histamine antibodies may be used depending on the fluorophore used as a probe.

Histidine can be obtained from Sigma Chemical Co. (St. Louis, MO).

Source of HDC enzyme

a) Recombinant - In the preferred embodiment of the invention the human HDC enzyme is used. It is contemplated that either the full length protein be used or a truncated form such as the 53 KD form can be used so long as the truncated form retains the histidine decarboxylase activity. Preferably, the human HDC protein (SEQ ID. 1) or a fragment thereof is used. The HDC protein can also be fused to protein tags, such as glutathione S-transferase (GST), to facilitate the purification.

b) Purified - The method of the invention can be practiced using a purified HDC enzyme. As used in the text herein the term partially purified is meant to include a HDC enzyme that has been partially purified to a greater extent than the HDC enzyme would be found in the human cell. Purification procedures for HDC are known in the art and are taught by Watabe A, Fukui T, Ohmori E, Ichikawa A: Purification and properties of L-histidine decarboxylase from mouse stomach. Biochem. Pharmacol. 1992;43:587-593 the contents of which are incorporated herein.

Standard assay for detecting inhibitors

In the standard assay, HDC, diluted in a HDC buffer containing a reducing agent such as DTT and the enzyme co-factor PLP, is added to a sample plate. A test compound in HDC Buffer plus a suitable amount of DMSO or buffer alone is added to the plate. Fluorescently labeled -histamine and histidine are combined in FP Buffer and transferred to the plate in 10μL. Finally, 90 nM anti-histamine antibody is added in 20μL of FP Buffer. Thus, the final concentrations in the assay are: HDC 25 to 50nM, FITC-histidine 3 to 6nM, histidine 300 to 600uM , anti-histamine antibody 25 to 50nM, and DMSO 1 to 5 %. The plate is then incubated at 37 ^{0} C. for at least 15 minutes. The fluorescence polarization signal is read on a suitable intstrument for reading fluorescence polarization such as an LJL Analyst (Molecular Devices, Sunnyvale, CA) with excitation at 485nm, emission at 530nm, a fluorescein dichroic mirror at 505nm and G factor set to 1. A decrease in fluorescence signal is an indication that the test compound inhibits the activity of the HDC.

Modifications of standard procedure for detecting candidate inhibitors –

Optimization of parameters

The present invention provides an HDC assay for determining HDC modulating activity of a candidate compound and a diagnostic method for determining HDC levels in patient samples. It is contemplated that a person skilled in the art may practice the invention in a manner in which a number of parameters may be altered in order to adapt the assay for their own use. Some of the parameters that may be altered include: the anti-histamine antibody concentration, the concentration of the substrate histidine, the probe concentration, the source and concentration of the HDC enzyme, the test compound concentration, the order of additions of the components, the volume of the individual components, the total volume of reaction, addition of a pre-incubation step of HDC with the test compound before the reaction, the duration of the reaction, the temperature of the reaction, the type of assay plates, a cooling or heating step, addition of a step to stop the enzymatic reaction (ex: acid, base, salt, known HDC inhibitors or else), the type of instrument and related parameters used to read the fluorescence polarization signal.

Selectivity of the fluorescent probe for its receptor target molecule.

In one embodiment of the invention the selectivity of the anti-histamine antibody for histamine over histidine is greater than 100X. A suitable candidate inhibitor would be expected to have an IC50 of <10 μm.

Standard assay for diagnosing diseases

Methods to extract and prepare tissues or serum from normal or diseased samples to measure HDC activity are known in the art. For example: (1)1- Sieja K, Stanosz S, von Mach-Szczypinski J, Olewniczak S, Stanosz M. Concentration of histamine in serum and tissues of the primary ductal breast cancers in women. Breast. 2005 Jun;14(3):236-41; (2) E. Masini,V. Fabbroni, L. Giannini,A.Vannacci1, L.Messerini, F. Perna, C. Cortesini and F. Cianchi Histamine and histidine decarboxylase up-regulation in colorectal cancer: correlation with tumor stage Inflamm. res. 54, *Supplement 1* (2005) S80–S81; (3)

3- Fogel WA, Dudkowska M, Wagner W, Grzelakowska-Sztabert B, Manteuffel-Cymborowska M.Ornithine and histidine decarboxylase: activities in hypertrophic and hyperplastic mouse kidney. Inflamm Res. 2005 Apr; 54 Suppl 1:S62-3.

These methods could be applied to prepare samples for testing in the fluorescent HDC assay described herein. Our method would allow for high throughput testing of HDC activity in the target normal or diseased tissue or plasma or blood samples.

EXAMPLES

Reagents

FITC-Histamine (Thiourea, N-[3',6'dihydroxy-3-oxospiro[isobenzofuran-1 (3H),9'-[9H]xanthene-5(or 6-yl)-N'-[2-(1H-imidazol-4-yl)ethyl]-2,4-dimethyl-, disodium salt) was obtained from Molecular Probes (Eugene, OR). Histamine monoclonal antibody 22.12 was obtained from Argene (Varilhes, France). L-Histidine, histamine, potassium phosphate (1M mono- and dibasic solutions), polyethylene glycol 400 molecular weight, ethylene glycol tetraacetic acid (EGTA), dithiothreitol, pyridoxal-5- phosphate, and sodium chloride were from Sigma Chemical Co. (St. Louis, MO). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Pierce Chemical Co. (Rockford, IL). Dimethylsulfoxide was from Baker Chemical Corp. Black opaque polystyrene 384-well plates were obtained from Corning-Costar. The known HDC inhibitors, histidine-methyl ester, and His-Phe were from Sigma Chemical Co and alpha-fluoromethylhistidine was obtained from the Boehringer Ingelheim Pharmaceuticals compound library.

Buffers

HDC Buffer is comprised of 200mM potassium phosphate (pH 6.8), 2% PEG-400, 0.2mM EGTA, and 0.03% CHAPS. FP Buffer is 16.6mM Tris-HCl (pH 7.5) and 50mM NaCl.

HPLC Determination of Histamine Content

HPLC separations were performed on an Agilent 1090M equipped with a diode array detector. A Delta-Pak HPI C4 300Å, 2.0 x 150mmcolumn (Waters) was used. The mobile

phase consisted of 20ml PIC® B-8 Low UV Reagent (Waters) in 1000ml 10mM triethylamine phosphate, pH 3.0. All separations were carried out at room temperature (22°C) with a flow rate of 0.2ml/min and were monitored at a wavelength of 215nm.

Histamine concentrations were calculated using standard curves for concentration vs. area response. The standard curves were generated using duplicate injections of histamine in control buffer at concentrations of 0 to 200 or 600μM. Six equally spaced concentrations were used. The standard curves were calculated for concentration in μM vs. area response using linear regression on a TI-68 calculator. Correlation coefficients were greater than 0.999.

Cloning of human HDC

The cDNA corresponding to the truncated 53 Kd form of full length human HDC (accession number: NM_002112) was amplified by PCR using total RNA from the human mast cell line HMC-1 (Maeda K, Taniguchi H, Ohno I, Ohtsu H, Yamauchi K, Sakurai E, Tanno Y, Butterfield JH, Watanabe T, Shirato K: Induction of L-histidine decarboxylase in a human mast cell line, HMC-1. *Exp Hematol.* 1998;26:325-31.). The primers used were: 5'-atgatggagcctgaggagtacagag and 3'-acactactgactcaggatgagagt. The HDC cDNA (1.5 Kb) was cloned into the pcDNA4.1 vector. Clone pcDNA 4.1-HDC was used as template to obtain a PCR product containing the first 1431 bases (amino acid residues 1-477) of HDC and incorporating a thrombin cleavage site 5' and adjacent to the first base. This PCR product was cloned into pDEST 20 using Gateway Cloning Technology (Invitrogen Life Technologies) following the manufacturer's protocols. DNA purified from the final expression clone was sequenced confirmed and then transformed into DH10Bac E.coli for transposition into the bacmid. Recombinant bacmid DNA was purified from single colonies and transposition was verified by PCR analysis.

Baculoviral expression and purification of GST-HDC

A 20 L volume of SF900II-SFM (Invitrogen cat# 10902-088) was sterile filtered into a 30 L MBR stirred tank bioreactors. The MBR bioreactor was equipped with pH, dissolved oxygen and temperature probes and control set points were: pH 6.2, DO 50%, Temp 27°C,

RPM 110. Four 1 L shake flasks of Sf9 cells were grown to a cell density between $2.5x10^6$ and $3x10^6$ cells/mL and were used to inoculate the bioreactor, resulting in 24 L of media with an approximate cell density of $4 x10^5$ to $5x10^5$ cells/mL. The inoculated bioreactor was sampled daily for cell density, viability, and cell diameter using a Cedex cell counter (Innovatious). Nutrient (glucose, glutamine) and waste (ammonia) analysis was also carried out daily using a Bioprofile 100 analyzer (Nova Biomedical). Twenty-four hours after the initial cell inoculation, the bioreactor was infected with GST-HDC baculovirus to achieve an MOI of 0.1. A 1.5 mL cell supernatant sample (centrifuged, decanted and frozen) was taken pre-infection and every 24 hours until the run was harvested for SDS-PAGE and WESTERN analysis. Twenty-four hours after infection, 25 mg of Leupeptin was dissolved in SF900II-SFM media, filter sterilized and injected into the bioreactor. Runs were harvested 48 hours post-infection. The infected cells were pelleted in a 12 L centrifuge (Sorvall BP12) @ 3000 rpm, 4°C for 10min per spin. The pellets were the combined into one centrifuge bottle and given a final spin at 3500 rpm for 10 minutes, at 4°C. Pellets were weighted and frozen at -80°C until use. The standard pellet yield was 300 grams.

For protein purification, all buffers were prepared from distilled and deionized water and all procedures were carried out at 4°C. The cell pellet was mixed with lysis buffer (20 mM Hepes, pH 7.5, 150 mM KCl, 10% glycerol, 2 mM DTT, 1 mM EGTA, Roche protease inhibitor cocktail tablets, and 0.01 mM PLP) at a ratio of 5 ml/g of cell pellet. The cells were homogenized on ice using PolyTron PT 2100 (Kinematica AG, Switzerland), then sonicated 3 times for 5 min with a Branson Sonifier 450 (Converter, USA) at 50% duty cycle. The cell lysate was centrifuged at 18,600g for 30 min followed by 225,071g for 60 min. The clarified lysate was loaded directly onto a 50 mL Glutahione Sepharose 4B column (Amersham, Sweden) using a AKTA Prime chromatography system (Amersham, Sweden). After loading, the column was washed with 4 column volumes (CV) of wash buffer (20 mM Hepes, pH 7.5, 150 mM KCl, 10% Glycerol, 2 mM DTT, 0.5 mM EDTA, 0.5 mM PMSF and 0.01 mM PLP), then was eluted with 10 CV of elution buffer (20 mM GTH reduced, 2 mM DTT and wash buffer, pH 8.0). The HDC product eluting from the column was pooled according to visual inspection of an SDS-PAGE gel. A standard yield

was 33 mL of HDC at 1.8 mg/mL. The pooled product was dialyzed into 5 L of buffer 1 (20 mM Hepes, pH 7.5, 0.1 mM EGTA, 0.2 mM PMSF, 0.25 mM DTT, 10% Glycerol and 0.01mM PLP) for 4 hours, then dialysis into 5 L of buffer 2 (0.1 M K Phosphate, pH 7.5, 2% PEG-400, 0.1 mM EGTA, 2 mM PMSF, 10% Glycerol and 0.02 mM PLP) for 17 hours. The final product was aliquoted, snap frozen in liquid nitrogen, and stored at -80°C.

Histidine Decarboxylase Assay

In the standard assay, HDC, diluted to 90nM in HDC Buffer plus 0.9mM DTT and 99μM PLP, was added to a black opaque 384-well plate in 20μL. Test compound in HDC Buffer plus 6% DMSO or buffer alone was added to the plate in 10μL. 36nM FITC-histidine and 3.6mM histidine were combined in FP Buffer and transferred to the plate in 10μL. Finally, 90 nM anti-histidine antibody was added in 20μL of FP Buffer. Thus, the final concentrations in the assay were: HDC 30nM, FITC-histidine 6nM, histidine 600μM, anti-histamine antibody 30nM, DMSO 1%. The plate was incubated at 37°C for 90 minutes. The fluorescence polarization signal was read on an LJL Analyst (Molecular Devices, Sunnyvale, CA) with excitation at 485nm, emission at 530nm, a fluorescein dichroic mirror at 505nm and G factor set to 1. A 96-well plate version of the assay, used in assay development, was performed as described above with twice the volumes indicated for 384-well plates.

High Throughput Screening

The assay was automated on a Zymark Allegro™ robotics system (Caliper-Zymark, Hopkinton, MA), using a Multidrop to add enzyme, the Sciclone to add substrate/probe and test compound, and a Multidrop to add the antibody. The plates were incubated at 37° C in a humidified environment, and fluorescence polarization was read on an LJL Analyst integrated into the Allegro system using the settings described above. Compounds were screened at a concentration of 5 μg/mL. The POC values were calculated relative to an assay blank containing complete reaction minus HDC, and a 100% control containing HDC buffer with 1% DMSO in place of compound.

RESULTS

The structure of the HDC enzyme product (histamine) and the histamine probe (FITC-histamine) are shown in FIG. 1. We first set out to examine the interaction of several commercial anti-histamine antibodies to the FITC-histamine probe (data not shown). One of these antibodies (D22.12), showed strong probe binding as measured by fluorescence polarization and was retained for further characterization.

We first determined the affinity of the probe for the antibody and the specificity of the interaction, both of which are crucial to constructing a robust assay in a competitive mode. FIG. 2 shows a binding curve of probe and antibody as determined by measurement of anisotropy. The probe concentration was held at 6nM and the antibody concentration varied over approximately 3 logs. A dissociation constant of 3.9nM was determined upon fitting the data. The specificity of the antibody for histamine over histidine was demonstrated as shown in FIG. 3. Probe and antibody concentrations were held constant at 6 and 50nM, respectively, while the concentrations of histamine and histidine were varied as shown. Histidine was unable to compete with FITC-histamine for binding to the antibody over the 5 log range examined. Histamine, however, freely competed with the probe for antibody binding, yielding an IC50 of 135μM. Thus, the tight and specific binding of FITC-histamine to the histamine monoclonal antibody and the ability to compete for that binding with the product of the enzymatic reaction suggests that it is possible to develop a robust competitive FP assay for HDC.

FIG. 4A shows a time course of the enzymatic reaction at various concentrations of HDC. Histidine, at 600uM, was approximately twice the reported Km of 200-400uM (Watabe et al 1992). At HDC concentrations >100nM, the reaction is linear for only about 30 minutes. In order to balance the size of the assay window with the enzyme requirements for a large scale screen and linearity of the reaction, we chose to use 25-50nM HDC and an incubation time of 90 minutes in the standard assay. This was further refined in FIG 4B, which shows a titration of HDC at a 90 minutes incubation performed on the Allegro robotic system. From this experiment we arrived at an enzyme concentration of 30nM. Thus, the final assay conditions were set at 30nM HDC, 30nM antibody, and 6nM FITC-

histamine in a total volume of 60μL for 90 minutes at 37°C. The PLP concentration was set at 33μM to maintain saturation of the enzyme.

FIG. 5 shows the behavior of 3 known HDC inhibitors: methyl ester histidine, α-fluoromethyl histidine, and the dipeptide histidine-phenylalanine in the standard assay. We determined IC50's of 7.7uM, 1.4uM, and 228.1uM for the 3 compounds, respectively. These values are in good agreement with those obtained using the HPLC assay).

The assay as described was then used to screen compounds from a library at a final concentration of 5 μg/mL. 384-Well plates were set up to contain 352 compound wells, 16 control wells (no compound) and 16 blank wells (no enzyme) per plate. Compounds, in neat DMSO, were diluted in buffer to give a final DMSO concentration of 1% in the assay. This concentration of DMSO was shown to have no effect on the enzymatic activity or stability (data not shown). The assay was fully automated on the Allegro robotic system, enabling throughputs of approximately 100 plates per day. FIG. 6 shows a scatter plot of blank and control wells for a single screening run of 90 plates, with an average Z' of 0.6 and an assay window of 80-100mP. In excess of 600,000 compounds were screened in the assay with a confirmed hit rate of 0.05% using 60% of control as the hit criteria. Confirmed hits were subsequently assayed in 10 point dose response to assess potency.

DISCUSSION

An important parameter contributing to the performance of an FP assay is the affinity of the fluorescent probe for its receptor or target molecule. As a general rule, the Kd for probe binding to its receptor is inversely proportional to the fraction bound. Thus, high affinity binding allows for an optimal fluorescent-ligand/receptor stoichiometry and a robust FP signal. We screened multiple anti-histamine antibodies in order to find one that had a suitable affinity for our histamine-fluorescein probe. Only one of these antibodies, D22.12, had an affinity that was high enough for the development of an FP assay. The D22.12 antibody was generated by immunizing mice with 2-histaminyl-1,4-benzoquinone coupled to albumin (Guesdon et al; 1986) whereas all the other antibodies we tested were generated by immunization with histamine or acetylated histamine coupled to albumin.

The high binding affinity of D22.12 for histamine-fluorescein could result from a structural homology between the immunogen used to obtain D22.12 (histaminyl benzoquinone) and the histamine-fluorescein probe.

The Km of HDC (54 Kd form) for its substrate histidine is 275 μM (data no shown). Thus, an important requirement for the development of an HDC assay is the selectivity for histamine over histidine. Our FP assay shows over a 100-fold selectivity for histamine over histidine. However, as shown in Figure 3, the histidine concentration should be kept below 2 mM due to non-specific increase in the FP signal. This limitation should not be an issue in most applications to measure HDC activity given that the Km of the enzyme is much lower than the maximal amount of histidine tolerated by the assay.

Decarboxylases form a large family of enzymes playing important physiological roles (Christen et al; 2001). For example, DOPA decarboxylase is responsible for the synthesis of the key neurotransmitters dopamine and serotonin via decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan respectively. Current methods used to measure transmitters such as serotonin and dopamine are analogous the histamine detection techniques. Thus, the assay we describe here for HDC could be applied to related enzymes such as dopa decarboxylase and allow for the development of new inhibitors with better pharmacological characteristic.

CLAIMS:

1. A fluorescence polarization assay for determining the HDC modulating activity of a candidate compound comprising the steps of:

a) providing a reaction mixture comprising a HDC, histidine, a fluorescently labeled histamine probe, a candidate compound and an anti histamine antibody having selectivity for histamine at least 10 fold greater than histidine;

b) incubating the reaction mixture;

c) determining whether inhibition of HDC has occurred in the presence of the test compound, wherein an increase in fluorescence polarization signal is an indication that the test compound inhibits the activity of the HDC.

2. The assay of claim 1 wherein the anti histamine antibody has a selectivity for histamine of at least 100 fold greater than histidine.

3. The fluorescence polarization assay of claim 1 wherein the reaction mixture is incubated for more than 15 minutes.

4. The fluorescence polarization assay of claim 3 wherein the reaction mixture is incubated for between 60 and 120 minutes.

5. The fluorescence polarization assay of claim 4 wherein the reaction mixture is incubated for about 80 to 100 minutes.

6. Method of claim 1 wherein the HDC is polypeptide of SEQ ID. No. 1.

7. Method of claim 1 wherein the HDC is a recombinant enzyme.

8. Method of claim 1 wherein the HDC is partially purified.

9. Method of claim 1 wherein the histamine probe has an greater affinity than 1μm for the anti histamine antibody.

10. The method of claim 1 wherein the antihistamine antibody used is generated by immunizing mice with a histamine bridged by a linker region to a carrier and where said linker is structurally homologous to the fluorescein probe.

11. The method of claim 10 wherein the linker region is 1,4-benzoquinone coupled to a carrier.

12. The method of claim 10 wherein said carrier is albumin

13. The method of claim 1 wherein the fluorescently labeled histamine probe is chosen from, FITC, rhodamine, teramethylrhodamine and Cy5.

14. The method of claim 1 wherein the histidine concentration is between 10μM to 5 mM.

15. The method of claim 10 wherein histidine concentration is between 100μm and 1mM.

16. A method for detection of HDC activity in a patient sample as a diagnostic tool for a disease, wherein said method comprises:

a) contacting said sample with a reaction mixture comprising, histidine, a fluorescently labeled histamine probe and an anti histamine antibody having selectivity for histamine at least 10 fold greater than histidine;

b) incubating the reaction mixture;

c) determining whether an increase in HDC activity has occurred in the patient sample compared to the level of HDC activity in control sample, wherein a decrease in fluorescence polarization relative to the control sample is an indication that the patient sample is at risk for the disease.

17. The method of claim 16 wherein the antihistamine antibody used is generated by immunizing mice with histamine bridged by a linker region to a carrier wherein said linker is structurally homologous to the histamine - fluorescein probe.

18. The method of claim 16 wherein the disease is chosen from cancer, asthma, mastocytoma, immunological disorders, bone disorders and gastrointestinal disorders.

19. The method of claim 18 wherein the disease is cancer.