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Development of a high-throughput assay for aldosterone synthase inhibitors using high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Aldosterone plays a key role in the pathogenesis of hypertension, congestive heart failure, and chronic kidney disease. Aldosterone biosynthesis involves three membrane-bound enzymes: aldosterone synthase, adrenodoxin, and adrenodoxin reductase. Here, we report the development of a mass spectrometry-based high-throughput whole cell-based assay for aldosterone synthesis. A human adrenal carcinoma cell line (H295R) overexpressing human aldosterone synthase cDNA was established. The production of aldosterone in these cells was initiated with the addition of 11-deoxycorticosterone, the immediate substrate of aldosterone synthase. An automatic liquid handler was used to gently distribute cells uniformly to well plates. The adaption of a second automated liquid handling system to extract aldosterone from the cell culture medium into organic solvent enabled the development of 96- and 384-well plate formats for this cellular assay. A high-performance liquid chromatography–tandem mass spectrometry method was established for the detection of aldosterone. Production of aldosterone was linear with time and saturable with increasing substrate concentration. The assay was highly reproducible with an overall average Z' value = 0.49. This high-throughput assay would enable high-throughput screening for inhibitors of aldosterone biosynthesis.

Aldosterone is a steroid hormone produced from the adrenal glands. It binds to the mineralocorticoid receptor (MR)[2], a member of the nuclear hormone receptor family. Traditionally, the main target organ of aldosterone is the kidney, where activation of MR in the distal convoluted tubule results in increased sodium reabsorption and potassium excretion, leading to volume expansion and increased blood pressure [1]. Clinical studies have demonstrated that aldosterone blockade with MR antagonists could reduce cardiovascular-related hospitalization and mortality in patients with congestive heart failure [2]. MR antagonists have also been shown to slow the progress of chronic kidney disease [3]. However, the use of MR antagonists is associated with increased risk of hyperkalemia, especially in patients with compromised renal function [4,5]. Additionally, there is a compensatory increase of aldosterone production during long-term treatment with MR antagonists [6]. This could worsen the MR-independent effects of aldosterone in the vascular wall and heart [7].

Blocking the production of aldosterone represents an alternative approach to MR antagonism. Aldosterone is synthesized from cholesterol in the outermost layer of the adrenal cortex (zona glomerulosa) through a cascade of steroid hydroxylase and oxygenase enzymes [8]. Aldosterone synthase (CYP11B2) catalyzes the last and rate-limiting steps in aldosterone synthesis. CYP11B2 is a member of the cytochrome P450 family and is located on the membranes of mitochondria. The action of CYP11B2 requires two other mitochondria proteins, adrenodoxin and adrenodoxin reductactase for electron transfer. This complex enzyme system provides several challenges for the development of a high-throughput biochemical assay for the screening of aldosterone synthase inhibitors.

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[2] *Abbreviations used:* angII, angiotensin II; CRC, concentration–response curve; CYP11B2, aldosterone synthase; DMSO, dimethyl sulfoxide; DOC, 11-deoxycorticosterone; EIA, enzyme immunoassay; IS, internal standard; MR, mineralocorticoid receptor.

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An immunocompetition-based assay is commonly used for measurement of aldosterone in cell culture medium or bodily fluids [8,9]. This method depends on the specificity of the monoclonal antibody against aldosterone. However, all of these antibodies display some degree of cross-reactivity toward other steroids, including cortisol and corticosterone. Cortisol and corticosterone are the major glucocorticoids in human and rodents, respectively. Their concentrations in serum and cell culture medium are 100- to 1000-fold higher than that of aldosterone. Thus, although anti-aldosterone antibodies display only weak cross-reactivity with cortisol or corticosterone, their presence could still compromise the accuracy of aldosterone measurement of the immunoassays. A cellular aldosterone assay employing conversion of radiolabeled substrate has been published [10]. These authors used HPTLC to separate and quantitate aldosterone. In addition, a high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the measurement of aldosterone has been reported [11]. These methods overcome the cross-reactivity associated with the immunoassays and the MS method has become widely used for the measurement of aldosterone in clinical samples. However, the published method is not suitable for high-throughput screening of small molecule inhibitors due to the time required for the assay.

We sought to develop a whole cell-based screening assay for aldosterone synthase using an LC–MS/MS detection method. The human adrenal cellular carcinoma cell line H295R is widely used for the study of aldosterone and cortisol production [9]. However, the aldosterone production rate is low in these cells. The baseline aldosterone concentration in the cell medium of H295R cells is below 40 pg/ml, the detection limit for our LC–MS-based measurement. While stimulation of the cells with angiotensin II increased aldosterone concentration several fold, to approximately 200 to 400 pg/ml, this still represents a narrow window size over baseline aldosterone concentration, and is not ideal for a high-throughput screening assay. To overcome these issues, we elected to establish H295R cells constitutively expressing the human aldosterone synthase gene. The use of this cell line enabled the establishment of a high-throughput screening assay for aldosterone production.

Materials and methods

Materials

Human adenocarcinoma cell line H295R was purchased from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's-Ham's F12 medium (DME/F12), and a cocktail solution of insulin, transferrin, and selenium (ITS) and neomycin were from Invitrogen (Carlsbad, CA). Geneticin G418 was from Life Technologies. Nu serum was from BD biosciences (San Jose, CA). Aldosterone, 11-deoxycorticosterone (DOC), and other chemicals were from Sigma Chemicals (St. Louis, MO). Aldosterone-d7 was from CDN. Tissue culture plates and polypropylene plates were from Corning Life Sciences (Edison, NJ). An enzyme immunoassay (EIA) kit for aldosterone was from Cayman Chemicals (Ann Arbor, MI).

Generation of H295R cells overexpressing human CYP11B2

The full-length cDNA clone of human CYP11B2 was generated through a polymerase chain reaction approach using public nucleotide sequence information. The cDNA was subsequently subcloned into mammalian expression vector pcDNA3.1/neomycin (Invitrogen).

H295R cells were maintained in DME/F12 medium supplemented with insulin (1 ng/ml), transferrin (1 μg/ml), selenium (1 ng/ml), and 2.5% Nu serum. Cells were transfected with the cDNA construct containing human aldosterone synthase with Lipofectamine reagent (Invitrogen) following the recommended protocol. Individual neomycin-resistant cell clones (500 μg/ml geneticin) were selected and tested for their ability to produce aldosterone. Cell clones that displayed the highest aldosterone production ability were expanded and used for the development of 96-well and 384-well plate format assays.

Aldosterone production assay

For a 96-well plate format assay, H295R cells or H295R cells overexpressing human aldosterone synthase grown in flasks were collected by trypsin-EDTA, counted, centrifuged, and then resuspended in culture medium containing 10 μM DOC at a concentration of 200,000 cells/ml. Cells were dispersed with a multichannel pipette into 96-well culture plates at 30,000 cells per well (150 μl). Test compounds were diluted in a separate 96-well plate in cell culture medium at 6× final concentration. The diluted compound solution (30 μl) was then transferred into the cell plates. The cells were incubated at 37 °C plus 5% CO_2 for 24 h. One hundred microliters of cell medium from each well was collected into a polypropylene plate, sealed, and store at −80 °C until use.

An automatic liquid handler (TECAN) was used to set up the 384-well plate format aldosterone production assay. Cells were trypsinized and resuspended in cell culture medium containing DOC at a concentration of 250,000 cells/ml. The cell suspension was positioned on top of a shaker (BT Banstead Thermolyne, Roto Mix, Type 50800) during dispensing to ensure a homogeneous cell suspension. A small-bore needle disposable tubing assembly (Matrix, WellMate) was employed. The tubing was primed with distilled H_2O and again with cell suspensions. Forty-five microliters of cell suspension (11,250 cells/well) was dispersed into each well of a 384-well plate at a slow dispense rate to ensure uniform cell numbers in each well. Test compounds were diluted in a separate 384-well plate in cell culture medium at 10× final concentration. The diluted compound solution (5 μl) was then transferred into the cell plates using the automatic liquid handler. The cells were incubated at 37 °C plus 5% CO_2 for 24 h. Forty microliters of cell medium from each well was collected into a polypropylene plate, sealed, and stored at −80 °C until use.

Aldosterone measurement in the medium was performed with the EIA kit following the manufacturer's recommendation or with the LC–MS/MS method (described below).

Aldosterone measurement by high-performance liquid chromatography–tandem mass spectrometry—routine analysis

A Beckman Coulter FX liquid handling system (modified for use of organic solvents) with a 96- or 384-tip head was used to add an internal standard solution to each well (10 μl of 100 ng/ml d7-aldosterone, CDN, in 15% ACN/water). The wells were then extracted 3× with ethyl acetate (150 μl for 96 well or 40 μl for 384 well) using the FX, combining the organic layers in a new plate. The solvent was dried in a GeneVac HT-4 or under nitrogen. The FX was then used to reconstitute the samples in 60 μl 15% ACN/water and the plates were heat-sealed. For routine analysis, an Agilent LC with a binary pump produced a gradient of water and ACN, each containing 0.1% formic acid, with a flow rate of 1 ml/min over a Betasil 2.1 × 10 mm C18 column. A 25 μl aliquot of the sample was injected and a gradient from 20 to 100% ACN+0.1% formic acid in 1 min was initiated. Aldosterone was eluted at 0.7 min. Starting conditions were held for 1 min to reequilibrate the column. Detection employed an ABSciex 4000 for MS/MS analysis in negative ion mode. The MS/MS method monitored two multiple reaction monitoring transitions for sample