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Rapid, convenient method for screening imidazole-containing compounds for heme oxygenase inhibition

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ABSTRACT

Introduction: Sensitive assays for measuring heme oxygenase activity have been based on the gas-chromatographic detection of carbon monoxide using elaborate, expensive equipment. The present study describes a rapid and convenient method for screening imidazole-containing candidates for inhibitory activity against heme oxygenase using a plate reader, based on the spectroscopic evaluation of heme degradation. **Methods:** A PowerWave XS plate reader was used to monitor the absorbance (as a function of time) of heme bound to purified truncated human heme oxygenase-1 (hHO-1) in the individual wells of a standard 96-well plate (with or without the addition of a test compound). The degradation of heme by heme oxygenase-1 was initiated using L-ascorbic acid, and the collected relevant absorbance data were analyzed by three different methods to calculate the percent control activity occurring in wells containing test compounds relative to that occurring in control wells with no test compound present. **Results:** In the cases of wells containing inhibitory compounds, significant shifts in λ_{max} from 404 to near 412 nm were observed as well as a decrease in the rate of heme degradation relative to that of the control. Each of the three methods of data processing (overall percent drop in absorbance over 1.5 h, initial rate of reaction determined over the first 5 min, and estimated pseudo first-order reaction rate constant determined over 1.5 h) gave similar and reproducible results for percent control activity. The fastest and easiest method of data analysis was determined to be that using initial rates, involving data acquisition for only 5 min once reactions have been initiated using L-ascorbic acid. **Discussion:** The results of the study demonstrate that this simple assay based on the spectroscopic detection of heme represents a rapid, convenient method to determine the relative inhibitory activity of candidate compounds, and is useful in quickly screening a series or library of compounds for heme oxygenase inhibition.

1. Introduction

Heme oxygenase continues to generate considerable interest, especially as regards its role in biology and medicine (Otterbein & Zuckerbraun, 2005; Kinobe, Dercho & Nakatsu, 2008). The action of heme oxygenase (HO) on the substrate, heme, produces carbon monoxide, biliverdin, and ferrous iron (Fe^{2+}). The two active HO isozymes are HO-1 (inducible) and HO-2 (constitutive). Our laboratory has been actively engaged in the design and synthesis of selective inhibitors of the two isozymes. This work has been successful in developing inhibitors highly selective for HO-1 based on small molecules containing an imidazole moiety (Vlahakis et al., 2005; Vlahakis, Kinobe, Bowers, Brien, Nakatsu & Szarek, 2006; Roman et al., 2007). The method employed for assaying heme oxygenase activity has commonly involved the detection of the produced carbon monoxide using gas-chromatographic techniques (Vreman & Stevenson, 1988; Cook et al., 1995; Vlahakis et al., 2005). The method is reliable and sensitive but somewhat laborious and requires specialized equipment. Here we report a rapid and convenient method for testing a series of imidazole-containing compounds for activity against a truncated form of human heme oxygenase-1 (hHO-1) using an assay based on the spectroscopic detection of heme and heme-containing adducts using a standard 96-well plate reader and L-ascorbic acid as the requisite co-factor. With a source of purified heme-bound HO protein in hand, the method involves using relatively simple techniques of visible spectroscopy to determine the relative inhibitory activity of candidate compounds, and is useful in rapidly screening a series or library of compounds. The two diagnostic

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parameters are the shift in λ_{max} attributable to the binding of an inhibitor to the heme–hHO-1 complex and the diminution of the rate of heme degradation.

2. Methods

The assay utilizes pure hHO-1, heme, L-ascorbic acid, and candidate HO inhibitors. Heme–hHO-1 absorbance spectra are measured before and after the addition of test compounds. The heme degradation is initiated by the addition of L-ascorbic acid and is monitored by visible spectroscopy. Finally the data analysis, and the estimates of heme–hHO-1 degradation based on the overall percent drop in absorbance (0–90 min), the initial rate of reaction (0–5 min), and on the calculation of the estimated pseudo first-order rate constant (0–90 min), are performed. Following a summary of material preparation, the details of the assay are exemplified by a description of its application to compound **7**.

2.1. Test compounds and reagents

The candidate imidazole-containing HO inhibitors (**1**–**16**) were synthesized in our laboratories; synthetic preparations have been reported elsewhere (Roman et al., 2007; Vlahakis et al., 2005; Vlahakis, Kinobe, Bowers, Brien, Nakatsu & Szarek, 2006; Vlahakis, Kinobe, Nakatsu, Szarek & Crandall, 2006). All of the other reagents were obtained from Sigma-Aldrich. The identities of the test compounds were not revealed to the operator of the assay until final results were presented, thus maintaining an unbiased blind assay system.

2.2. Expression and purification of hHO-1

For crystallization purposes, a truncated, soluble version of hHO-1 was used that contains 233 amino acids (hHO1-t233) and has been employed successfully to solve the high-resolution crystal structure for hHO-1 (Schuller, Wilks, Ortiz de Montellano & Poulos, 1998; Schuller, Wilks, Ortiz de Montellano & Poulos, 1999; Lad et al., 2005). Bacterial expression and purification of hHO-1 were based on published protocols (Wilks & Ortiz de Montellano, 1993; Wilks, Black, Miller & Ortiz de Montellano, 1995; Wang et al., 2004; Rahman et al., 2008). Briefly, DH5α cells transformed with the hHO1-t233/pBace expression plasmid (Wilks, Black, Miller & Ortiz de Montellano, 1995; Wilks, Medzihradszky & Ortiz de Montellano, 1998) (a generous gift from Dr. Ortiz de Montellano, University of San Francisco) were grown in 4 L (4×1 L) LB media supplemented with 100 mg/L ampicillin following inoculation from a mini-culture. After 18 h of growth, cells were harvested and centrifuged resulting in green pellets which were stored at −80 °C. Pellets were subsequently thawed on ice, resuspended in lysis buffer (100 mM Tris(hydroxymethyl)aminomethane, pH 8.0; 2 mM EDTA; 2 mM phenylmethanesulfonyl fluoride), and lysed by sonication. Cell debris was separated by centrifugation for 40 min at 15,000 rpm (Beckman JA-20 rotor) at 4 °C. A 35–60% ammonium sulfate pellet was prepared by adding ammonium sulfate to 35% saturation and rocking at 4 °C for 40 min followed by centrifugation for 20 min at 15,000 rpm. Ammonium sulfate was added to the supernatant to a final concentration of 60% saturation and the mixture was rocked for 1 h at 4 °C followed by centrifugation as before. The 35–60% saturated ammonium sulfate pellet was cleared of ammonium sulfate by dialysis against 10 mM potassium phosphate (pH 7.4) prior to further purification. Up to this point, the hHO-1 could be monitored by the appearance of a green pigment indicative of biliverdin bound to the enzyme, owing to conversion of endogenous heme by the recombinant protein. The protein was purified further using fast protein liquid chromatography (FPLC) over a MonoQ (HR 10/10, Amersham Biosciences) anion-exchange column using a gradient of 0–100 mM KCl in 10 mM potassium phosphate (pH 7.4). Fractions containing the apo hHO-1, as monitored by absorbance (280 nm) and SDS-PAGE analysis, were pooled and the protein concentration determined using the Bradford method (Bio-Rad) (Bradford, 1976; Stoscheck, 1990).

2.3. Preparation of a heme–hHO-1 complex stock solution

The apo hHO-1 protein obtained in Section 2.2 was combined with 1.2 equivalents of hemin (Fluka), and the mixture was rocked at 4 °C for 20–30 min, and then stored at −20 °C. The excess heme was removed by passage over a PD-10 size-exclusion column (Amersham Biosciences) which had been equilibrated with 20 mM potassium phosphate (pH 7.4). Protein concentration was determined by the spectrophotometric measurement of absorbance at 405 nm ($\varepsilon_{405} = 140{,}000\ M^{-1}\ cm^{-1}$) (Wilks, Black, Miller & Ortiz de Montellano, 1995; Yoshida & Kikuchi, 1979). Purity was assessed by measurement of the R_z ratio ($A_{405}/A_{280} > 2.1$) (Lad et al., 2003) and by SDS-PAGE analysis.

2.4. Solutions of test compounds and assay preparations

The compounds to be tested for HO-1 inhibitory activity were placed individually in small vials; each compound (~1 mg) was weighed exactly in each vial. To each vial distilled water (500 µL) was added. Dilution to 200 µM was then performed (guided by a spreadsheet created in Microsoft Excel) by adding a 20-mM potassium phosphate buffer (pH 7.4) solution (1000 µL) to a new capsule, removing a calculated amount of the buffer, and adding a calculated amount of the concentrated inhibitor solution from the vial. The capsule was shaken vigorously resulting in a 200-µM stock solution of the test compound. This protocol was followed for each compound to be tested. The frozen heme–hHO-1 stock solution (431 µM) prepared as in Section 2.3 was taken from the freezer and kept on ice, then centrifuged at room temperature just before use. Calculations were performed to ensure a final concentration of the test compound to be 100 µM after all of the reagents had been added, including the L-ascorbic acid solution (see below). The calculated volume of buffer (which was added first) required in each well varied, whereas the volume of the 431-µM human HO-1 stock solution was always 2.32 µL in each well, and was added second and the solution was stirred using the tip of the pipette. A specified amount of the 200-µM solution of the test compound was then added. Finally, 10 µL of a 10-mM solution of L-ascorbic acid was added to each well to initiate the reaction. All of the experiments were performed in quadruplicate.

2.5. Heme–hHO-1 absorbance spectra (A vs λ) before the addition of test compounds

For completeness, an absorbance versus wavelength spectrum (300–700 nm, in 1-nm increments) of the contents of each well (containing buffer and heme-hHO-1 stock only) was taken before the addition of the test compounds (for comparison later) using a PowerWave XS (Bio-Tek Instruments) plate reader controlled using program KC4 (Version 3.3, Rev. #10). This process took approximately 1 min/well, but could be bypassed in future experiments.

2.6. Heme–hHO-1 absorbance spectra (A vs λ) after the addition of test compounds

To particular wells 50 µL of the relevant 200-µM stock solution of test compound were added and each mixture was stirred with the tip of the pipette, resulting in solutions of test compounds near a concentration of 100 µM. Again, an absorbance versus wavelength spectrum (300–700 nm, in 1-nm increments) of the contents of each of the wells was taken. This process took approximately 1 min/well.