A HIGH THROUGHPUT SCREENING ASSAY FOR NOS USING SPA

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Introduction

The formation of nitric oxide (NO) by the action of nitric oxide synthase (NOS) has been implicated in a wide range of physiological and pathological processes. These functions include maintenance of vascular tone, neuronal signalling and host response to infection[1,2,3]. In addition, sustained synthesis of NO has been implicated in the aetiology of endotoxic shock[4], inflammation-related tissue damage[5] and neuronal pathology [2].

NOS exists in three distinct isoforms. Two are constitutively produced, and are classified according to cellular location as endothelial (eNOS) and neuronal (nNOS). The third isoform is inducible (iNOS) and can be produced by a wide range of cell types in response to a variety of stimuli[6]. All three isoforms catalyze the formation of NO by conversion of arginine to citrulline, with the concomitant release of NO. Enzyme activity is dependent on a series of co-factors (NADPH, FAD, FMN, calmodulin and tetrahydrobiopterin).

Amersham Biosciences has developed a high throughput assay (TRKQ7160) suitable for the screening of potential inhibitors of iNOS activity (Figure 1).
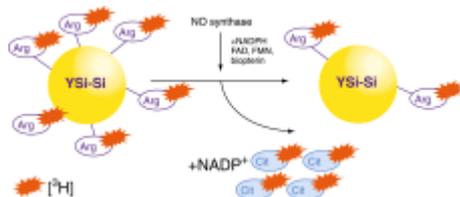

Figure 1. Assay schematic.

The assay takes advantage of the discriminatory binding characteristics of a proprietary coating# applied to yttrium silicate (YSi) SPA beads. In the presence of iNOS activity, arginine is converted to citrulline and NO. The radiolabelled citrulline is unable to bind to the beads, hence iNOS activity can be measured in proportion to the reduction of SPA signal. In the absence of iNOS activity, arginine is not converted to citrulline. The beads are able to bind arginine and thus a signal is generated by the addition of those beads. The assay method has been optimized for a 384 well format, and consists of a short (10-20 minute) liquid phase assay, followed by the addition of bead in a 'stop' solution to generate the signal. This is stable for a minimum of 24 hours.
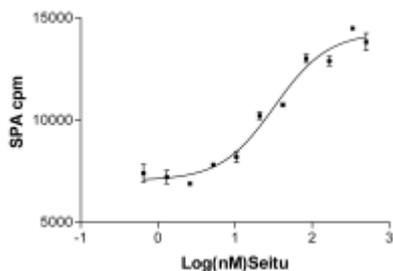

Figure 2. Inhibition of NOS activity by 2-ethyl-2-thiopseudourea hydrobromide (Seitu). Enzyme (0.038 units) was incubated for 20 minutes in assay buffer (50 mM Tris.HCl, pH 7.5, containing 1.0 mM NADPH, 380 mM FMN, 380 mM FAD, 380 mM tetrahydrobiopterin, 2.9 mM dithiothreitol, 2 mM L-arginine) and 100,000cpm [3H]arginine (diluted in

reaction buffer) at room temperature. Reactions were terminated by the addition of 'stop' reagent (0.5 mg SPA bead suspended in 20 ml 50 mM NaOH). Inhibitor added in 1 ml DMSO. Results are means ±SEM, n=3. IC_{50}: 32.47 nM. 95% confidence limits: 23-45 nM.

Screening for iNOS inhibitors in the LOPAC library

To show the utility of this assay it was tested in a semi-automated screening assay in a 384-well format.

The compounds of the LOPAC™ library were dissolved in DMSO to a final concentration of ~4 mg/ml. A Beckman Multimek™ was used to reformat the library from 96 to 384 well, and to dilute the compounds further, to a final concentration of ~0.4 mg/ml.

For the screen, 1 μl compound was transferred to assay plates using a CyBio CyBi™-Well 384-well dispenser. This gave a final assay concentration of ~0.02 mg/ml for each compound. Assay components were set up as two bulk stocks, enzyme and substrate, both in buffer. Enzyme was used at 0.038 U/well; substrate was a mix of both labelled and unlabelled arginine, as Figure 2. These were dispensed to the assay plates using a single aspiration, four dispense (standard Z for quadrant dispensing) protocol using the Multimek. Tips were washed between the dispensing of the two bulk stocks. Assay plates were incubated at room temperature for 20 minutes, prior to manual addition of SPA bead in 'stop' solution, using an Eppendorf™ repeater pipette. The stopped reactions were incubated overnight at room temperature prior to counting (1 minute per well using a Wallac MicroBeta™ Trilux). Data was exported to ActivityBase™ for both storage and analysis.
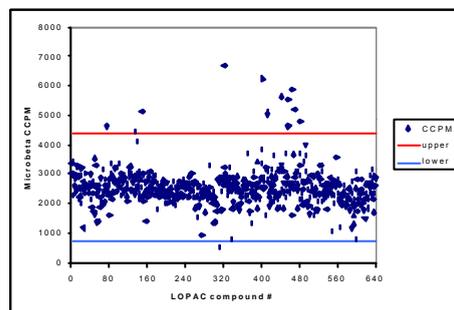

Figure 3. Analysis of primary screening data. The cut off levels were set at ±3 SD of the mean of all the data.

Using ±3 SD as a cut off value, a total of 13 (2%) potential 'hits' were identified. These 'hits' were picked manually from the library master plate for retesting.

Retesting of putative 'hits'

The 13 'hits' were retested by both SPA and an ion exchange resin method. For each test, the assay methodology was identical (see Figure 3 legend for details), the only difference was in the signal generation, and the actual signal measured - SPA measures the loss of arginine whereas the ion exchange method is designed to measure the accumulation of citrulline. Thus, for iNOS inhibitors, the SPA format will return a high signal and the ion exchange method will return a low signal. A fourteenth sample was tested alongside the 'hits', based on its potential to be an activator of iNOS, as identified by a signal from the screening plate that was 3 SD below the mean of the data from both plates.
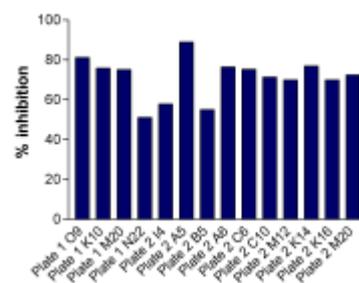

Figure 4. SPA retest data. General protocol as Figure 2. Samples were identified by plate and well from the original 384-well screening plate.
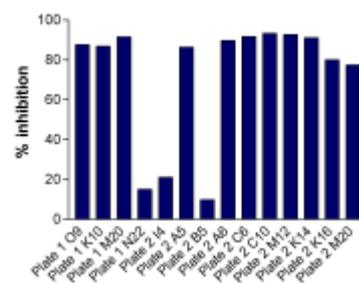

Figure 5. Ion exchange column data. Assay protocol as Figure 3. Stop solution (20 mM Hepes/1 mM EDTA/1 mM Seitu, pH7.5). Columns (Bio-Rad 731-6213, pre-filled with AG^a 50W-X8 resin) were converted to the Na^+ form by washing 4 x 2 ml 1M NaOH, then neutralised ready for use by washing 4 x 2 ml H_2O, checking that the eluate pH was below 8.0. Samples were applied to the column and eluted with 2 ml H_2O. 100 ml aliquots were analyzed by liquid scintillation counting.

Both methods were able to identify the same true and false 'hits' in the retesting. The putative 'enhancer' was shown to be a screening artefact, again by both methodologies. Interrogating the database supplied with the LOPAC library identified a total of 15 compounds as being inhibitors of at least one form of NOS. The screen was able to identify 13 of the 15. Of the two compounds missed, one, aminoguanidine hemisulphate, has a very high IC_{50} (~250 μM) and thus would not be expected to show up as a hit at the concentrations used. On checking, the other compound, N'G,N'G dimethylarginine hydrochloride was found to have a solubility problem in our master library plate.

References

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CONCLUSION

This SPA offers a simple, rapid, automatable method for the screening of compound libraries, in a 384 well microplate format for activity against the iNOS isoform. This speed and simplicity offers an enablement over existing assay formats.