Comparison of the IMAP Fluorescence Polarisation Assay with the Scintillation Proximity Assay for Phosphodiesterase Activity.

David R. Mobbs, Joanne O. Wiseman, John Dawson, Richard A. Williamson

Introduction

Inhibition of phosphodiesterase (PDE) isoforms represent drug targets for a range of diseases. The Molecular Devices Corporation have developed IMAP* technology that is able to measure PDE activity in an homogenous, non-radioactive assay format. The technology relies on the ability of immobilised trivalent cations to bind the phosphate group that is produced on the hydrolysis of fluorescein-labelled cyclic adenosine mono phosphate (Fl-cAMP) to the non-cyclic Fl-AMP form. Binding of Fl-AMP product to the beads slows their rotation and leads to an increase in the fluorescence polarisation ratio of parallel to perpendicular light. Inhibition of PDE prevents this signal increase.

We have compared the IMAP assay with the Scintillation Proximity Assay** (SPA) that is already established for PDE4B and PDE4D. We found that there is less than 0.5-log unit difference in pIC50 value between the two assay formats across a range of biological potency and chemical diversity. The linear regression coefficients were 0.966 for PDE4B, and 0.971 for PDE4D.

Materials and Methods

- PDE4B (human recombinant, in-house)
- PDE4D (human recombinant, in-house)
- Black 384 NUNC plates (code 262260) for IMAP
- White 96 Wallac Isoplates (code 1450-514) for SPA

Compound Handling

For both the IMAP and SPA methods, 100 test compounds were preincubated at ambient temperature in microtitre plates with PDE enzyme in assay buffer for 10-30 minutes.

For inhibition curves, 10 concentrations (1.5nM - 30uM) of each compound were assayed.

IMAP assay

The IMAP Explorer kit (code R8062, 8000 wells) was purchased from Molecular Devices. The kit contains;

- 5X reaction buffer 10mM Tris HCl pH 7.2, 10mM $MgCl_2$, 0.1% bovine serum albumin, 0.05% NaN_3
- 5X binding buffer (proprietary)
- IMAP binding reagent (proprietary)
- Fl-cAMP substrate

Stock buffers were diluted 5-fold using reverse osmosis water. The substrate was thawed and diluted 50-fold in reaction buffer. The IMAP binding reagent was diluted 400-fold in binding buffer. The enzyme stocks were thawed on ice and diluted appropriately in reaction buffer.

Serial dilutions of test compounds were prepared in DMSO. Aliquots were dispensed into plates.

All reagents were dispensed using the Labsystems Multidrop™.

10ul of diluted enzyme or reaction buffer for controls.

10ul of diluted substrate to all wells.

Plates were shaken on an orbital shaker for 1 minute then incubated at room temperature for 40 minutes.
60ul of diluted IMAP binding reagent was added to all wells.

The plates were left to stand for 1 hour before reading (although the signal was found to be stable overnight). The IMAP FP ratio of parallel to perpendicular light was measured using an Analyst™ plate reader.

Enzyme dose response curves were produced to determine the enzyme dilution factor, as shown in Figure 1 for PDE4B.

Time-course analyses were carried out at the appropriate enzyme dilution (PDE4B 1:400, PDE4D 1:800) to determine the duration of the linear reaction as shown in Figure 2 for PDE4B.

Typically reactions were found to be linear to a background corrected signal of window of 150mP units.

SPA

Reagents purchased from Amersham Biosciences:

- Phosphodiesterase Yttrium Silicate beads (RPNQ 0150)
- [5',8-^{3}H]adenosine 3',5'-cyclic phosphate (code TRK.559)

Additional material;
Assay buffer 50mM Tris pH 7.5, 8.3mM $MgCl_2$, 0.05% BSA, 1.7mM EGTA

The Multidrop was used to dispense [5',8-^{3}H]adenosine 3',5'-cyclic phosphate to give 0.05µCi per well at ~ 10nM final concentration. Plates were shaken on an orbital shaker for 5 minutes and incubated at ambient temperature for 40 minutes.

Phosphodiesterase SPA beads were added using manual pipetting (~1mg per well) to terminate the assay. Plates were sealed, shaken and allowed to stand at ambient temperature for 1hour to allow the beads to settle. Bound radioactive product was measured using a Wallac Trilux 1450 MicroBeta scintillation counter.

Results

Curves were analysed using ActivityBase™ and XLfit™.
Results were expressed as pIC50 values.
A regression analysis was carried out to compare the IMAP v SPA results for each enzyme tested. The results are shown in Figures 3 (PDE4B) and 4 (PDE4D).

IMAP assay

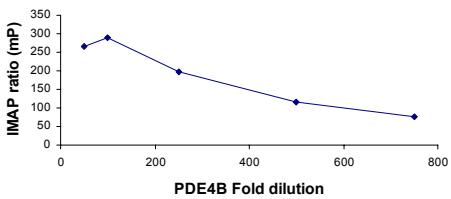

Figure 1. Enzyme dose-response for PDE4B (IMAP) Corrected for background

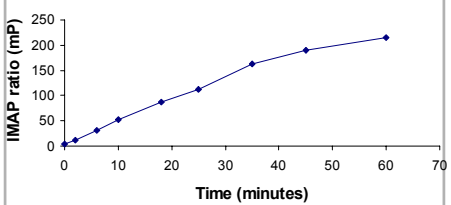

Figure 2. Time-course for PDE4B (IMAP) Corrected for background

Comparison of IMAP with SPA

IMAP	SPA
Non-radioactive	Radioactive
Beads do not settle rapidly	Dense beads settle rapidly
Natural substrate plus fluorescein	**Natural substrate plus tritium**
Can be miniaturised to 1536	Difficult to miniaturise (can use LEADseeker™)
384-well plate read time 5 minutes	96-well plate read time 10 minutes (can use LEADseeker)

Table 1. Relative advantages of IMAP and SPA formats

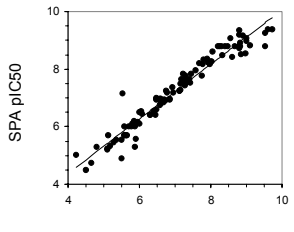

Figure 3. PDE4B regression analysis of pIC50 values for SPA v IMAP. r^2 0.966, gradient 0.92

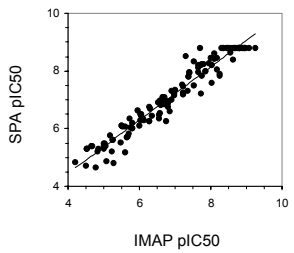

Figure 4. PDE4D regression analysis of pIC50 values for SPA v IMAP. r^2 0.971, gradient 0.92

The pIC50 value achieved for the standard compound roflumilast agrees with the literature value of 9.2

Discussion and Conclusion

The IMAP pIC50 values of 100 test compounds were found to agree to within 0.5 log units of the SPA values for PDE4B and PDE4D.

We checked the parallel intensity of the diluted substrate before each assay run. Typical values are 1 to 4 million parallel counts. We found it necessary to vortex mix the stock substrate before dilution, especially after freeze/thaw cycles.

We routinely use the IMAP assay to screen compounds against PDE4B and PDE4D. The technology has reduced the turn-around time for compound testing by 25% compared to the SPA. This is due to the following advantages of the IMAP assay; the use of 384-well plates rather than 96-well plates, the shorter read time and the ability to use the Multidrop to dispense all reagents. The non-radioactive nature of IMAP has been a major improvement too. It has reduced the need for radioactivity swabbing of laboratories, and has reduced plate disposal costs.

Work is in progress to convert PDE3, PDE4A and PDE7 to the IMAP format. Molecular Devices sell a fluorescein-labelled cGMP substrate for use in assay of enzymes such as PDE5, PDE6 and PDE9. We have carried out a limited study on PDE5, and intend to revisit this work in the future.

*Molecular Devices Corporation, Sunnydale, CA, USA.

**Amersham Biosciences UK Ltd, Pollards Wood, Chalfont St Giles, Bucks, HP8 4SP, UK

Trademarks;
Multidrop - Thermo Labsystems Oy, Ratastie 2, PO Box 100, Vantaa 01620, Finland

Analyst - Molecular Devices Corporation, Sunnydale, CA, USA

ActivityBase and XLfit - ID Business Solutions Limited, 2 Ocean Court, Surrey Research Park, Guildford, Surrey, GU2 7QB, UK

LEADseeker - Amersham Biosciences UK Ltd, Pollards Wood, Chalfont St Giles, Bucks, HP8 4SP, UK

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