A Fast and Simple Method For Measuring P-Glycoprotein (Pgp) Inhibition

Donald L. Melchior, The Fluorosome Company, Worcester, MA 01605, USA
Frances J. Sharom, Department of Molecular and Cellular Biology, University of Guelph, Guelph ON Canada N1G 2W1
E.J. Dell, BMG LABTECH, 13000 Weston Parkway, Cary NC 27513, USA

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- The Fluorosome®-*trans*-pgp fluorescence intensity assay specifically measures the inhibition of P-glycoprotein multidrug transporter
- BMG LABTECH´s FLUOstar Omega with onboard injectors used to easily set up fluorescence kinetic measurements

Introduction

P-glycoprotein (Pgp; ABCB1), a member of the ATP-binding cassette (ABC) superfamily, exports structurally diverse hydrophobic compounds from the cell driven by ATP hydrolysis.[1] Pgp expression has been linked to the efflux of chemotherapeutic drugs in human cancers leading to multidrug resistance. Pgp activity can also result in low oral absorption and poor brain penetration. Interaction of drugs with the Pgp may also cause an increase in toxicity of co-administered compounds.

Interaction of drugs with active transporters such as Pgp is of increasing interest to the pharmaceutical industry based, in large part, on new draft FDA guidelines requiring knowledge of whether a drug candidate is a substrate and/or inhibitor of Pgp.

The Fluorosome Company's Fluorosome®-*trans*-pgp assay, together with BMG LABTECH's FLUOstar Omega, provides a rapid, sensitive and specific reconstituted Pgp liposome assay system for identification of compounds that interact with the transporter. The assay measures the ability of a compound to compete with a Pgp substrate for transport, and determines the IC_{50} value. The FLUOstar Omega's ability to inject at the point of measurement assures that no data is lost. Up to 50 readings per second taken concurrently upon injection, provides ample data for calculation of first order transport rates. Single concentration inhibition measurements can be made very rapidly, and an IC_{50} determination carried out in only 10 minutes thereby.

Assay Principle

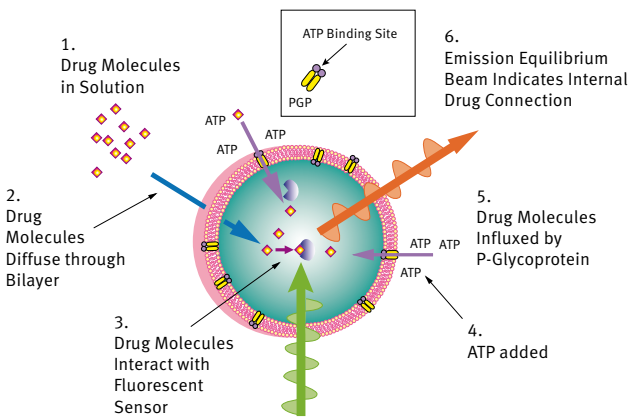
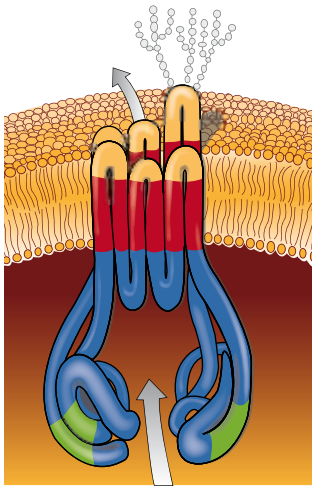


Fig. 1: Principle of the Fluorosome®-*trans*-pgp Assay

Materials and Methods



The Pgp is a 170 kDa intrinsic membrane protein that effluxes a wide range of drugs from the cell. Pgp is also an outwardly directed flippase for fluorescent phospholipid and glycosphingolipid derivatives, which suggests that it may also translocate drug molecules from the inner to the outer membrane leaflet. The membrane lipid bilayer plays and important role in Pgp function and may regulate both binding and transport of drugs.

Fig. 2: The Pgp Efflux Pump

Isolation and Purification of Pgp

Purified Pgp (›90%) is isolated from Pgp-overexpressing CH^RB30 cells by detergent (CHAPS) extraction followed by affinity chromatography on Con A-Sepharose.[2] Micellar Pgp is reconstituted into lipid bilayers by mixing in solution with CHAPS-solubilized micellar phospholipid, and the detergent is removed by gel exclusion chromatography. The resulting reconstituted Pgp is aliquoted and stored frozen at -70°C.

Manufacture of Fluorosome-trans-pgp

Frozen reconstituted Pgp is thawed and mixed in buffer with fluorophore (BSA-fluorescein) then converted into Fluorosome-*trans*-pgp by extrusion. The resulting unilamellar vesicles are separated from unencapsulated fluorophore by gel exclusion chromatography. The fluorosome-*trans*-pgp is then subjected to Quality Control Criteria; size, fluorescence, passive permeability, and ATP-stimulated transport of a control Pgp substrate.

The Fluorosome-trans-pgp assay procedure.

1. 98 µl of Fluorosome-*trans*-pgp in buffer containing 3 µM of the substrate S-HR was added to each well of a 96-well half-well plate.
2. A 2 µl aliquot of a DMSO solution of test compound was added. Each aliquot contained test compound at one of 7 concentrations to give, after dilution into the well, the desired concentration of compound. Blank, reference wells (no compound) had 2 µl of DMSO added to them.
3. The plate was placed in the FLUOstar Omega, and the fluorescence was monitored at 485/520 for 60 seconds as follows:
   a. a fluorescence baseline was established during the first 18 seconds;
   b. at 18 seconds, 5 µl of a solution of ATP in buffer was injected into each well (final concentration 2 mM), and the fluorescence was monitored for 42 seconds;
   c. the slope from 30 to 60 seconds was immediately calculated by BMG LABTECH's MARS data analysis software.

A typical picture of fluorescence signals measured over time is given in Figure 3.

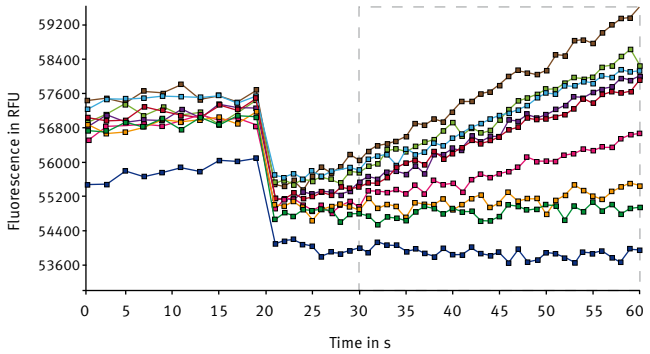


Fig. 3: Fluorescence kinetic signal curves for different samples

Results and Discussion

The slope for each well (Fig.3) was plotted against concentration of test compound, and IC_{50} values were calculated by using a robust fit for a single order decay (Fig. 4 and 5).

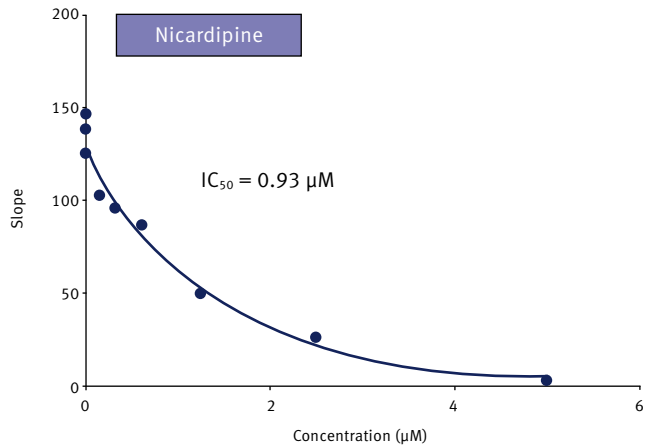


Fig. 4: Single order decay fit with Nicardipine as test compound

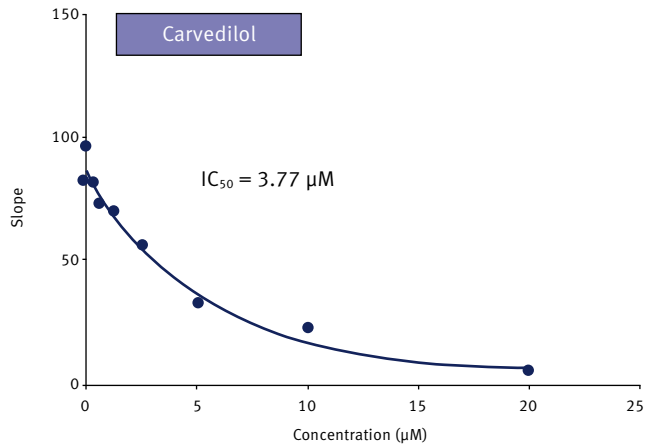


Fig. 5: Single order decay fit with Carvedilol as test compound

Correlation of Fluorosome-trans-pgp IC_{50} Data and Cell Monolayer IC_{50} Data

IC_{50} values are given as µM concentrations. Bidirectional transport inhibition studies were conducted on the same set of compounds as were measured with the Fluorosome-*trans*-pgp assay, examples of which are shown in Fig. 6.

Bidirectional transport inhibition studies employed LLC-MDR1 cell monolayers and [^{3}H]digoxin (0.1 µM) as the substrate essentially as described in.[3]

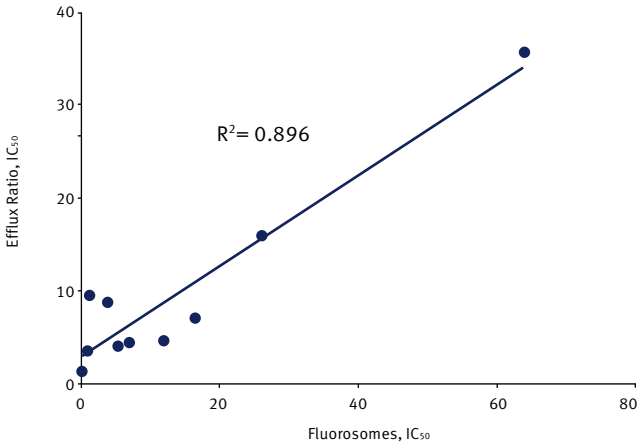


Fig. 6: Cell monolayer IC_{50} values reported as Efflux Ratio, i.e. changes in net transport Papp B-A - Papp A-B.

Results using the Fluorosome-*trans*-pgp assay are in excellent agreement with those obtained by cell culture methodology.

Conclusion

We report a novel assay specific for measuring inhibition of the P-glycoprotein multidrug transporter (Pgp; ABCB1) and an ideal instrument for its use.

- Simple assay procedure: No sterile conditions, non-compound specific, no radiolabel or LC-mass spectroscopy
- Specificity: Fluorosome®-*trans*-pgp contains only the P-glycoprotein transporter
- Low Sample Requirement: A 100 µl volume of Fluorosome®-*trans*-pgp Reagent per assay requires 1 nanomole drug
- Speed: 1 minute per inhibition assay - e.g. an 8 point IC_{50} determination + 2 references takes only 10 minutes
- Popular format: 96 well half-well or 384 well microplates - one well per inhibition assay

References

1. Eckford and Sharom (2009) *Chem Rev.* **109**, 2989-3011.
2. Liu and Sharom (1996) *Biochemistry* **35**, 11865-11873.
3. Chu et al. (2007) *J. Pharmacol. Exp. Ther.* **321**, 673-683.

Germany:	**BMG LABTECH GmbH**	**Tel: +49 781 96968-0**
Australia:	BMG LABTECH Pty. Ltd.	Tel: +61 3 59734744
France:	BMG LABTECH SARL	Tel: +33 1 48 86 20 20
Japan:	BMG LABTECH JAPAN Ltd.	Tel: +81 48 647 7217
UK:	BMG LABTECH Ltd.	Tel: +44 1296 336650
USA:	BMG LABTECH Inc.	Tel: +1 877 264 5227
Internet:	**www.bmglabtech.com**	**applications@bmglabtech.com**