



# Reconstituted P-glycoprotein in Fluorosome® lipid bilayer vesicles - basis for an *in vitro* P-glycoprotein assay.

Donald L. Melchior<sup>1</sup>, Frances J. Sharom<sup>2</sup>, George E. Wright<sup>1</sup>, Steven E. Wright<sup>1</sup>, Ronghua Liu<sup>2</sup>.

<sup>1</sup>GLSynthesis Inc., 1 Innovation Drive, Worcester, MA 01605 U.S.A; <sup>2</sup>Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1.

## 1. Introduction

The need for a simple, direct method to characterize P-glycoprotein (Pgp, MDR1) substrates and inhibitors led us to combine two methodologies:

Fluorosome®-trans;  
Pgp isolation and membrane reconstitution.

to produce a prototype *in vitro* Pgp assay -  
"Fluorosome-trans-pgp".

Fluorosome®-trans is a fluorophore-containing liposome developed to measure the passive diffusion (permeability) of drugs across membrane bilayers. The incorporation of reconstituted Pgp into the bilayers of Fluorosomes results in a system capable of measuring active, Pgp mediated, ATP-dependent transport of small molecules and to characterize inhibitors of the Pgp.

## 2. The Fluorosome-trans-vesicle

1. Drug Molecules in Solution
2. Drug Molecules Diffuse through Bilayer
3. Drug Molecules Interact with Fluorescent Sensor
4. Emission Beam Indicates Internal Drug Concentration

Excitation Beam

Fluorescence quenching of Fluorosome-trans and Soluble fluorescent probe

## 3. The Fluorosome-trans Assay Trans-membrane Passive Drug Diffusion

- 1. Instrumentation**  
The Fluorosome® Solution, FS-1  
Custom designed cuvette based spectrophotometer with FS1.4 acquisition and analysis software  
High Throughput Format  
Injecting Plate Reading Spectrophotometer with auto injecting capability
- 2. Measurement**  
A. Fluorosome®-trans in cuvette  
B. Inject Drug
- 3. Analysis**  
In a typical experiment, k is the first order rate constant for flux of the test compound across the membrane:  
1. Fit curve to obtain k in sec<sup>-1</sup>.  
2. Calculate the permeability constant, PC, from k by:  
 $PC = k / C_o$   
Where C<sub>o</sub> is ratio of vesicle surface area to encapsulated volume
- 4. Typical Results**  
Correlation between permeability measured with Fluorosome-trans-pc and human oral absorption of 47 drugs

## 4. The Pgp Molecule

P-glycoprotein (Pgp, MDR1, ABCB1) is a member of the ATP-binding cassette (ABC) superfamily. It is a 170 kDa intrinsic membrane protein. Structural models suggest that 2 nucleotide-binding domains are closely associated to form a nucleotide sandwich dimer. Pgp is 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 ATPase catalytic cycle of the protein is thought to proceed via an alternating site mechanism, although the details are not clear. The membrane lipid bilayer plays an important role in Pgp function and may regulate both the binding and transport of drugs<sup>1</sup>.

<sup>1</sup>Frances J. Sharom in "Drug Transporters: Molecular Characterization And Role in Drug Disposition" eds. G. You, M.E. Morris, Chapter 10, pgs. 223-262, Wiley Interscience, 2007.

## 5. Fluorosome-trans-pgp manufacture

**Isolation and Purification of Pgp**

Pgp is isolated from Pgp-overexpressing CHRB30 cells by detergent (CHAPS) extraction followed by affinity chromatography on Con A-Sepharose. This micellar form of Pgp is reconstituted into lipid bilayers by being mixed in solution with CHAPS solubilized micellar phospholipid and the detergent removed by gel filtration. 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, ATPstimulated control substrate transport.

## 6. The Fluorosome-trans-pgp Vesicle

1. Drug Molecules in Solution
2. Drug Molecules Diffuse through Bilayer
3. Drug Molecules Interact with Fluorescent Sensor
4. ATP Added
5. Drug Molecules Influxed by P-Glycoprotein
6. Emission Equilibrium Beam Indicates Internal Drug Concentration

ATP Binding Site  
PGP

## 7. The Fluorosome-trans-pgp Assay Pgp-mediated active Drug Transport

P-glycoprotein mediated influx of vinblastine in the absence and presence of the ATPase inhibitor sodium orthovanadate (1 mM)

**Assay Procedure**

1. Establish baseline for Fluorosome-trans-pgp solution.
2. Inject drug. Change in fluorescence reflects the passive diffusion of drug into Fluorosome-trans-pgp particle.
3. Baseline is re-established. Drug concentration is in equilibrium between inside and outside of Fluorosome-trans-pgp particle.
4. Inject ATP (final concentration 2 mM). After an equilibration period, the slope in fluorescence reflects the ATP stimulated Pgp pumping of drug into the Fluorosome-trans-pgp vesicle.
5. After an equilibration period, the change in fluorescence at a given time, Δ, reflects a drug's substrate capacity relative to the Pgp

## 8. Fluorosome-trans-pgp Assay Controls

**Fluorosome-trans-pgp. ATP stimulated drug influx is mediated by the Pgp pump.**

Active drug uptake is observed only for Pgp substrates in the presence of ATP.

Δ is the reduction in fluorescence resulting from ATP-dependent influx of a compound into Fluorosome-trans-pgp. Measurement begins after an equilibration interval following injection of ATP.

**Drug uptake is not observed for Pgp-substrates in the absence of ATP**

ATP Control

Fluorescence change with time upon injection of 2mM ATP for Fluorosome-trans-pgp (a) not preincubated with a pgp substrate (basal activity) (b) preincubated with 2.5 μM of the pgp substrate bisbenzamide

**Drug uptake is not observed for Pgp substrates with a non-hydrolyzable ATP analogue**

ATP-AMP-PNP Control

Fluorescence change with time of Fluorosome-trans-pgp preincubated with 12.5 μM of the pgp substrate bisbenzamide (a) upon addition of 2 mM ATP (b) upon addition of 2 mM of the non-hydrolyzable ATP analog 5'-adenylyl-β-γ-imidodiphosphate, (AMP-PNP).

**Drug uptake is not observed for Non-Pgp substrates in the presence of ATP**

Amiloride Control

Fluorescence change with time of Fluorosome-trans-pgp upon addition of 50 μM of the pgp non-substrate amiloride (passive diffusion) (a) upon injection of 2 mM ATP after the passive diffusion of amiloride into the Fluorosome-trans-pgp particle has reached equilibrium

## 9. Fluorosome-trans-pgp as a Pgp inhibitor assay

**The transport of Bisbenzamide by Pgp in the presence and absence of Cyclosporin A**

**The use of Fluorosome-trans-pgp to assay for Pgp Inhibition by Valinomycin and Cyclosporin A**

1. Fluorosome-trans-pgp was preincubated with 2.5 μM bisbenzamide
2. Valinomycin and Cyclosporin A were added to aliquots of the above
3. The above solutions were placed in a spectrophotometer; after an equilibrium baseline obtained, ATP was added.
4. Bisbenzamide transport was observed and compared to bisbenzamide transport in the absence of inhibitor

**"Use of bisbenzamide as a "test" substrate allows for the testing of inhibitors that affect the "H transport site" of the Pgp. Use of tetramethylrhodamine as a "test" substrate allows for the testing of inhibition at the "R transport site" of the Pgp.**

<sup>1</sup>Frances J. Sharom in "Drug Transporters: Molecular Characterization and Role in Drug Disposition" eds. G. You, M.E. Morris, Chapter 10, pgs. 223-262, Wiley Interscience,

For further information E-mail: [Fluorosomes@glsynthesis.com](mailto:Fluorosomes@glsynthesis.com)

Phone: 508-754-6700 Exts. 102/105 Fax: 508-754-7075

Web: [www.fluorosomes.com](http://www.fluorosomes.com)