

Abstract

We have developed a luminescent method for detecting drug-dependent changes in the ATPase activity of the multi-drug transporter P-glycoprotein (Pgp, MDR1 or ABCB1). Pgp is an ATP-dependent efflux pump for a wide range of drugs that plays an important role in multi-drug resistance and certain adverse drug-drug interactions. Drugs that are transported by Pgp can be identified as stimulators of its ATPase activity. Our luminescent method relies on the ATP-dependence of the light-generating reaction of firefly luciferase. After a pool of ATP is first exposed to the Pgp ATPase ATP consumption is detected as a decrease in luminescence from a second reaction with firefly luciferase. The quantity of ATP consumed can be interpolated from a luciferase/ATP standard curve and from this the rate of ATPase activity calculated. We used a stabilized mutant of the luciferase enzyme in a formulation that provided stable, glow-style signals from multi-well plates. The method detected the dose-dependent stimulation of recombinant human Pgp ATPase activity by verapamil and inhibition of this activity by cyclosporin-A. This simple add and read format is well suited for screening applications.

General Assay Method

•Perform Pgp ATPase Assay

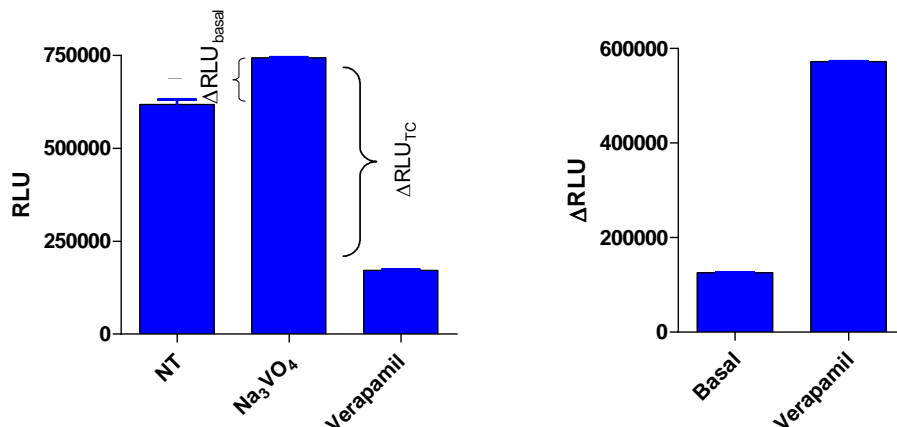
An insect cell plasma membrane fraction with recombinant Pgp over-expression and Na_3VO_4 treated controls are incubated in a reaction buffer with ATP in the presence or absence of test compounds. In the presence of Na_3VO_4 ATP consumption by Pgp is negligible and without Na_3VO_4 Pgp consumes ATP to a greater or lesser extent dependent on the effect of the test compounds.

•Add Luciferase and Read

A firefly luciferase reaction mixture is added after the Pgp ATPase incubation to stop Pgp activity and initiate an ATP-dependent luminescent reaction. Signals are measured on a luminometer and can be converted to ATP concentrations by interpolation from a luminescent ATP standard curve.

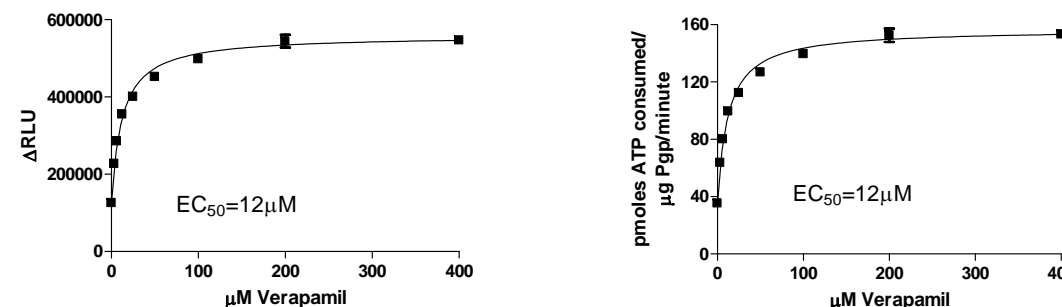
The difference between Pgp and Pgp+ Na_3VO_4 reactions represents the ATP consumed by Pgp. Test compounds that increase this difference are scored as Pgp substrates.

Pgp-dependent Changes in Luminescence



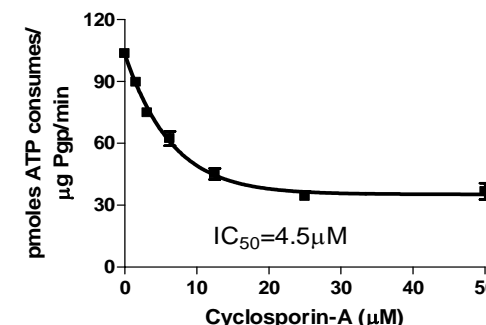
Pgp-dependent changes in luciferase luminescence: Pgp reactions (40 min/37°C) were performed with no treatment (NT), plus the Pgp inhibitor Na_3VO_4 or with 200 μM verapamil (left panel). The Pgp-dependent changes in relative luminescent units (ΔRLU , replotted in left panel) reflect basal and verapamil-stimulated ATPase activity.

Stimulation of Pgp ATPase



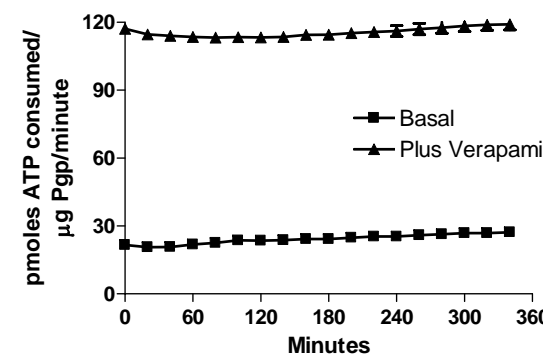
Recombinant Pgp (+/ Na_3VO_4) was incubated with verapamil for 40 minutes at 37°C before initiating luminescence. The data is presented as ΔRLU (left panel) or as pmoles ATP consumed/ μg Pgp/minute (right panel). ΔRLU is the difference between reactions with Pgp and those with Pgp plus Na_3VO_4 and is proportional to the quantity of ATP consumed by Pgp. For the right panel reaction rates were calculated after interpolation of the ATP content of each reaction from a luminescent ATP standard curve.

Inhibition of Pgp ATPase Activity by Cyclosporin-A



Verapamil-stimulated (200 μM) Pgp ATPase activity was measured in the presence of increasing concentrations of cyclosporin-A. Reaction rates were calculated after interpolation of ATP concentrations from a luminescent ATP standard curve.

Luminescent ATPase Assays Provide for Stable ATPase Activity Measurement



Recombinant Pgp reactions were incubated for 40 minutes at 37°C plus or minus Na_3VO_4 with 200 μM verapamil or without verapamil (basal). Pgp ATPase activity was then stopped and ATP-dependent luminescence initiated and measured at 20 minute intervals for 340 minutes. ATP quantities consumed by Pgp and ATPase activities were calculated by comparing luminescent assay signals to an ATP luminescent standard curve measured in parallel with Pgp assays. The results show that luminescence can be read immediately or after a delay without a substantial change in apparent ATPase activities.

Conclusion

The luminescent method described detects drug-dependent changes in Pgp ATPase activity. The simple add and read format uses stable reagents to produce stable readouts. The method is amenable to high through put single dose applications and multi-dose application for quantitative analyses.