Application Note

SCREENING OF GPCR-ANTAGONISTS USING THE CALCIUM MOBILISATION ASSAY

Accurate and reliable characterization of potential antagonists for pharmacological studies

Abstract

The nucleotide receptor family P2Y comprises of G protein-coupled receptors that show potential as drug targets for various disorders. Targeting the $P2Y_2$ subtype is particularly promising, since antagonists could be effective against tumour metastasis, excessive inflammatory reactions, atherosclerosis, kidney disorders, and osteoporosis [1-3]. The precise role of the $P2Y_2$ receptor in these conditions is often not clear, which can be partially attributed to the limited availability of potent and selective antagonists that are required for pharmacological studies [4]. To characterise potential antagonists, three commercially available compounds were screened for $P2Y_2$ receptor inhibition using the calcium mobilisation assay with two different fluorescent dyes measured on a TriStar² LB 942 plate reader. Reliable and consistent results were obtained.

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Introduction

Measurement of a fluorescence intensity signal is a common technique frequently employed for *in vitro* assays in drug discovery laboratories. It is a simple, versatile, and cost-effective method that has been used for several decades. Using ratiometric assays, side effects such as photobleaching or uneven dye loading can be avoided, generally resulting in an increased reliability and reproducibility. A large selection of fluorescent probes is available on the market with different excitation and emission wavelengths for various needs. The two fluorescent dyes used in this study were fluo-4 and fura-2.

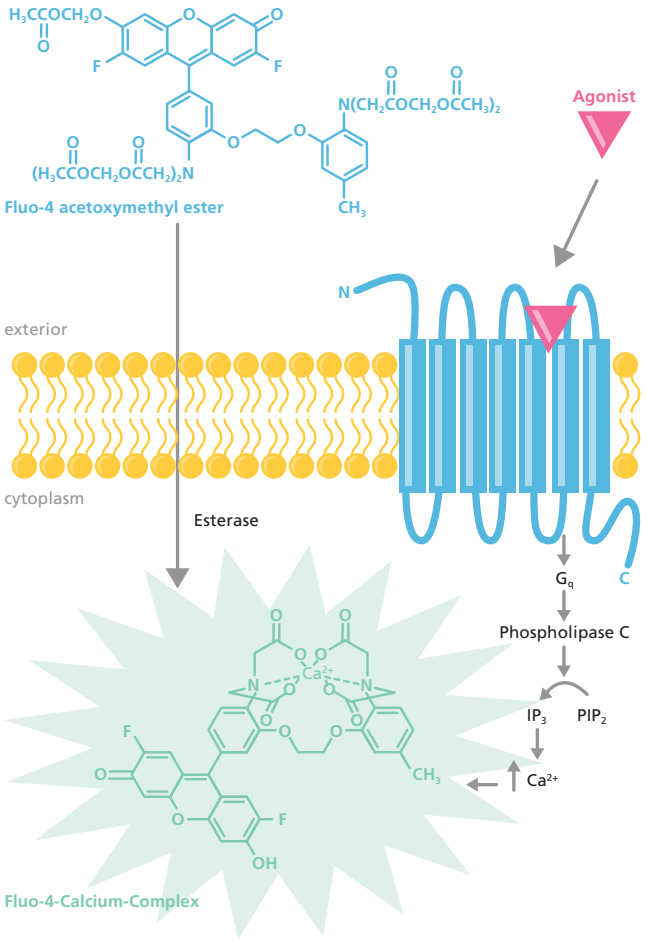

Fig. 1: Basic principles underlying the calcium mobilisation assay. Shown here is the fluorescent dye fluo-4 as an example.

THE CALCIUM MOBILISATION ASSAY

The $P2Y_2$ receptor is Gq-coupled and initiates an intracellular cascade of events that lead to an increase in the cytosolic concentration of calcium ions. If the cell has been loaded with a suitable fluorescent dye, the rise in intracellular Ca^{2+} concentration can be detected and quantified. In these experiments, activation of the receptor was achieved through the application of its endogenous agonist UTP. The presence of a test compound should prevent the rise in cytosolic [Ca^{2+}] if it acts as an antagonist for the $P2Y_2$ receptor. This principle is schematically illustrated in Fig. 1.

The dyes used here were fluo-4 and fura-2. Both are commercially available in the form of an acetoxymethyl ester (AM). They are sufficiently lipophilic to diffuse across plasma membranes into the cell. The acetoxymethyl ester precursor itself emits little fluorescence. However, once inside the cell, the acetoxymethyl ester group that masks the calcium ion binding structure is cleaved by non-specific esterase enzymes. The resulting anionic dye is now capable of binding Ca^{2+}, whereby the emitted fluorescence intensity increases. As a result of the negative charge at physiological pH, the dye is unable to diffuse back out of the cell.

Fluo-4 is an analogue of fluo-3, which has been in use for various types of experiments involving the detection of Ca^{2+}. The two chlorine atoms were substituted with fluorine for fluo-4 in order to increase the excitation at a wavelength of 488 nm and thereby the emitted light intensity at 520 nm [5].

Fura-2 was introduced in 1985 by Molecular Probes® and has since been applied in a wide variety of cells. As opposed to fluo-4, fura-2 measurement is ratiometric, as the excitation maximum shifts towards shorter wavelengths at increasing concentrations of calcium ions. The ratiometric measurement minimises the effects of photobleaching, uneven dye loading, or leakage of dye [6]. In this study, fluo-4 was compared against fluo-2 to analyse the impact of these side effects using the TriStar² LB 942 plate reader.

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- Fluorescence Polarization
- Top and Bottom Reading
- Incubation
- Gas control

Fig. 2

Materials, Methods, and Settings

MATERIALS

- 1321N1 astrocytoma cells stably transfected with the $P2Y_2$ receptor
- Dulbecco's Modified Eagle Medium (Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% foetal calf serum (Sigma-Aldrich, Munich, Germany), G418 (200 µg/ml; Applichem, Darmstadt, Germany), penicillin (100 U/ml), and streptomycin (0.1 mg/ml; Life Technologies GmbH, Darmstadt, Germany)
- Hank's balanced salt solution (HBSS) buffer
- Phosphate-buffered saline (PBS)
- Trypsin-EDTA (Life Technologies GmbH, Darmstadt, Germany)
- 10 mM compound stock solutions in 100 % dimethyl sulfoxide (DMSO)
- 100 µM stock solution of the endogenous agonist UTP (Sigma-Aldrich, Munich, Germany) in HBSS buffer
- Fluorescent dyes fluo-4 and fura-2 (Life Technologies GmbH, Darmstadt, Germany)
- Pluronic F-127 (Sigma-Aldrich, Munich, Germany)
- Sterile, black, flat, clear bottom, surface-coated 96-well microplates with lids (Corning 3340, Corning, Tewksbury, Massachusetts, USA)
- Berthold Technologies TriStar² LB 942 Multidetection plate reader (see Fig. 2)

METHODS

1321N1 human astrocytoma cells stably transfected with the coding sequence for the $P2Y_2$ receptor and cultured at 37 °C and 10% CO_2 in the nutrient medium described above were used. Approximately 24 h prior to testing, the nutrient medium was discarded and the cells rinsed with phosphate-buffered saline before detachment using trypsin-EDTA. The cells were subsequently suspended in DMEM with the supplements described above and dispensed into sterile, black, flat, clear bottom, surface-coated 96-well polystyrene microplates with lid (Corning 3340) at 50,000 cells per well. The microplates were incubated overnight at 37 °C in humidified air with 10% carbon dioxide, during which the cells adhered to the coated bottom of the wells.

Test compounds were investigated by measuring their inhibition of $P2Y_2$ receptor-mediated intracellular calcium mobilisation using a TriStar² LB 942 (Berthold Technologies, Bad Wildbad, Germany) plate reader. At the start of the assay, the plated cells were loaded with fluo-4 acetoxymethyl ester or fura-2 acetoxymethyl ester for 1 h. Excess dye was subsequently removed and HBSS buffer given to the cells. Afterwards, the cells were pre-incubated with the test compound for 30 min prior to the injection of the physiological agonist UTP at a final concentration of 250 nM, which corresponded to the EC80 value at the respective cell line. The final volume was 200 µl per well.

INSTRUMENT SETTINGS

- Excitation filters: 485 nm, 340 nm (10 nm bandwidth), 380 nm (10 nm bandwidth)
- Emission filters: 535 nm, 510 nm (40 nm bandwidth)

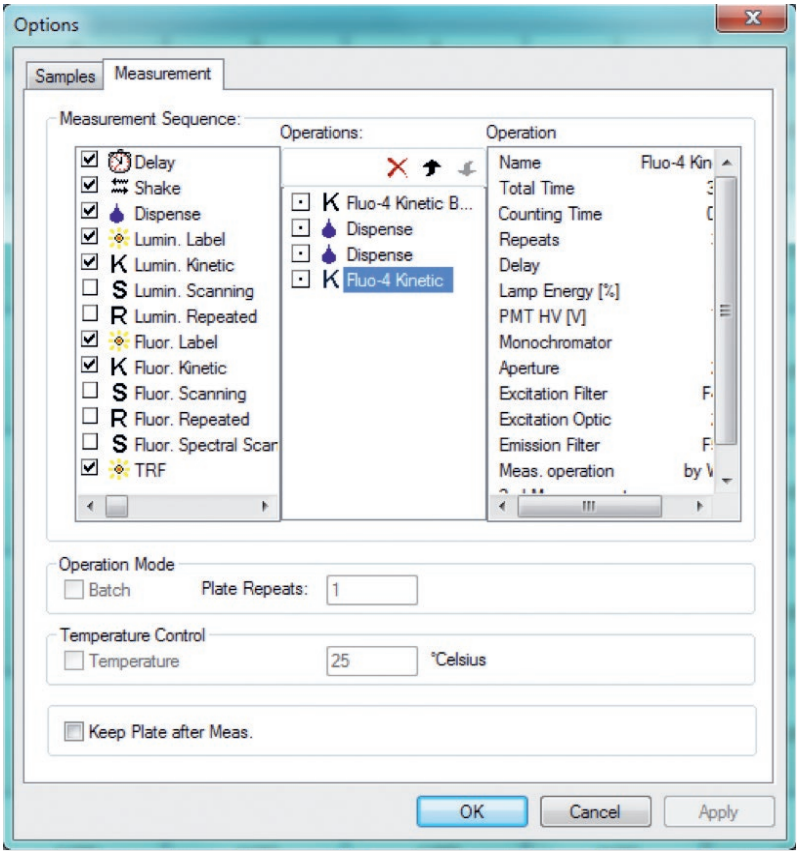

Fig. 3: Screenshot of the instrument settings dialogue in the MikroWin 2010 software for the fluo-4 measurement. For fura-2, it is analogous. The first measurement step is to determine the background fluorescence prior to addition of the agonist. Subsequently, there are two dispense steps with identical settings (see Fig. 4) but for two different injectors: one for the agonist, while the other pump injects buffer into different wells as negative control.

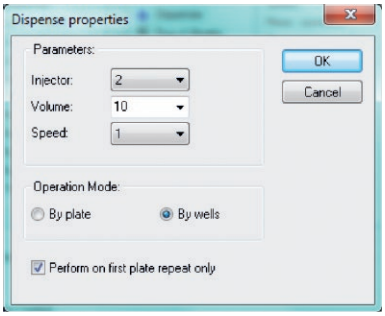

Fig. 4: Screenshot of the dispense properties dialogue in the MikroWin 2010 software for the fluo-4 and fura-2 measurements

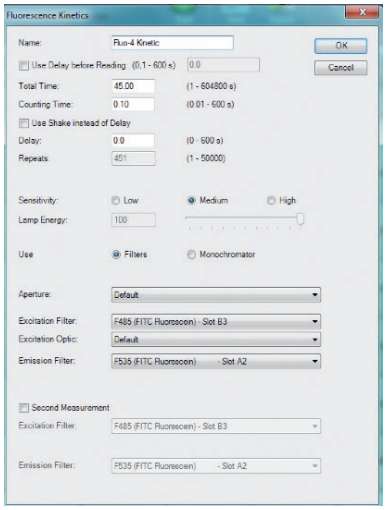

Fig. 5: Screenshot of the instrument settings dialogue in the MikroWin 2010 software for the fluo-4 measurement

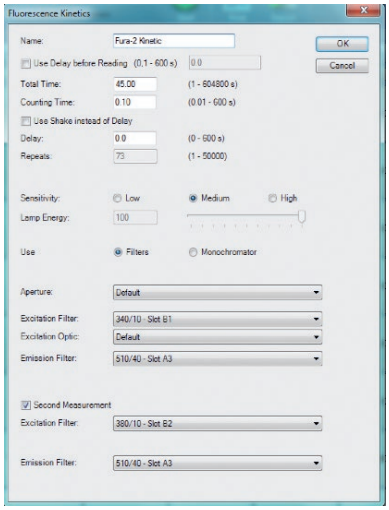

Fig. 6: Screenshot of the instrument settings dialogue in the MikroWin 2010 software for the fura-2 measurement

Results

The percentage inhibition of the commercially available, non-selective P2Y receptor antagonists reactive blue-2 (RB-2) and suramin, as well as the selective $P2Y_2$ receptor antagonist AR-C118925 was determined using the calcium mobilisation assay with the fluorescent dyes fluo-4 and fura-2. The results are summarised in Fig. 7.

The inhibition values for the fluo-4 and fura-2 measurements correlate well with each other for all of the assessed compounds. AR-C118928 shows complete inhibition at 50 µM while suramin and reactive blue 2 are weak antagonists. This is in accordance with data in the literature.

Conclusions

This study demonstrates that the TriStar² LB 942 plate reader can be used to accurately screen test compounds for antagonism at the $P2Y_2$ G protein-coupled receptor using two commonly-used, commercially-available fluorescent dyes. Consistent results were obtained for the direct and the ratiometric assay format. This demonstrates the precision and high reproducibility of fluorescence measurements using the TriStar² LB 942 plate reader, confirming the suitablilty of the plate reader for the realiable characterisation of potential antagonists for pharmalogical studies.

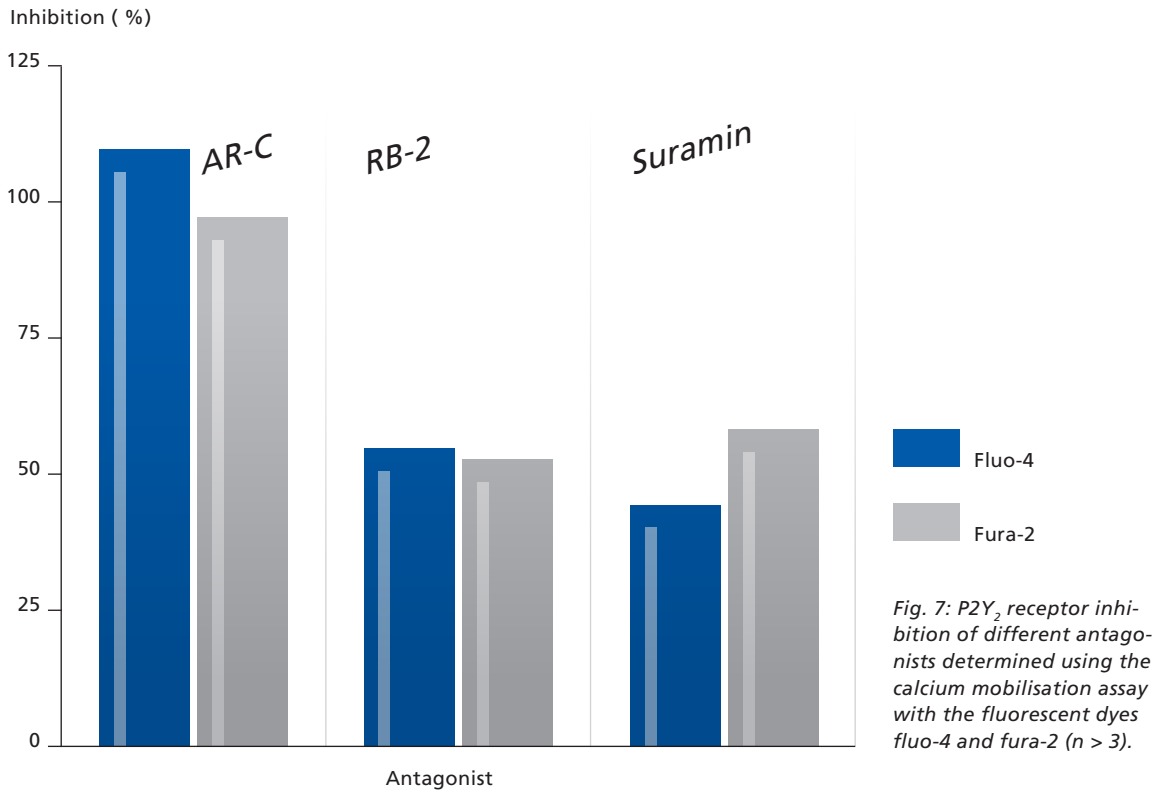

Fig. 7: $P2Y_2$ receptor inhibition of different antagonists determined using the calcium mobilisation assay with the fluorescent dyes fluo-4 and fura-2 (n > 3).

References

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