APPLICATION NOTE

SCREENING FOR $P2Y_2$ RECEPTOR ANTAGONISTS USING THE BERTHOLD TECHNOLOGIES MITHRAS² LB 943 MONOCHROMATOR MULTIMODE READER – AN EXEMPLARY APPLICATION

Abstract

The nucleotide receptor family P2Y comprises G protein-coupled receptors that show potential as drug targets for a range of 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 Mithras² LB 943 plate reader. Reliable and consistent results were obtained; thus, the Mithras² LB 943 plate reader is a suitable instrument for this application.

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. 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 here are fluo-4 and fura-2.

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 was 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 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

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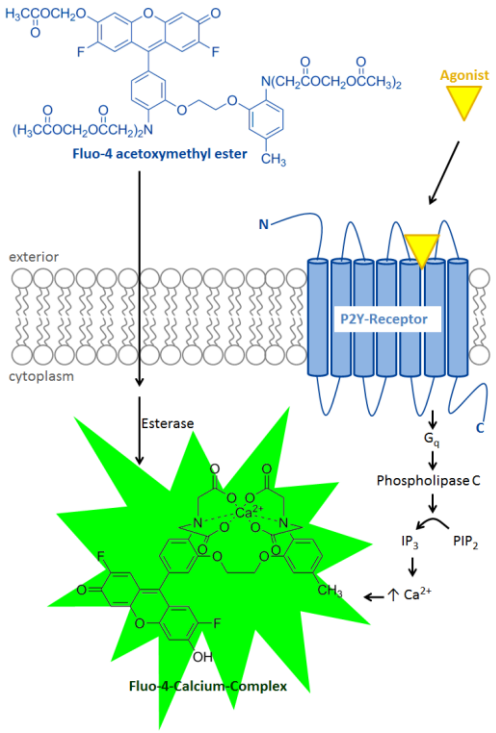

Fig. 1: Basic principles underlying the calcium mobilisation assay. Shown here is the fluorescent dye fluo-4 as an example.

The dyes used here were fluo-4 and fura-2. Both are commercially available in the form of an acetoxymethyl ester (AM). They are thereby sufficiently lipophilic to readily 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. Owing to 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]. The fluorescence absorption and emission spectra for fluo-4 are shown below:

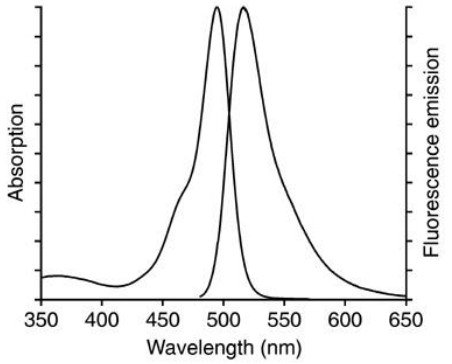

Fig. 2: Fluorescence absorption and emission spectra of fluo-4 [5]

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 (Fig. 3). The ratiometric measurement minimises the effects of photobleaching, uneven dye loading, or leakage of dye [6].

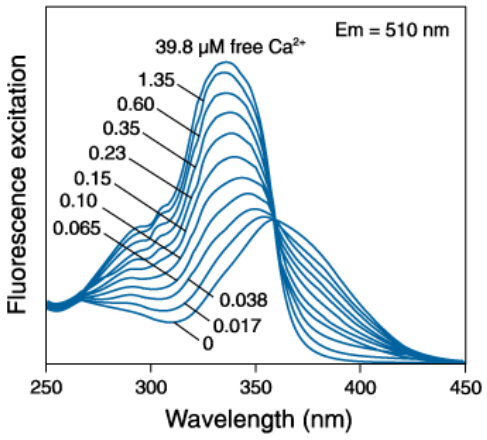

Fig. 3: Fluorescence excitation spectra of fura-2 with different calcium concentrations [6]

The Berthold Technologies Mithras² LB 943 Monochromator Multimode Reader

The Mithras² LB 943 is a high-end microplate multimode reader based on monochromator technology with excellent performance. Characterized by its sensitivity and robustness, especially in luminescence and BRET measurements, the reader supports all important reading technologies:

- Luminescence
- BRET and BRET2
- Fluorescence
- FRET
- Fluorescence Polarisation
- UV/VIS absorbance
- AlphaScreen® and AlphaLISA®
- Time-resolved fluorescence
- TR-FRET

Mithras² additional features:

- Monochromator & filter technology
- Detectors: 2 low-noise PMTs (up to 850 nm) and ultra-low noise PMT operated in single photon counting mode, photo diode (200 – 1000 nm)
- Top & bottom reading
- Automatic plate height adjustment (Z optimization)
- Up to 4 JET injectors (98% accuracy & precision over entire volume range)
- All microplate formats up to 1536-well
- Shaking and incubation up to 45°C

Different reading technologies have their own demands on the optical system for optimal performance. In contrast to conventional multi-technology instruments, the Mithras multimode microplate reader has been designed with a proprietary optical system consisting of separate optical paths for different reading technologies (mDOPS). The separated light paths of the mDOPS optical system ensure that the needs for high sensitivity and a wide dynamic range are met for each reading technology. This results in high-end performance that typically can be achieved with dedicated instruments only.

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 Mithras² LB 943 monochromator multimode reader

Instrument settings

- Fluo-4 excitation filter: ID-Number 40271-01*
- Fluo-4 emission filter: ID-Number 40273-01*
- Fura-2 excitation filters: ID-Number 40086-01 (340 nm), 40087-01 (380 nm)
- Fura-2 emission filter: ID-Number 40094-01

* Included by default in some Mithras² configurations.

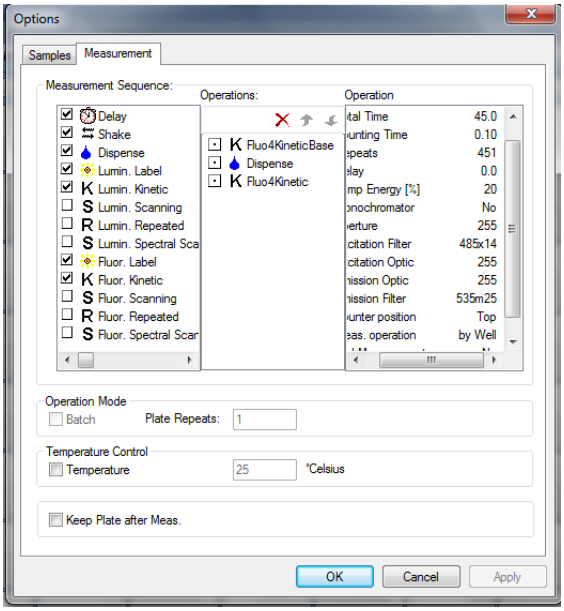

Fig. 4: Screenshot of the instrument settings dialogue in the MikroWin 2010 software for the fluo-4 and fura-2 measurements

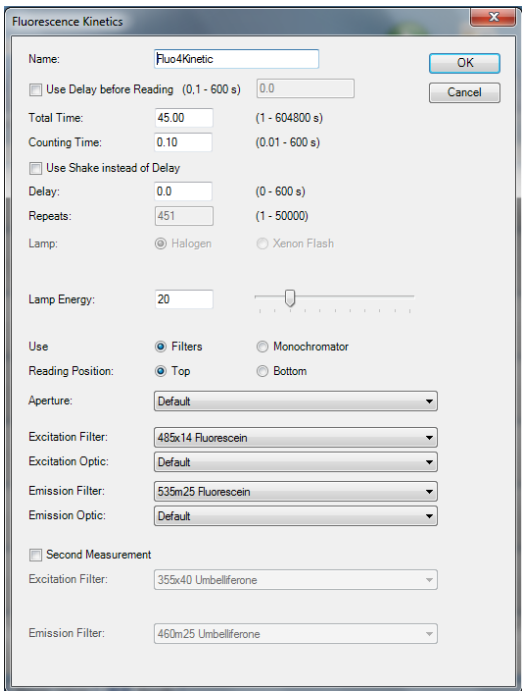

Fig. 5: Screenshot of the instrument settings dialogue in the MikroWin 2010 software for the fluo-4 measurement.

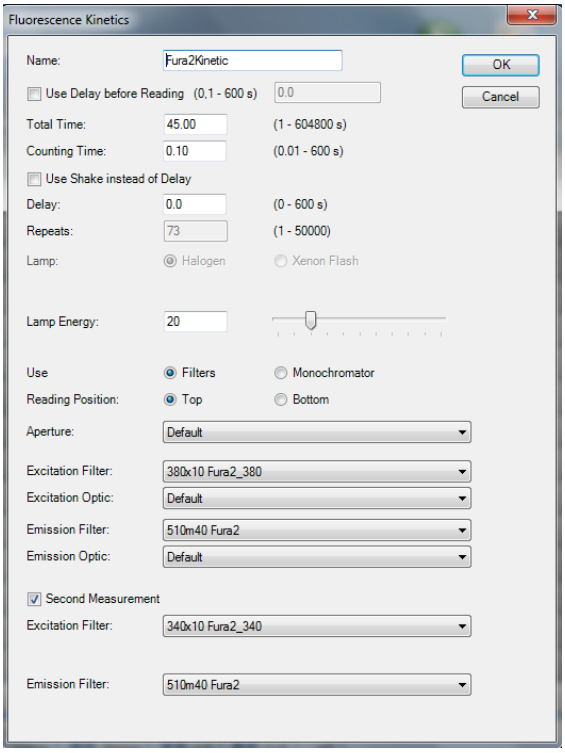

Fig. 6: Screenshot of the instrument settings dialogue in the MikroWin 2010 software for the fura-2 measurement.

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 then 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 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 Mithras² LB 943 (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.

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.

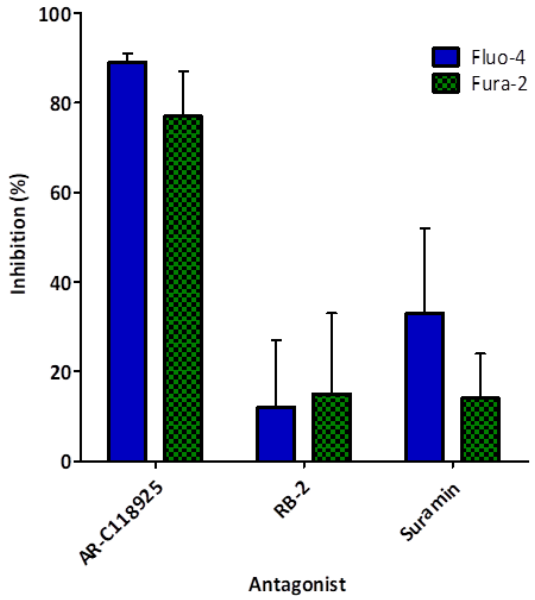

Fig. 7: $P2Y_2$ receptor inhibition of different antagonists determined using the calcium mobilisation assay with the fluorescent dyes fluo-4 and fura-2 (n = 3).

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 strong inhibition while suramin and reactive blue 2 are weak antagonists. This is in accordance with data in the literature.

Conclusions

Test compounds were screened for antagonism at the $P2Y_2$ G protein-coupled receptor using two commonly-used, commercially-available fluorescent dyes measured on the Mithras² LB 943 plate reader. Reliable and consistent results were obtained, confirming the suitability of the Mithras² LB 943 plate reader for this application.

References

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