**Assessment of proteasome activity in live cells**

Introduction

Aim

This protocol describes the analysis of how to assess the proteasome activity of live LUHMES cells (human neuronal).

Purpose

Proteasomal activity is an essential process of cellular homeostasis. It can be directly inhibited, e.g. by proteasomal inhibitors, or decreased as a consequence of e.g. energy depletion, since the proteasome is ATP-dependent.

Limitations

The proteasome has three major catalytic activities: the β1 subunit has a caspase-like activity (i.e. cleaves after acidic amino acids), the β2 subunit has a trypsin-like activity (i.e. cleaves after basic amino acids) and the β5 subunit has a chymotrypsin-like activity (i.e. cleaves after hydrophobic amino acids). The used substrate (MeOSuc-Gly-Leu-Phe-AMC) is thus most likely mainly cleaved by the β5 subunit, while the activity of the other subunits might not be assessed to a similar extent. The positive control (e.g. MG-132) is an inhibitor which impairs all catalytic subunits.

Additionally, for proper cellular proteasomal protein degradation, also the ubiquitin system has to work functionally, which is not assessed by this assay.

During the assay (while the proteasomal substrate is incubated), no treatment substances are on the cells, thus treatment substances might be diluted out of the cells during assessment of proteasomal activity.

On top, other intracellular proteases could cleave the substrate. This background/ non-blockable cleavage should be assessed by controls, e.g. broad-spectrum proteasome inhibitors.

Method outline

Cells are cultured normally according to established lab protocols in 96 well plates. Around the time of endpoint assessment, the medium is removed from the wells and the assay mix with the proteasomal substrate is added to replace the medium. The proteasomal substrate is cell-permeable and diffuses into the proteasome, as well as the cleavage products diffuse out of the proteasome.

The proteasome activity dye consists of a fluorophore which is linked to a small peptide chain which quenches the fluorescence activity. Upon proteasomal cleavage, the fluorescence increases. Thus the increase in fluorescence activity between directly after addition of the substrate and 2 h later is correlated with the proteasomal activity.

Method description

Chemicals and buffers

* Proteasome substrate: MeOSuc-Gly-Leu-Phe-AMC from Bachem (I1430).  
  Prepare 10 mM Stock in DMSO, store at -20°C.
* HBSS buffer
* Cells in 96 well plates (otherwise adapt the volumes)
* Fluorescence plate reader

Preparation upfront

* Prepare working mix of proteasome substrate: dilute 1:400 in HBSS, final concentration 25 µM

Experimental procedure

* Remove medium from the wells (2 h before the endpoint should be determined)
* Add 100 µl/well working mix (25 µM proteasome substrate in HBSS)
  + Measure fluorescence directly after addition (=baseline measurement; ex. 360 nm, em: 465, fixed gain)
* Incubate the cells in their standard incubation conditions, thereby wait for 1-3 h, depending on the cellular activity
  + Measure fluorescence again (=end measurement; ex. 360 nm, em: 465, fixed gain – same as for baseline)
  + Time course measurements can be performed to find the best waiting time
* Use assay positive controls: cells treated with a sufficient concentration of a proteasome inhibitor, e.g. MG-132 (alternatively: lactacystin, bortezomib, epoxomicin or a combination of them)

Data analysis

* Subtract from each value of the end measurement its baseline value
* Calculate fluorescence intensity relative to control
  + Positive controls can be used to determine the amount of not proteasome inhibitor blockable substrate cleavage