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Vital Inhibitor-Based Cytochemical Procedure for Proteasome Visualization

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Abstract

The fluorescent-inhibitors can be used for cytochemistry of enzymes and this technique gives and interesting alternative for classical immunolabeling in both microscopy and flow cytometry. Here we discuss the published data for proteasome inhibitor is BSc2118 that can be easily conjugated with BODI-PY fluorochromes and describe this novel cytochemical procedure. The direct vital pre-fixed inhibitor-based proteasome staining can be efficiently combined with post-fixed classic immunocytochemical detection of other cellular components after ethanol-based fixation. The fluorescent BSc2118 can be used as aggresome marker in aggresome formation with the same effectiveness as FK2 antibody for polyubiquitinated proteins.

Fluorochrome-tagged derivates of BSc2118 can also be used for visualization of inhibitor distribution in organs and tissues in animal model. We found this technique as promising as fast and effective for aggresome formation studies.

Keywords: Fluorochrome; Proteasome; Aggresome

Introduction

The histochemical localization of cellular compounds for confocal microscopy involves mainly staining of them with use of monoclonal antibodies conjugated with fluorochrome. However in the case of enzymes also use of fluorescent-inhibitors for cytochemistry is possible. This technique give and interesting alternative for classical immunolabeling in both microscopy and flow cytometry and reduces possible antibody cross-reactivity esp. with multi-color staining [1,2].

The vital inhibitor-based staining procedure of proteasomes (BSc2118-FL prior to fixation) combined with subsequent ethanol based fixation enables efficient proteasome staining [3-5]. Moreover, inhibitor based detection of proteasomes in biosensors enables proteasome and immunoproteasome quantitative measurement in the serum (Patent Pending PL: P.396170; P.396171; P.417435).

Ubiquitin-Proteasome System in Protein Degradation

There are two key pathways for protein degradation within cells: Lysosomal-based degradation and Ubiquitin-Proteasome System (UPS) [6,7]. The majority of cytosolic and nuclear proteins are destroyed by UPS-degradation [8]. The proteasome is a multicatalytic enzyme complex and it can be found in the in the cytoplasm and in the nucleus of all eukaryotic cells. The whole complex of 26S proteasome is composed of 20S core particle and two 19S regulatory subunits. Regulatory particles regulate entrance of the substrate into the core and they are localized at both ends of the 20S core particle. Each 20S core is a barrel-shaped catalytic part of the 26S complex and it contains 4 rings: Two outer α rings and two inner β rings. In each ring there are 7 globular subunits, α1-α7 within α-ring and β1-β7 within β-ring, respectively. The β-rings are responsible for enzymatic activities of the proteasome. These activities are: Chymotrypsin-like localized within the β5 subunit; trypsin-like (within the β1 subunit) and caspase-like (within the β2 subunit) [9-12]. The Ubiquitin (Ub) is a highly conserved polypeptide, 76 acids in length, which acts as a molecular label in cellular protein trafficking. Substrates for degradation by 26S proteasome are conjugated with polyubiquitin chain in the process named ubiquitination by cascade of three enzymes [13]. The UPS system is schematic displayed in Figure 1.

Proteasome Inhibitor BSc2118 and Its Fluorescent Derivates

The first proteasome inhibitor approved for human therapy was Bortezomib. The evaluation of several new inhibitors for their therapeutic potential is ongoing. The new proteasome inhibitor

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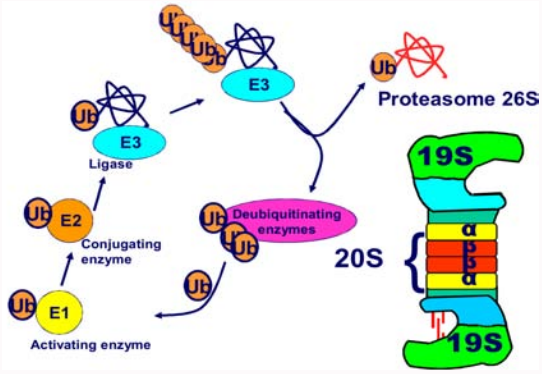

Figure 1: Schematic presentation of the ubiquitin-proteasome system [6].

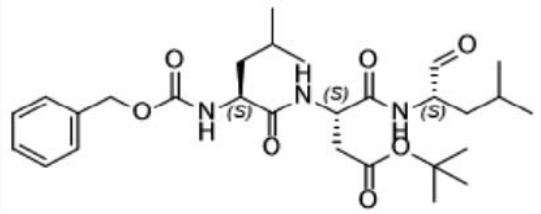

Figure 2: The chemical structure of BSc2118.

is BSc2118 (patent no T30305) which is a derivative of the classical proteasome inhibitor MG132 (Figure 2) [14]. BSc2118 displays a better proteasome inhibition profile than MG132 and it inhibits all three proteolytic activities and it was shown to interact with all active sites of 20S proteasome in X-ray crystallography, thus BSc2118 in not subunit specific at the concentrations more than (10 mM) [14].

BSc2118 was shown to promote the accumulation of polyubiquitinated proteins in aggresomes, induce apoptosis, cell cycle arrest at G2/M, and prevent NF-kappaB activation [14,15]. Moreover, BSc2118 has systemic anti-myeloma and local anti-melanoma activity in the animal models [5,16,17].

Nowadays BSc2118 is commercially available in US and it can be easily conjugated with BODI-PY fluorochromes. The red fluorescent variant of BSc2118 can be synthesized as follows: To the 0.01 mM solution of the BSc2118 in 0.5 ml Dimethylformamide DMF (pH=8.5) should be given Bodipy-581/591 SE (Molecular Probes) to the final concentration of 0.01 mM. The reaction mixture should be stirred overnight in darkness and then purified by HPLC. The synthesized fluorescent inhibitor can be dissolved in DMSO to obtain 1 µg/ml stock solution of BSc2118 for further use.

Fluorochrome-tagged derivates of BSc2118 were designed for visualization of inhibitor distribution in organs and tissues in animal model [5,18]. It was shown that fluorescent forms of BSc2118 are suitable for analysis of the inhibitor distribution of BSc2118 in mouse tissues after systemic administration (given in to a dose of 60 mg/kg body weight) [5]. Fluorescent distribution pattern of BSc2118-FL correlates with inhibition of proteasomal activity in cells or mouse tissues [5,15].

Inhibitor-Based Staining of Proteasomes

Fluorochrome-tagged derivates of BSc2118 can be adapted to cytochemical procedure for vital proteasome staining in cells *in vitro*. It was confirmed that this staining procedure with green fluorescent

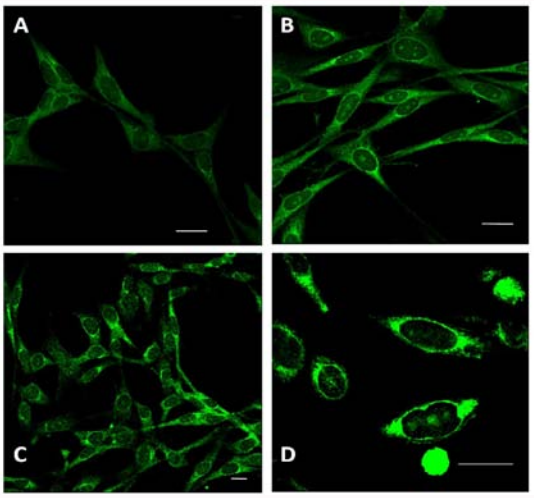

Figure 3: The dynamics of aggresome formation in NIH3T3 cells using green fluorescent BSc2118-FL [3].
Cells were incubated with BSc2118-FL (1 µM) for 5 min (A) of for 2 h (B), 6 h (C) 24 h (D). Scale Bars 10 µM.

BSc2118-FL and red BSc2118-TR is fully comparable with indirect immunocytochemistry method [4,5]. The staining of proteasomes in NIH3T3 cells by green fluorescent Bsc2118-FL is shown in Figure 3.

Furthermore, in the novel cytochemical approach it was confirmed that this direct vital pre-fixed inhibitor-based proteasome staining can be efficiently combined with post-fixed classic immunocytochemical detection of other cellular components after ethanol- based fixation. And fluorescent BSc2118 can be used as aggresome marker in aggresome formation with the same effectiveness as FK2 antibody for polyubiquitinated proteins [4,15].

A Practical Approach of Vital Inhibitor-Based Proteasome Detection

Cell culture

For all cytochemical procedures cells should be cultured onto multi-chamber slides (Becton Dickinson, US).

Basic proteasome staining protocol

Add BSc2118-TR to the culture media for 3 min at the final concentration of 5 µM. After washing fix cells (as described in next section), rehydrate and if required perform classic post-fixed immunostaining for protein of your interest.

Fixation

Use ice-cold buffered 70% ethanol (pH 7.5; phosphate buffer) for 3 minutes and directly rehydrated (pH 7.5; phosphate buffer).

Antibodies and immunocytochemistry

Use the antibodies at dilution according to manufacturer instruction. As the secondary antibodies it is recommended to use the DyLight Affinity-Purified Fab Anti-Fc Specific (Jackson ImmunoResearch, US) at 1:10 000 dilution for 1h in 37°C in humidified atmosphere. Both prior to and after the secondary antibody incubation, wash the sides tripled in phosphate-buffered saline (pH 7.4) with 0.1% Tween 20 (Sigma, US). The nonspecific antibody binding should be blocked for 60 min in 10% BSA at RT.

Aggresome formation assay

Use with BSc2118-TR in concentrations 0.5 µM to 2.5 µM for

1.5 h to 16 h to induce aggresome formation. Always incubate the controls with DMSO solvent prior to fixation in the corresponding concentration and for at least the same time.

Embedding for laser scanning confocal microscopy

Subsequently to cytochemical procedures, slides should be embedded in Vecta Shield (if necessary containing 4′,6-diamidino-2-phenylindole-DAPI) (Vector Laboratories, US). The analysis and images can be made using a confocal laser microscope equipped with appropriate lasers of florescent microscope with appropriate filters. During analysis put a special attention to exclude cross-talk effects.

Conclusion

Please note that there are also disadvantages of fluorescent-inhibitor technique. The high inhibitor concentration is required for initial staining and high concentration cannot be used for prolonged studies, as it leads to the inhibition of the target enzyme, which triggers severe physiological side effects (incl. cell death). Therefore, lower inhibitor concentration must be used for longer time-points in kinetics studies. High inhibitor concentration is required for basic proteasome staining (BSc2118-TR 5 μM for 3 min). Conveniently, 0.5 μM BSc2118-TR is sufficient to stain proteasomes and induces aggresome formation after 24 h.

To sum up, we found this technique as promising as fast and effective for aggresome formation studies. Studies on aggresome formation process and development of methods for their visualization can lead to invention of diagnostic tools enabling prediction if proteasome inhibitors can be effective in particular types of malignancy.

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