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Research paper

Induced prodrug activation by conditional protein degradation

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ABSTRACT

Enzyme prodrug therapies hold potential as a targeted treatment option for cancer patients. However, off-target effects can be detrimental to patient health and represent a safety concern. This concern can be alleviated by including a failsafe mechanism that can abort the therapy in healthy cells. This feature can be included in enzyme prodrug therapies by use of conditional degradation tags, which degrade the protein unless stabilized. We call this process Degradation-Directed Enzyme Prodrug Therapy (DDEPT). Herein, we use traceless shielding (TShld), a mechanism that degrades a protein of interest unless it is rescued by the addition of rapamycin, to test this concept. We demonstrated that TShld rapidly yielded only native protein products within 1 h after rapamycin addition. The rapid protection phenotype of TShld was further adapted to rescue yeast cytosine deaminase, a prodrug converting enzyme. As expected, cell viability was adversely affected only in the presence of both 5-fluorocytosine (5-FC) and rapamycin. We believe that the DDEPT system can be easily combined with other targeting strategies to further increase the safety of prodrug therapies.

1. Introduction

Enzyme prodrug therapies are an attractive alternative to conventional chemotherapies due to their potential to elicit a localized, targeted toxic effect at the tumor site (Altaner, 2008; Andrady et al., 2011). One example is use of yeast cytosine deaminase (yCD), which converts a non-toxic prodrug, 5-fluorocytosine (5-FC), to the clinically prevalent cytotoxic drug, 5-fluorouracil (5-FU), for the treatment of glioblastoma (Polak et al., 1976; Zhang et al., 2015). Proposed methods for introducing prodrug converting enzymes (PCEs) require the targeted delivery of the enzyme to the cancer, as any off-target activity would kill the benign cell upon addition of the prodrug (Biela et al., 2003; Fong et al., 2011; Tian et al., 2013; Wang et al., 2015). While many methods have tested successful for targeting tumors *in vitro*, none are completely free of risk and safety concerns. Activation based on targeting extracellular cancer markers may lack the required specificity as these markers are presented at certain levels on healthy cells as well (Bildstein et al., 2011). More importantly, we lack any means of regulating the intracellular protein levels after delivery as there is no innate mechanism for clearing the PCE in an event of promiscuous delivery.

One way to address this issue is to impose an additional layer of cell-specificity by controlling intracellular enzyme levels in a process we coin "Degradation-Directed Enzyme Prodrug Therapy (DDEPT): Healthy cells would quickly degrade the PCE and remain unscathed while cancerous cells, through a targeted activation mechanism, would preserve the PCE towards a therapeutic outcome. DDEPT is most conveniently executed by simply grafting a conditional degradation domain (DD) to the PCE (Bonger et al., 2011; Caussinus et al., 2012; Chung et al., 2015; Iwamoto et al., 2010; Pratt et al., 2007), which under normal circumstances is recognized and swiftly eliminated by the proteasome, but the DD can be stabilized by the introduction of a chemical cue. This approach is simple but leaves behind a DD-PCE fusion protein, which may affect its endogenous biological activity. An improved technology termed Traceless Shielding (TShld) was recently reported to generate native proteins, in which a chemical cue is used to both shield the target proteins from degradation and trigger their release from the DD (Lau et al., 2010). Briefly, TShld consists of two separate constructs that function together to rescue the protein of interest. On the first construct, the protein payload is flanked by a conditional DD, FKBP, which is stabilized by the small molecule rapamycin (Banaszynski et al., 2006; Banaszynski et al., 2008), and the C-terminus of ubiquitin (UbC, residues 35–76). In the absence of rapamycin, FKBP destabilizes the complex containing the payload, resulting in its degradation. In addition to stabilizing FKBP, rapamycin also induces complementation between FKBP and the second construct

Abbreviations: yCD, yeast cytosine deaminase; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; PCE, prodrug converting enzymeprodrug converting enzyme; DDEPT, degradation-directed enzyme prodrug therapy; DD, degradation domain; TShld, traceless shielding; FKBP, FK506 binding protein; FRB, FKBP12-Rapamycin Binding Domain; mTOR, mechanistic target of rapamycin

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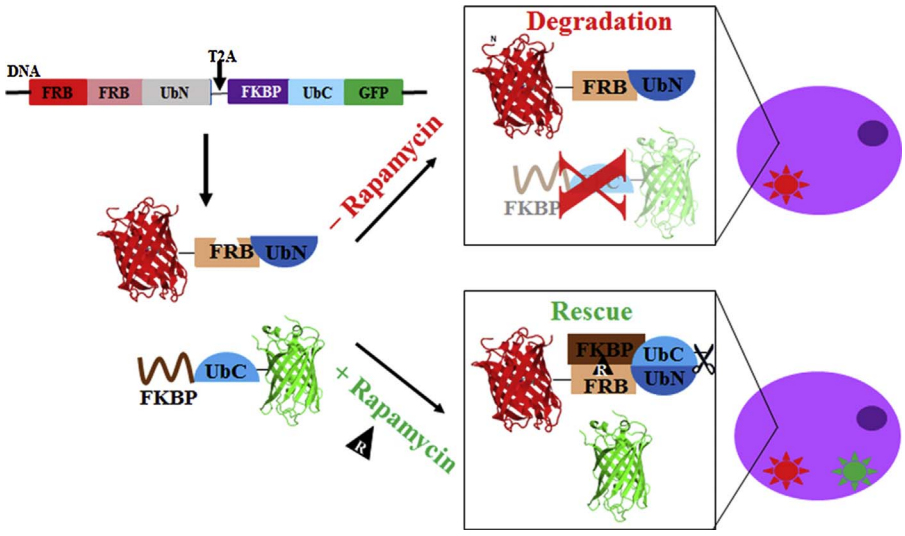

Fig. 1. Schematic representation of mCherry TShld GFP protein product. The transcribed DNA includes the entirety of the protein under one promotor. A T2A site between the proteins forces the synthesis of two separate proteins due to ribosome skipping at the Gly-Pro junction of the T2A peptide (left). In the absence of rapamycin, FKBP is destabilized, targeting itself and its fusion partners, including GFP, towards degradation. The first part of the construct, including mCherry, does not contain a degradation tag and is not subject to induced degradation (top right). In the presence of rapamycin (black triangle containing the letter "R"), FKBP is stabilized and FRB is recruited to FKBP, promoting a close proximity between UbC and UbN. GFP is cleaved from the construct, rescuing it from possible degradation. Both mCherry and GFP can now be detected (bottom right).

consisting of FRB, a domain from the mTOR protein, and the N-terminus of ubiquitin (UbN, residues 1–37), resulting in reconstitution of the split ubiquitin and separation of the protein of interest from the rest of the complex via ubiquitin hydrolases (Fig. 1) (Johnsson and Varshavsky, 1994; Stagljar et al., 1998). Intrigued by the dual capability of TShld to provide conditional PCE rescue in a native conformation, we demonstrated the principle of DDEPT using TShld for the conditional rescue of yCD and controlled prodrug activation as an initial step towards a novel therapeutic direction.

2. Materials and methods

2.1. Plasmid construction

All constructs were prepared using standard molecular cloning techniques. pEntry TShld-GFP was a gift from Matthew Pratt (Addgene plasmid #53211). TShld GFP was cloned into pcDNA3.1 (Invitrogen), and mCherry was cloned onto the N-terminus. For cell viability experiments, GFP TShld yCD was cloned as follows: yCD was substituted for GFP and GFP was substituted for mCherry as the global protein expression control.

2.2. Cell culture

HeLa cells were maintained in T150 tissue culture flasks (Thermo Fisher) in Minimum Essential Media (MEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Corning) and 1% penicillin/streptomycin (HyClone) at 37 °C and 5% CO_2.

2.3. Transfection

Plasmid DNA was prepared using ZymoPURE™ Plasmid Midiprep Kit (Zymo Research) according to the manufacturer's protocol. HeLa cells were seeded at roughly 175,000 cells/well in 12-well plates (Corning) supplemented MEM as described above. One day after seeding, transfection was achieved with Lipofectamine® 3000 (Invitrogen) using 1 µg plasmid DNA per well and following the manufacturer's protocol. Transfection occurred for a minimum of 12 h.

2.4. Endpoint cell culture experiments

Transfected cells were treated with the appropriate amount of rapamycin (LC Laboratories, > 99% purity) and 5-FC (Sigma-Aldrich, > 99% purity) to achieve the desired final concentrations in a total volume of 1 mL for 24 h. Cells were washed twice in 1 mL pre-warmed imaging buffer (140 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM HEPES, pH 7.4). Cells were then incubated in 0.5 mL of pre-warmed imaging buffer throughout microscopy. For viability studies, 1 drop of NucRed® Dead 647 ReadyProbes® Reagent (Invitrogen) was added and allowed to incubate at room temperature for 2 min prior to imaging.

2.5. Fluorescent microscopy and image analysis

All images were captured using an Observer Z.1 Inverted Microscope (Zeiss) with GFP, mCherry, or Cy5 filter cube sets (Chroma). For image analysis, five images were captured in each well. Image analysis was conducted using the 'Measure' analysis in ImageJ with threshold set 10–255. Error bars represent the 95% confidence interval.

2.6. TShld time course experiments

HeLa cells were seeded in individual 35 mm tissue culture-treated culture dishes (Corning) and transfected as described above. Transfected cells were treated with 500 nM rapamycin in a total of 1 mL of media. Each hour, one plate was removed from the incubator, washed twice in 1 mL pre-warmed cell imaging buffer, and imaged in 0.5 mL of imaging buffer.

2.7. Western blotting

Following imaging, cells were incubated in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, pH 8.0) on ice for 20 min with protease inhibitor cocktail (Calbiochem). Cells were then removed from the plate with a cell scrapper (Genemate), and the lysate was clarified in a pre-cooled centrifuge at 12,000 rpm for 10 min at 4 °C. Total protein concentrations were normalized through a Bradford assay (Bio-Rad) with a BSA standard. 15 µg of lysate was mixed with a 5 x loading buffer and separated by 10% SDS-PAGE before being transferred to a nitrocellulose membrane (Bio-Rad).

Western blots were blocked in TBST (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 8.0) containing 5% non-fat milk overnight at room temperature with gentle shaking. Membranes were washed twice in TBST and incubated for 3 h in anti-GFP (1:5000 dilution, Covance) or anti-mCherry (1:2000 dilution, Novus) in TBS. The blots were then washed twice in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (GenScript) for 2 h in TBST. The blots were washed three times in TBST and developed using ECL reagents (GE) according to the manufactures protocol. Band intensities