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-fluouracil (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
consisting of FRB, a domain from the mTOR protein, and the N-terminal 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 TShdl to provide conditional PCE rescue in a native conformation, we demonstrated the principle of DDEPT using TShdl 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 TShdl-GFP was a gift from Matthew Pratt (Addgene plasmid #53211). TShdl GFP was cloned into pcDNA3.1 (Invitrogen), and mCherry was cloned onto the N-terminus. For cell viability experiments, GFP TShdl yCD was cloned as follows: yCD was substituted for GFP and yCD 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% CO₂.

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 CaCl₂, 1.0 mM MgCl₂, 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. TShdl 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 scraper (Genenate), 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
were quantified using ImageJ gel analysis tools.

3. Results

3.1. Appending a global protein expression marker to TShld

Previous reports on the TShld system have clearly indicated the release of target proteins 48 h after rapamycin addition. However, it was unclear whether the initial protection was mainly due to DD stabilization or DD release. Since the two protein complexes are expressed under one promoter and are translated into two individual constructs using the 2A viral "self-cleaving" peptide (Holst et al., 2006; Szymczak et al., 2004), we first fused mCherry to the N-terminus of FRB-UbN to allow direct monitoring of total protein synthesis. Similar to other reports, GFP was fused to the C-terminus of FKBP-UbC to enable easy tracking of protein rescue in a rapamycin dosage-dependent manner. HeLa cells were transfected with the mCherry-TShld-GFP construct. Cells were then cultured in a specified concentration of rapamycin ranging from 0 to 1000 nM in media for 24 h, and the abundance of mCherry and GFP was probed by both Western blotting and microscopy. As expected, the levels of mCherry fluorescence were unaffected by the rapamycin concentration in the media (Fig. S1). However, only a low level of GFP fluorescence was detected in the absence of rapamycin, which increased significantly in a dosage-responsive manner until 500 nM of rapamycin, at which point the maximum signal was observed (Fig. 2A, Fig. S2). Overall, a 11.2-fold difference in normalized GFP fluorescence was observed between 0 and 1000 nM of rapamycin (Fig. 2B).

3.2. Protein release from DD occurs rapidly

Western blotting was used to further probe the mechanism of GFP rescue. Only a small quantity of cleaved GFP product was detectable before rapamycin addition, indicative of efficient degradation and a low level of background cleavage. However, the DD tag cleavage via ubiquitin hydrolase was extremely rapid, and only cleaved GFP was detected even 1 h after rapamycin addition (Fig. 2C). The level of cleaved GFP increased slightly with time after the addition of rapamycin, while the level of mCherry-FRB-UbN remained constant (Fig. 2C and D). It is clear that the initial DD (FKBP) stabilization by rapamycin is also very rapid, a condition necessary for the subsequent quick release of GFP from the reconstituted ubiquitin by ubiquitin hydrolase.

3.3. DDEPT requires both protein rescue and prodrug to reduce cell viability

Having demonstrated the rapid rescue and release of native GFP by rapamycin addition, we next sought to execute DDEPT through the dynamic control of yCD levels using the TShld system in order to regulate the degree of 5-FC conversion to 5-FU and the corresponding cell viability. We first replaced mCherry with GFP as the expression marker because of the higher fluorescence intensity and yCD as the TShld payload. After transfection, the levels of GFP expression and cell viability were compared in the presence or absence of rapamycin ranging from 0 to 1000 nM and/or 300 μM 5-FC. To distinguish healthy cells from dead cells, we employed the NucRed® Dead 647 ReadyProbes® Reagent, which stains the genomic DNA of non-viable cells whose membrane integrity is compromised. No discernible difference in the GFP level was observed regardless of 5-FC and/or rapamycin addition.

![Fig. 2. mCherry serves as a marker for protein synthesis in the TShld system and can be used to probe the initial mechanism of protection. (a) Microscopy of HeLa cells demonstrates the dosage response of GFP levels to rapamycin while mCherry levels are maintained as constant. Images were obtained 24 h following the addition of rapamycin. (b) Quantification of fluorescent images showing statistically significant increases in GFP level relative to the mCherry transfection marker between all doses of rapamycin through 500 nM (n = 5, ** p < 0.01, *** p < 0.001). (c) Western blots probing for mCherry and GFP reveals constant mCherry-FRB-UbN expression with GFP protein levels quickly increasing over time. The response is rapid, leaving no uncleaved GFP detected, indicating that initial protection results from DD release. Quantification of the western blot band intensities shows the GFP time profile immediately following rescue initiation. The absolute intensity was different for the two proteins as mCherry-FRB-UbN was probed with anti-mCherry primary antibody and GFP was probed with anti-GFP antibody. (d) Fluorescent microscopy corroborates the findings of the western blot analysis.](image-url)
indicating their addition had no effect on protein expression. The addition of rapamycin by itself had no impact on cell viability across the spectrum of rapamycin added, and these levels were statistically similar to the group that received only 5-FC (Fig. 3A and C). These results again support the need of both 5-FC and active yCD for prodrug activation. In contrast, cell viability decreased in a rapamycin dosage-responsive manner in the presence of 5-FC as reflected by the higher number of purple fluorescent cells (Fig. 3B and C). Since the GFP level remained fairly constant independent of rapamycin and 5-FC addition, this observation implies that the increase in cytotoxicity is not a result of increased protein synthesis but a direct effect of yCD rescue by rapamycin addition.

4. Discussion

In summary, we reported here a new DDEPT approach to provide conditional prodrug activation based on controlled protein degradation. This approach capitalized on the well-known feature of the TShld system to both rescue the prodrug activating enzyme, yCD, from deactivation as previously reported (Lin and Pratt, 2014). For prodrug activation, this low level of background yCD rescue did not impact cell viability as even the introduction of 5-FC was not sufficient to cause cytotoxicity. Only in the presence of both 5-FC and rapamycin did we observe significant cell death. This approach allows the advantages of both targeted prodrug therapy in addition to a mechanism for clearing PCEs from healthy cells. This work represents an important first step in utilizing the DDEPT concept towards targeted prodrug activation. Looking forward, DDEPT would further benefit from autonomous switching such that the presence or absence of a specific cancer marker would determine the fate of the PCE thus rendering it independent of a small molecular trigger. For example, yCD has been engineered to accumulate only in cells that overexpress hypoxia-inducible factor 1α, common in many cancers (Wright et al., 2011). Furthermore, delivery platforms have been developed that specifically target unique markers such as human growth factor receptor 2 on the surface of breast cancer cells (Mann and Kullberg, 2016), allowing DDEPT to be used as a gene therapy combined with the additional failsafe of the PCE degradation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2017.09.005.

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