MONITORING CELL VIABILITY DURING GENE-DIRECTED ENZYME PRODRUG THERAPY WITH THE CELLTITER-GLO® ASSAY AND HUMAN NEURAL PROGENITOR CELLS

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Human neural progenitor cells (hNPC) can deliver neuroprotective molecules to sites of neurodegeneration and show great promise for neurodegenerative disease therapy. However, ex vivo gene therapy strategies raise significant safety issues associated with unanticipated adverse reactions. We are investigating the potential of gene-directed enzyme prodrug therapy (GDEPT) as a method for treating such adverse reactions. In the experiments presented, we assessed hNPC viability using the CellTiter-Glo® Luminescent Cell Viability and CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assays. The enhanced sensitivity provided by the CellTiter-Glo® Assay allowed us to evaluate the effectiveness of GDEPT strategies on hNPC.

Introduction

Human neural stem and progenitor cells are a multipotent subpopulation of cells that can be isolated from developing and adult brains and proliferate in culture in response to the mitogens EGF and FGF-2. In recent years, these cells have shown great promise for neuroprotective and repopulating therapies for neurodegenerative disease.

Cell survival and proliferation studies are important tools for identifying and characterizing human neural stem and progenitor cells (1,2). Classic assays, such as the MTS viability assay or LDH cytotoxicity assay, have been used successfully when assessing batch culture response. However, they often lack the sensitivity needed when attempting to characterize the response of a small subpopulation within a heterogeneous culture. Because human neural progenitor cells (hNPC) have very low transient transfection frequencies, functional analysis via wildtype or mutant gene overexpression is constrained by sensitivity of the detection method as well.

A leading therapeutic focus involves ex vivo gene therapy using hNPC to deliver neuroprotective molecules, promoting the survival of remaining neural connections in regions affected by neurodegenerative diseases, such as Parkinson or Huntington disease (3). Gene therapy approaches raise significant regulatory issues, especially in light of recent problems with ex vivo approaches to curing severe combined immune deficiency in children. Although the cure rate for the clinical trial was phenomenal, a significant side effect was clonal hematopoietic malignancy due to insertional mutagenesis as the result of delivering a functional gene to autologous mesenchymal stem cells (4).

One potential strategy to combat such side effects of ex vivo approaches is gene-directed enzyme prodrug therapy (GDEPT), which allows selective ablation of transplanted cells in the event of unanticipated adverse reactions. GDEPT involves administration of a prodrug that normal cells do not metabolize efficiently and, as a result, is not toxic. When the cells are engineered to overexpress an enzyme that catalyzes the activation of the prodrug ("suicide gene"), they become sensitive to its toxic effects (Figure 1). The most widely used combination is the herpes simplex virus thymidine kinase gene, which confers cytotoxicity in dividing cells that are expressing the gene in the presence of the drug gancyclovir. We have investigated the *E. coli* nitroreductase (NTR) gene/CB1954 prodrug combination since it does not require cell division to promote cytotoxicity (5). In addition, phase I safety trials have been completed in cancer patients (6).

We compared the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay[a] and the CellTiter-Glo® Luminescent Cell Viability Assay[b,c,d] for measuring hNPC viability, and present data using the CellTiter-Glo® Assay demonstrating increased sensitivity of hNPC to CB1954 when NPC are transiently overexpressing the NTR gene.

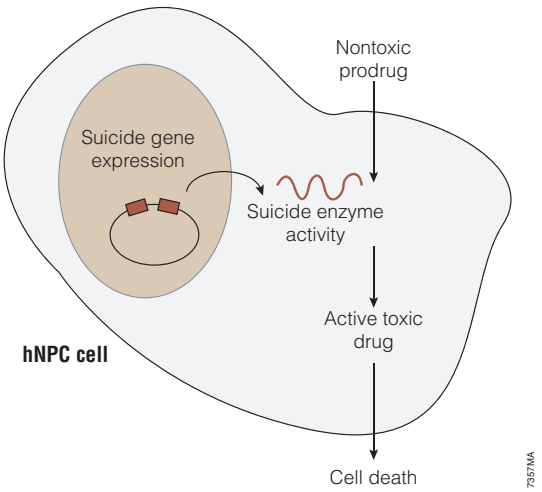

Figure 1. Gene-Directed Enzyme Prodrug Therapy (GDEPT) for hNPC. During GDEPT, hNPC are engineered to express a suicide gene, which encodes an enzyme that activates a prodrug via catalysis, making the hNPC sensitive to the drug's toxic effects. In the experiments described here, hNPC are transfected with a nitroreductase (NTR) gene and exposed to the prodrug CB1954. This approach allows ablation of transplanted cells in the event of adverse reactions to neuroprotective and repopulating therapies for neurodegenerative disease.

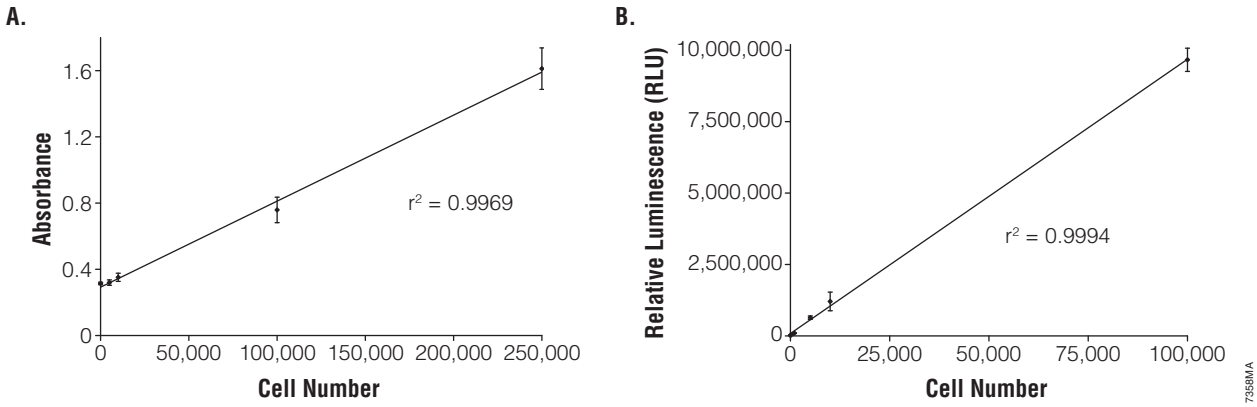

Figure 2. Detection of hNPC cell number by CellTiter 96® and CellTiter-Glo® Assays. Dilutions of hNPC were measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (**Panel A**) or CellTiter-Glo® Luminescent Cell Viability Assay (**Panel B**) according to the standard protocols. Data are the average ± standard deviation of 3–6 replicates.

Comparing the CellTiter-Glo® and CellTiter 96® Assays

We established the target cell number for subsequent drug titration experiments using two different assays for cell viability: CellTiter 96® AQ_{ueous} One Solution, which measures cell viability through quantitation of reducing equivalents such as NADH, and CellTiter-Glo® Luminescent Cell Viability Assay, which uses luciferase technology to measure directly the key energy metabolite, ATP. hNPC were maintained as proliferating neurosphere cultures as previously described (7). Spheres were collected by gravity sedimentation, dissociated gently with Accutase (Chemicon), counted on a hemacytometer and plated at increasing numbers on laminin-coated wells of a 96-well plate. Cells were lysed, and reducing equivalents or ATP were measured by simple incubation with the appropriate assay solution. For the colorimetric CellTiter 96® AQ_{ueous} One Solution protocol, the absorbance at 490 nm wavelength was measured and plotted against cell number (Figure 2, Panel A), while for the luminescent CellTiter-Glo® Assay protocol, relative luminescence as measured on a GloMax® 96 Microplate Luminometer (Cat.# E6501) was plotted against cell number (Figure 2, Panel B). The CellTiter-Glo® Assay produced higher r^2 values (the average of two experiments was 0.995 ± 0.006 versus 0.974 ± 0.032 for the CellTiter 96® AQ_{ueous} One Solution) and showed enhanced sensitivity as reflected in the fewer cells required to give signals above background levels. As few as 100 cells could consistently be detected with the CellTiter-Glo® Assay, while the CellTiter 96® AQ_{ueous} One Solution did not register 10,000 cells significantly above the medium-alone control.

CB1954 Treatment of hNPC

To test the effects of the prodrug CB1954 on hNPC, we gently dissociated spheres as described earlier (7) and plated 50,000 cells per well in a 96-well plate without growth factors. The cells were allowed to recover for 24 hours, and then they were treated for three days with increasing amounts of the prodrug. Cytotoxicity was assessed on the fourth day with the CellTiter-Glo® Assay (data shown in Figure 3; no DNA). Neural progenitors are relatively resistant to CB1954, showing a concentration of 1,000 µM for 50% lethality.

Using lipid-mediated transfection, we transiently transfected hNPC with constructs expressing NTR linked by an internal ribosome entry site (ires) either to GFP as a marker (NTRiresGFP) or to a neuroprotective gene, glial cell line-derived neurotrophic factor (GDNFiresNTR). Lipid-mediated transfection gives a low efficiency of transduction (10% or less) but is simple and not toxic to the progenitor cells (data not shown). When cells were transfected with the bicistronic message encoding NTR and GFP, the dose of prodrug required for 50% cytotoxicity was reduced tenfold compared to untransfected cells. This tenfold change in sensitivity compared to the relatively low number of cells expressing the NTR enzyme (~10%, data not shown) is quite similar to previously published reports in cell lines where cytotoxicity is thought to result from a "bystander effect" from release of toxic metabolites by NTR-transfected cells (8,9).

Interestingly, when a bicistronic message encoding GDNF and NTR was transiently transfected into hNPC (GDNFiresNTR construct), it showed a very similar transfection efficiency but only a slight trend toward increased cytotoxicity. Since we did not assess the amount of NTR protein produced in any of the experiments, and since the NTR message for this construct was located 3′ of the internal ribosome entry site, which typically reduces expression levels, these data may reflect lower enzyme levels. Alternatively, coexpression of a potent neuroprotective molecule such as GDNF could possibly confer some protective effect. Further experiments will answer this question.

Monitoring Cell Viability in hNPC

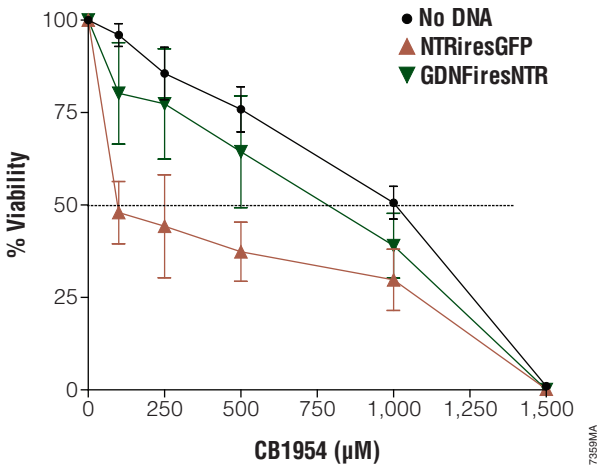

Figure 3. Effect of NTR expression on hNPC sensitivity to CB1954. hNPC were either untransfected (no DNA) or transfected with one of two NTR expression constructs: NTRiresGFP (a bicistronic message encoding both NTR and GFP) or GDNFiresNTR (a bicistronic message encoding the neuroprotective gene, GDNF, and NTR). Cells were then exposed to different doses of CB1954 for three days, and viability was measured using the CellTiter-Glo® Assay. Data are the average ± standard deviation of 6 replicates.

Summary

Both the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation and CellTiter-Glo® Luminescent Cell Viability Assays can be used to accurately measure hNPC viability. The superior sensitivity of the CellTiter-Glo® Luminescent Cell Viability Assay makes it a better choice for these experiments because only a subpopulation of the total cells was responsible for the response (due to low transfection efficiencies), and the sensitivity of the assay allowed fewer cells to be used. These characteristics of the assay allowed its use to monitor cytotoxicity in GDEPT in hNPC using a novel enzyme/prodrug combination.

References

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Protocols

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Technical Bulletin #TB245
(www.promega.com/tbs/tb245/tb245.html)

CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288
(www.promega.com/tbs/tb288/tb288.html)

Ordering Information

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay*	10 ml	G7570
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)*	1,000 assays	G3580
GloMax® 96 Microplate Luminometer	1 each	E6501

*For Laboratory Use. Additional sizes available.

[a]The MTS tetrazolium compound is the subject of U.S. Pat. No. 5,185,450 assigned to the University of South Florida and is licensed exclusively to Promega Corporation.

[b]U.S. Pat. No. 7,083,911, Australian Pat. No. 2002255553 and other patents pending.

[c]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312, 785294 and other patents and patents pending.

[d]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.