Appendix 1

Small-Molecule Toxicity Curves in CTLL-2 Cells

Introduction

The toxicities of various small molecules to CTLL-2 cells were measured to determine the optimal ligand input concentration for regulatory device evaluations and to determine whether certain small molecules of interest are sufficiently non-toxic to be useful candidates for aptamer selection. Specifically, theophylline, tetracycline, and doxycycline toxicities were measured to determine the maximum concentration that could be tolerated for characterization studies on theophylline- and tetracycline-responsive RNA switch devices. Phenobarbital, tamoxifen, folinic acid, and vitamin B_{12} (cyanocobalamin) are pharmaceuticals approved for clinical use by the United States Food and Drug Administration. Therefore, they are potentially suitable molecular inputs for RNA-based regulatory systems in therapeutic applications. The toxicity levels of these molecules were measured to assist in the choice of target ligands for the aptamer selection processes described in Chapter 6.

Results and Discussion

Theophylline at concentrations between 5 μM and 250 μM shows moderate toxicity to CTLL-2 cells (Appendix Figure 1.1). Toxicity increases significantly at 500 μM and above, but cells could still sustain growth at the highest concentration tested (1 mM) during the 5-day study. Based and these results, we determined 1 mM to be an appropriate concentration for use in transient transfection experiments, and 500 μM was chosen for long-term studies of cell lines stably integrated with RNA regulatory systems. Tetracycline causes severe toxicity even at low micromolar levels (Appendix Figure 1.2), and doxycycline shows very similar toxicity profiles (Appendix Figure 1.3). Although the

tetracycline aptamer has a reported K_D value of 0.8 nM[1], the concentration needed to trigger ribozyme switches may be higher, and the intracellular tetracycline concentration is likely to be significantly lower than the extracellular concentration. These conflicting constraints imposed by tetracycline toxicity and aptamer affinity preclude the determination of a specific input concentration for characterization studies. Therefore, transient transfection experiments were performed with a range of tetracycline input concentrations (Figure 3.3). T-cell growth regulation was impossible due to tetracycline's strong toxicity, thus tetracycline-responsive switches were not chosen for stable integration and subsequent characterizations.

Folinic acid is essentially nontoxic to CTLL-2 cells, and both phenobarbital and vitamin B_{12} can be administered to 1 mM or above without causing severe growth inhibition (Appendix Figures 1.4-1.6). Therefore, these small-molecule pharmaceuticals were chosen for aptamer selection studies (Chapter 6). In contrast, tamoxifen was found to be extremely toxic to CTLL-2 cells (Appendix Figure 1.7) and soluble only in organic solvents such as methanol, which also causes modest toxicity (Appendix Figure 1.8). As a result, tamoxifen was eliminated from the list of candidates for aptamer selection.

Materials and Methods

Theophylline toxicity curve. CTLL-2 cells stably expressing firefly luciferase (CffLuc cells) were cultured as described in Chapter 3. On Day 0, nine T25 tissue culture flasks were each seeded with 5 ml of cells at 0.15 x 10^6 cells/ml. A 40 mM stock of theophylline anhydrous (Sigma) dissolved in sterile water and filtered through a 0.22-μm syringe filter was used to bring each flask to the appropriate final theophylline

concentration. A 4 mM diluted stock was used to supplement the 5-μM sample well in order to avoid pipetting volumes less than 1 μl. Cell count in each sample was manually obtained every 24 hours using Trypan Blue stain (Gibco) and a hemocytometer. Cultures were fed 100 U/ml IL-2 every 48 hours and split as necessary to maintain cell densities between 0.15 x 10^6 cells/ml and 1.50 x 10^6 cells/ml. The relatively high cell density was chosen to allow more accurate manual cell count with a hemocytometer.

Tetracycline and doxycycline toxicity curves. CffLuc cells were cultured as described in Chapter 3. On Day 0, 24-well plates were seeded with cells at 0.03 x 10^6 cells/ml, 1 ml/well, 1 well for each ligand concentration tested. A 20 mM tetracycline hydrochloride (Sigma) stock and an 80 mM doxycycline hyclate (Sigma) stock were prepared by dissolving in sterile water and filtering through 0.22-μm syringe filters. Stocks were further diluted with water as necessary so a volume between 1 μl and 7.5 μl of dissolved ligand was added to each well to bring cultures to the appropriate final ligand concentrations. Starting on Day 1, 100 μl of each well was sampled every 24 hours for cell density measurements using a Quanta Cell Lab Flow Cytometer (Beckman Coulter). Viable populations were gated based on side scatter and electronic volume, and cell density was determined by viable cell count divided by total volume of sample analyzed on the flow cytometer. Cultures were fed 100 U/ml IL-2 every 48 hours, and samples with a measured density above 0.1 x 10^6 cells/ml on Day 4 were split ten-fold on Day 5 using media containing the appropriate ligand concentration.

Phenobarbital toxicity curve. CffLuc cells were cultured as described in Chapter 3. On Day 0, 24-well plates were seeded with cells at 0.02 x 10^6 cells/ml, 1 ml/well, 1 well for each ligand concentration tested. A 2 mM stock of phenobarbital (Sigma) was prepared by dissolving in RPMI-1640 media. Since a more concentrated stock could not be made due to phenobarbital's low solubility, large volumes of the dissolved ligand had to be added to samples at the high concentration points. Extensive dilution with water could have adverse effects on cell physiology, thus the phenobarbital stock was prepared in media. The stock was further diluted with media as necessary so >1 μl of dissolved phenobarbital was added to each well to bring cultures to the appropriate final concentrations. All wells were fed 100 U/ml IL-2 every 48 hours and split ten-fold on Days 3 and 5 using media containing the appropriate ligand concentration. Cell density was measured as described for tetracycline and doxycycline toxicity curves.

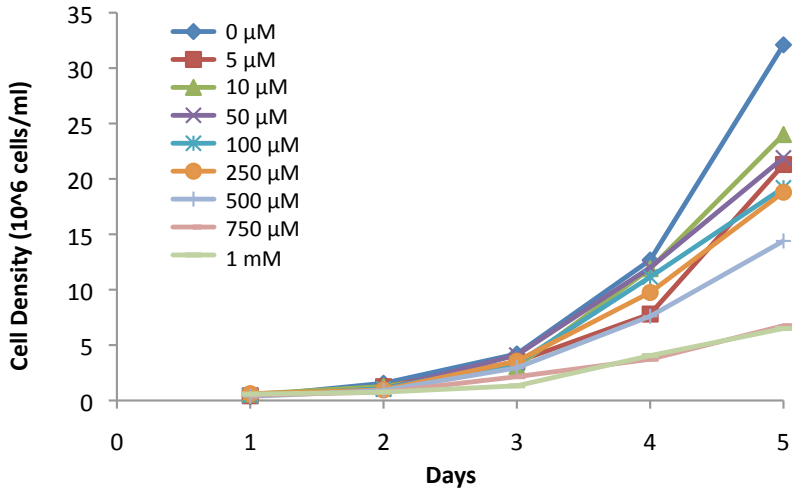
Folinic acid and vitamin B_{12} toxicity curves. CffLuc cells were cultured as described in Chapter 3. On Day 0, 24-well plates were seeded with cells at 0.05 x 10^6 cells/ml, 1 ml/well, 1 well for each ligand concentration tested. A 40 mM folinic acid calcium salt hydrate (Sigma) stock was prepared by dissolving in sterile water and filtering through 0.22-μm syringe filters. A 10 mM stock of vitamin B_{12} (cyanocobalamin, Sigma) was prepared by dissolving in RPMI-1640 media (see phenobarbital toxicity curve methods for the use of media instead of water). Stocks were further diluted as necessary so >0.5 μl of dissolved ligand was added to each well to bring the culture to the appropriate final ligand concentration. Cultures were fed 100 U/ml IL-2 every 48 hours, and all wells were split 6-fold on Day 2. The 2 mM folinic acid culture was found with bacterial

contamination on Day 3 and discarded. All remaining wells were subsequently supplemented with 50 U/ml penicillin:streptomycin. On Day 4, all folinic acid cultures were split ten-fold and all vitamin B_{12} cultures were split six-fold using media containing the appropriate ligand concentration. Cell density was measured as described for tetracycline and doxycycline toxicity curves.

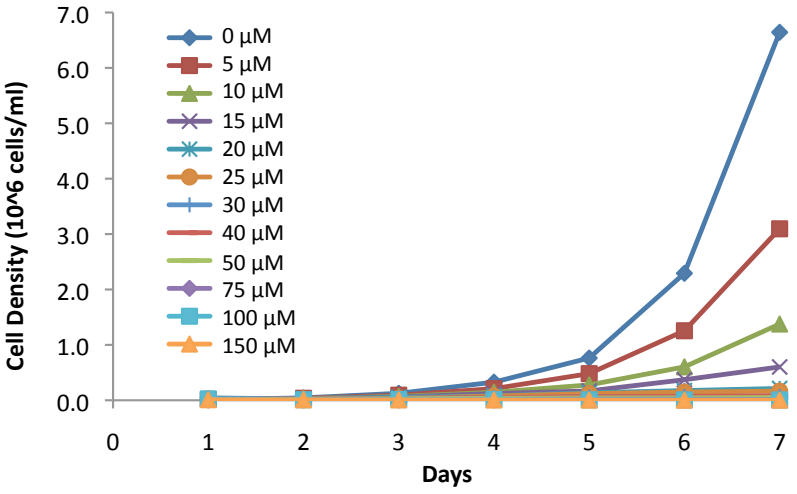
Tamoxifen and methanol toxicity curves. CffLuc cells were cultured as described in Chapter 3. On Day 0, 24-well plates were seeded with cells at 0.03 x 10^6 cells/ml, 1 ml/well, 1 well for each ligand concentration tested. Tamoxifen has extremely low solubility in aqueous solutions. Therefore, an 1 mM stock of tamoxifen citrate salt (Sigma) was prepared by dissolving in methanol with heating in a 37°C water bath and filtering through a 0.22-μm syringe filter. The stock was further diluted with methanol as necessary so a volume between 1 μl and 5 μl of dissolved ligand was added to each well to bring cultures to the appropriate final ligand concentrations. For the methanol toxicity curve, 100% methanol was used to supplement each cell culture to the appropriate final methanol concentration. Cultures were fed 100 U/ml IL-2 every 48 hours. All wells for the tamoxifen toxicity curve were split 11-fold on Days 2 and 4 using media containing the appropriate ligand concentration. Cultures for the methanol toxicity curve were never split. Cell density was measured as described for tetracycline and doxycycline toxicity curves.

Reference

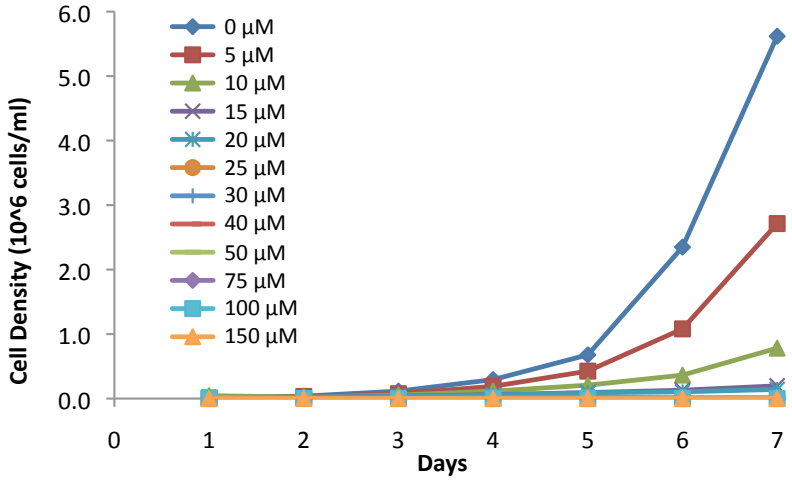
1. Weigand, J.E. & Suess, B. Tetracycline aptamer-controlled regulation of pre-mRNA splicing in yeast. *Nucleic Acids Res* **35**, 4179-4185 (2007).



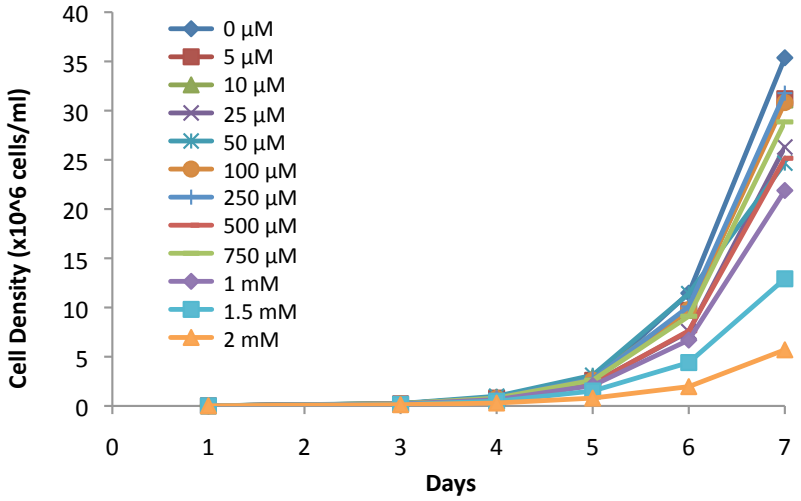
Appendix Figure 1.1. Theophylline toxicity curve in CTLL-2 cells.



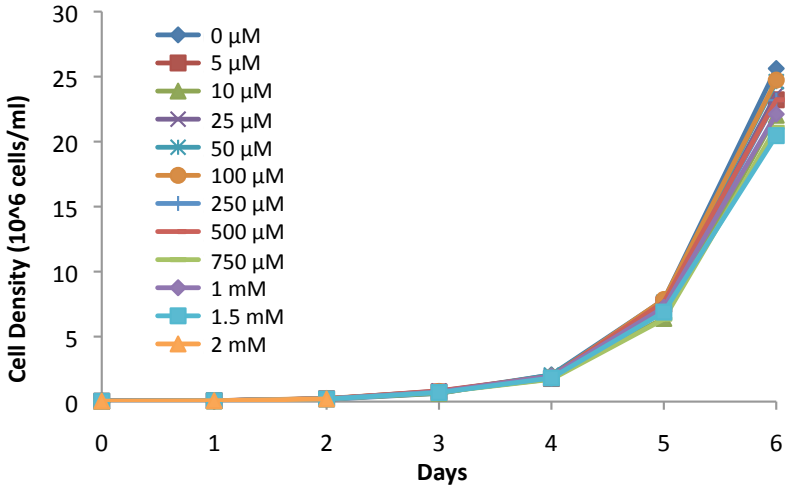
Appendix Figure 1.2. Tetracycline toxicity curve in CTLL-2 cells.



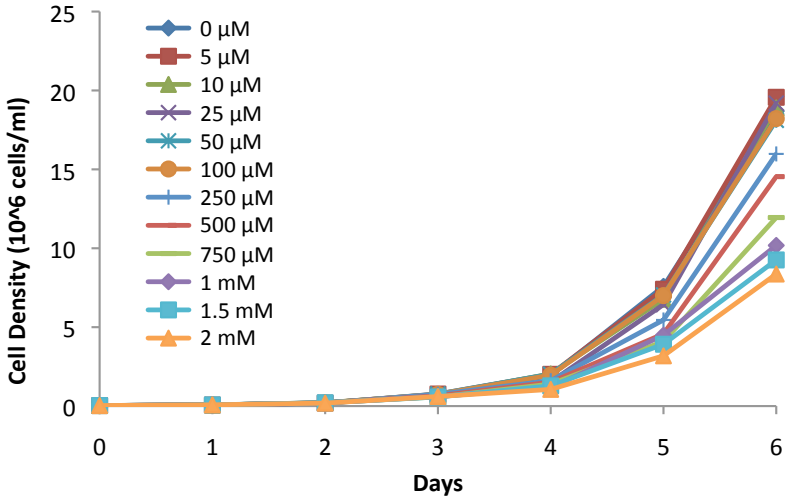
Appendix Figure 1.3. Doxycycline toxicity curve in CTLL-2 cells.



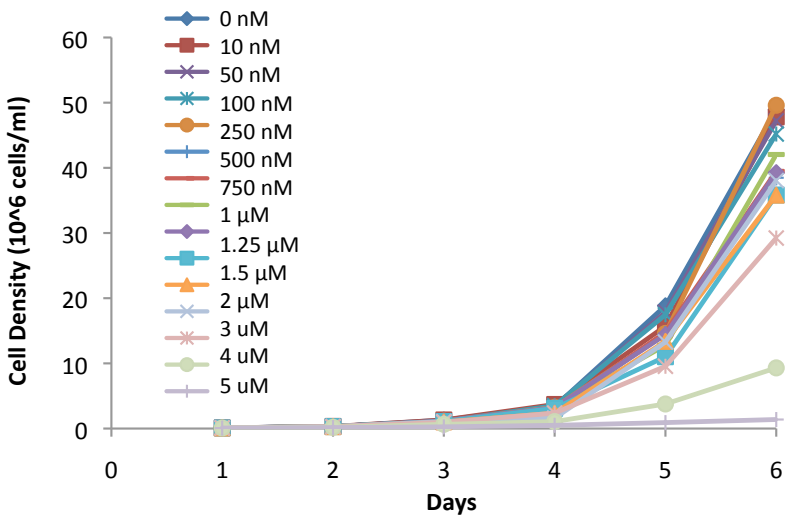
Appendix Figure 1.4. Phenobarbital toxicity curve in CTLL-2 cells.



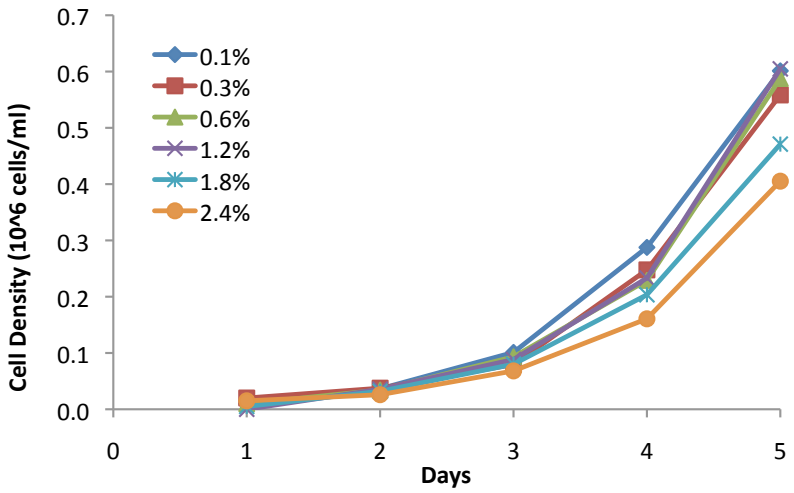
Appendix Figure 1.5. Folinic acid toxicity curve in CTLL-2 cells.



Appendix Figure 1.6. Vitamin B_{12} toxicity curve in CTLL-2 cells.



Appendix Figure 1.7. Tamoxifen toxicity curve in CTLL-2 cells.



Appendix Figure 1.8. Methanol toxicity curve in CTLL-2 cells.