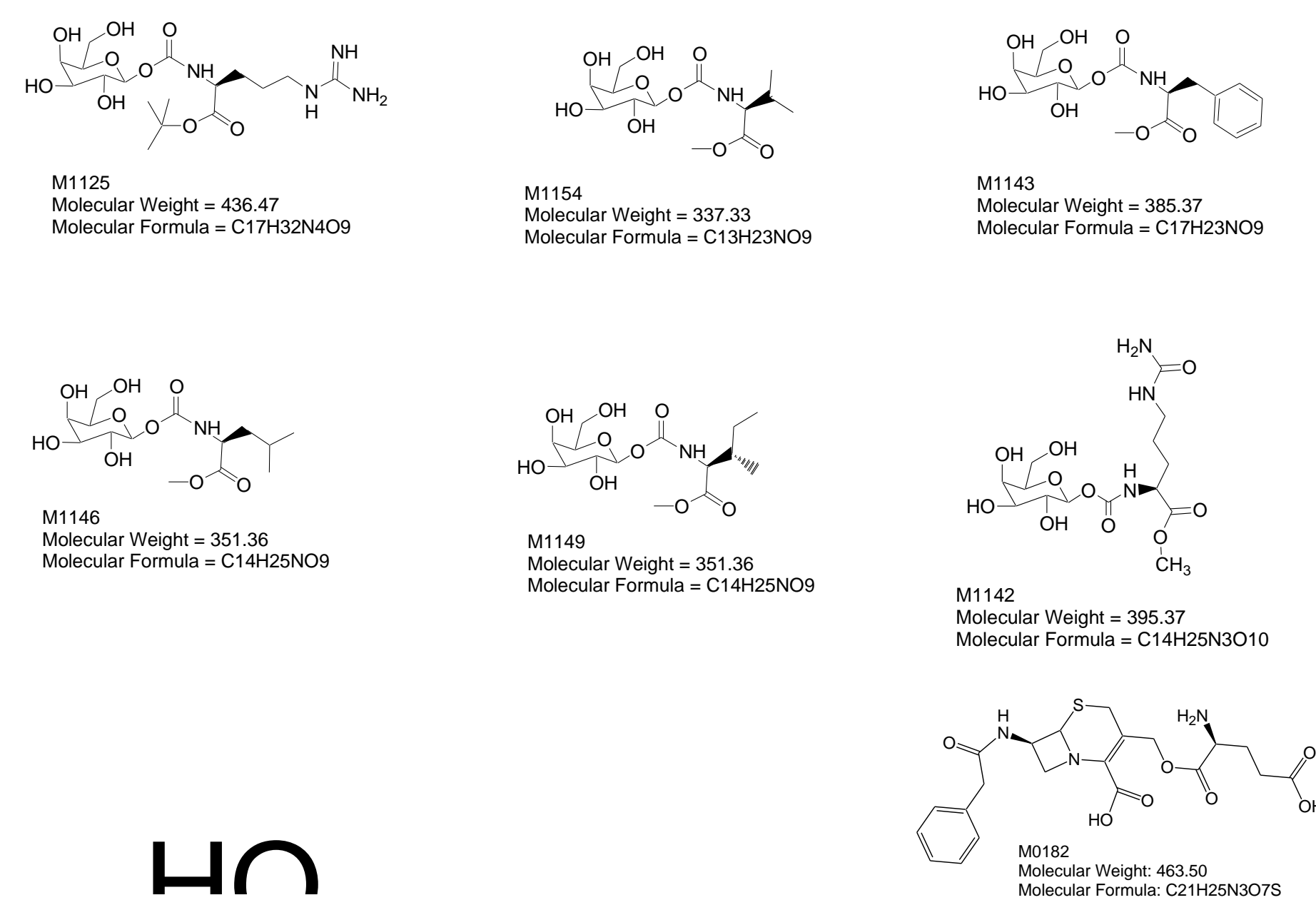


Expression of foreign genes in mammalian cells has become a standard biotechnology protocol, and co-expressed reporter genes are routinely used in these systems to track the expression levels of the simultaneously cloned genes. The complete lack of intrinsic enzyme activity in the resulting recombinant cells and the wide substrate specificities of the enzymes allow use of the marker gene for release of targeted conjugates at very low levels of expression. In an effort to impose a non-destructive selection mechanism on transgenic cells, we have prepared new β -galactosidase and Cephalosporin conjugates of common growth regulators, drugs and enzyme activators, for administration to animal cells or bacteria that contain either the beta-galactosidase (*lacZ*) or ampicillin resistance (beta-lactamase, *amp*) genes as gene fusion markers. These new conjugates were used to provide new methods of utilizing these fusion systems in transformed cells to select for transgenic cells in cell culture based upon expression levels of the marker genes. New conjugates of guanosine, L-arginine, L-citrulline, L-phenylalanine, L-leucine, L-isoleucine, L-valine, D-glucose and L-glutamine were prepared and assayed in both *E. coli* and in mammalian skin fibroblast (NIH3T3 and CREBAG2) cell lines grown in cell culture for their ability to cause specific and localized improvement of cell growth and to deliver the active agents in a cell- or co-culture specific manner. By combining the effect of certain metabolic inhibitors including mycophenolic acid (MPA) a GMP (guanosine monophosphate) synthetase inhibitor, or methotrexate, a dihydrofolate reductase inhibitor, with selection media lacking certain amino acids along with administration of a panel of conjugates, an 80-100% selection rate could be effected for *lacZ*-positive CREBAG2 cells over NIH3T3 (*lacZ*-negative) after incubation for 3 days in selection media followed by a 7 day recovery period. The use of these methods for selection of transgenic cell lines from progenitor and stem cell lines is currently underway. This work was supported by grant 1R43GM074315-01 from the NIGMS-NIH.

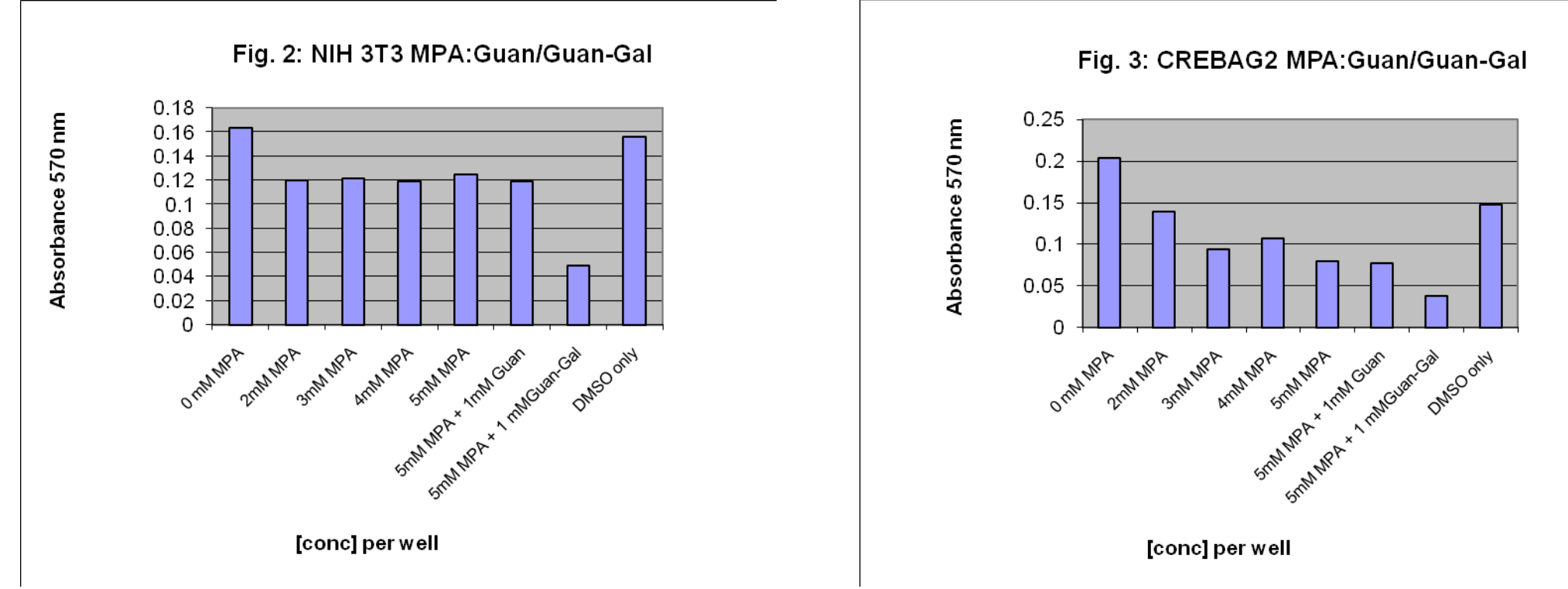
Figure 1: Enzyme Substrates



Materials and Methods

- E. coli* (strain JM109) and murine NIH3T3 and CREBAG2 fibroblast cell lines were obtained from the ATCC (Manassas, VA). Bacterial cells were grown in LB medium or M9 minimal media. Mammalian cell lines were cultured in Dulbecco's Modified Eagles Medium prior to selection studies.
- Under normal growth conditions, all mammalian cell culture media was supplemented with 9% FCS, 100U/mL penicillin, 100µg/mL streptomycin, and 0.25µg/mL amphotericin B and grown at 37°C humidified incubator in a 5-10% CO₂ environment.
- Selection media were prepared from a minimal media (Sigma D5796 or D5030) with added glucose, lactose, glutamine or amino acid conjugates.
- Cell growth was measured using the MTT assay. After removal of media, 0.8ml of HEPES Buffered Media and 0.2ml of 2mg/ml MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS solution was added to each well. Plates were incubated for 2.5 hours, the buffer removed and replaced by DMSO (0.5 mL) to develop the purple product and read in a Perkin-Elmer HTS7000 Bioassay microtiter plate reader, UV absorbance mode (@ 570nm).
- Percent selection of NIH3T3 and CREBAG2 cells grown in co-culture (50:50) was determined using the X-Gal staining assay. After removing the media and washing with PBS (3X) the cells were fixed by adding 1.0 mL cold Fixing Solution (37% formaldehyde and 25% glutaraldehyde) and incubating at room temp. (20 min.). Following washing (PBS, 3X) a solution of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, 1 mg/mL) in Staining buffer (0.1M phosphate buffer (pH 7.3) containing 2 mM MgCl₂, 5mM potassium ferrocyanide (K₄Fe(CN)₆·3H₂O and 5mM potassium ferricyanide (K₃Fe(CN)₆) was added and the cells incubated at 37°C for 2-16 hrs.
- Labeled cells were washed 3x with Phosphate Buffered Saline prior to imaging. Cells were imaged using a Nikon Labophot 2 fluorescence microscope and Sony Cyber-shot DSC-S85 digital camera. CellProfiler cell image analysis software was obtained from the Broad Institute at the Massachusetts Institute of Technology.

Figures 2 and 3: Mycophenolic Acid Inhibition with Guanosine-Galactoside



Figures 2 and 3. Mycophenolic acid is an inhibitor of guanosine biosynthesis. NIH3T3 and CREBAG2 cells were grown in 12 well plates with DME containing 9% FCS at 37°C with 10% CO₂. Cells were treated with DME (no FCS) containing mycophenolic acid (MPA) in the range of 0 to 5mM. Some wells with 5mM MPA were also treated with 1mM guanosine or 1mM guanosine galactoside. The plates were incubated overnight at 37°C with 10% CO₂. Cell growth was measured using the MTT assay. Relative growth levels are represented by the relative absorbance levels.

Figures 4 and 5: Methotrexate and Hypoxanthine Inhibition.

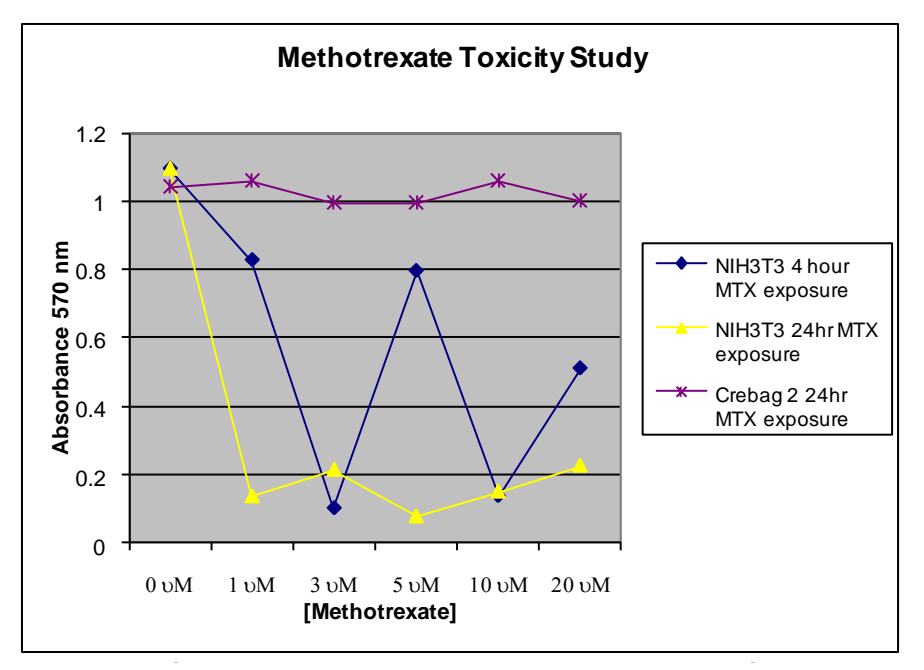


Figure 4. NIH3T3 and CREBAG2 cells were grown in 12 well plates with DME containing 9% FCS at 37°C with 10% CO₂. After 48 hours media was replaced and cells were treated with DME containing 0.5 mM methotrexate in DMSO for effective concentrations of 0, 1uM, 3uM, 5uM, 10uM, and 20uM methotrexate. Cells were incubated 4 hours or 24 hours before changing media back to DME with FCS. Cell growth was measured by MTT assay.

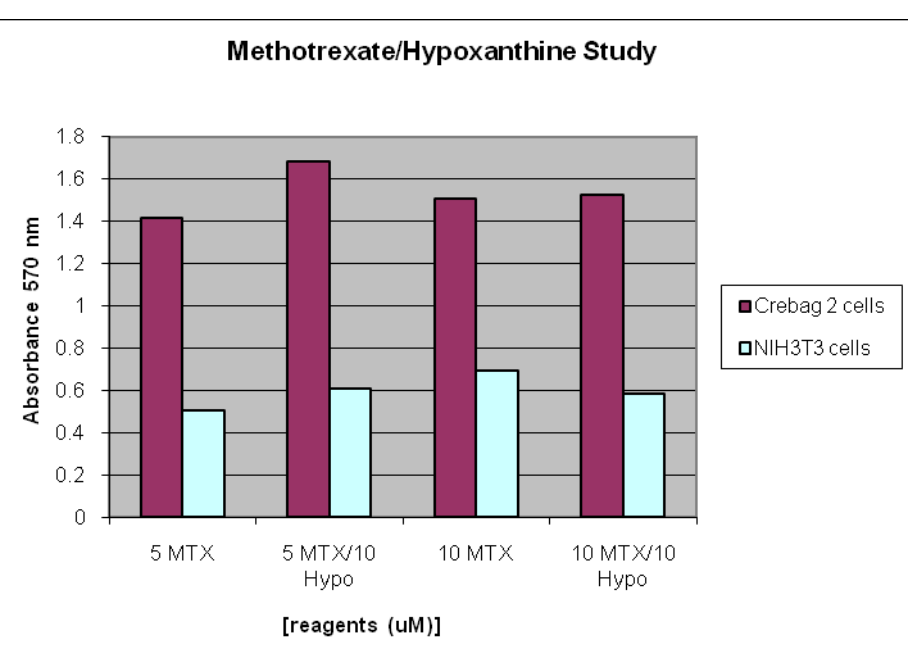


Figure 5. NIH3T3 and CREBAG2 cells were grown in 12 well plates with DME containing 9% FCS at 37°C with 10% CO₂. After 48 hours the cells were treated with DME containing 1.0 mM methotrexate for effective concentrations of 5 uM or 10 uM methotrexate. In addition some cells were also treated with a 1mM solution of hypoxanthine for an effective concentration of 10 uM. Cells were incubated for 24 hours then rinsed twice with fresh media and allowed to recover for a further 40 hours. Cell growth was measured by MTT assay.

Figure 6: Effect of Glucose-Free Media and Mycophenolic Acid.

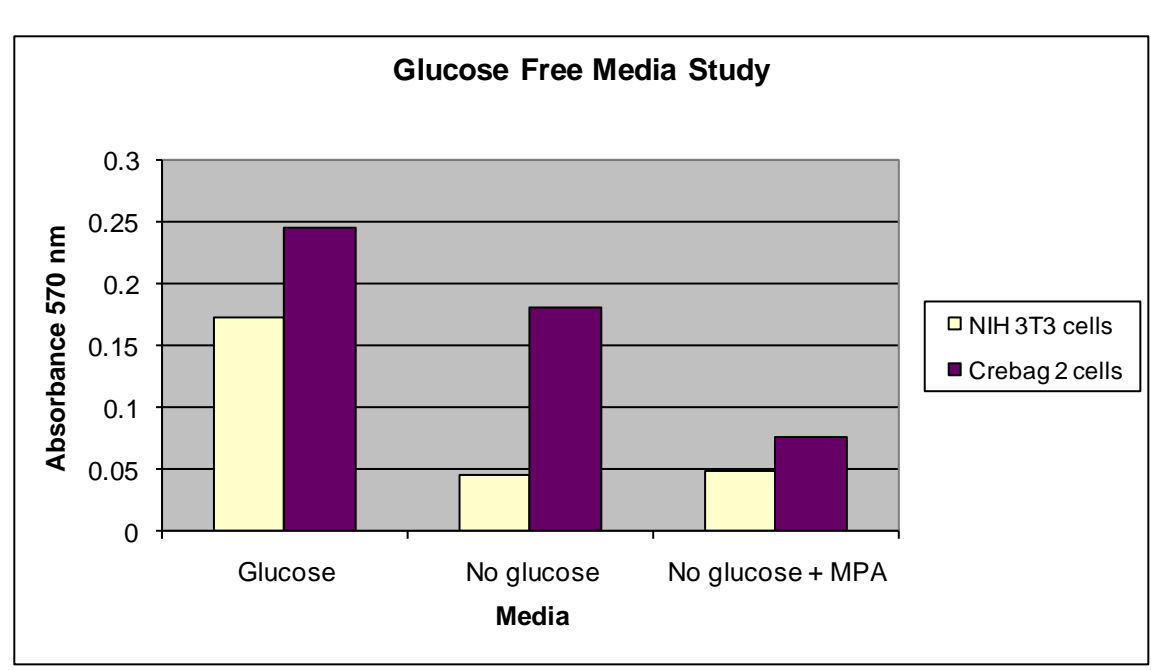


Figure 6. NIH3T3 and CREBAG2 cells were grown in 12 well plates with DME containing 9% FCS at 37°C with 10% CO₂. After 48 hours media was replaced with one of two media: DME (Sigma D5796) without added FCS, or DME without added FCS that was also without glucose and glutamine (Sigma D5030). Some CREBAG2 cells were also treated with mycophenolic acid at an effective concentration of 3 uM while NIH3T3 cells were also treated with mycophenolic acid at an effective concentration of 5 uM. Cells were incubated for 48 hours. Cell growth was measured by the MTT assay.

Figure 7: Effect of Glutamine and Lactose

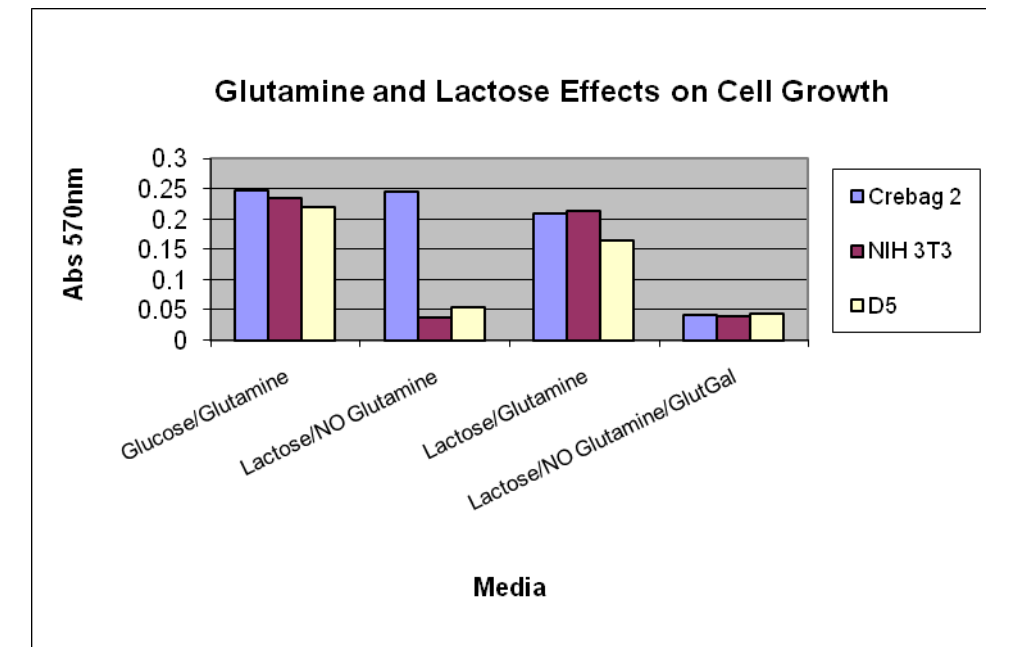


Figure 7. NIH3T3, CREBAG2, and D5 cells were grown in 12 well plates with DME containing 9% FCS at 37°C with 10% CO₂. After 24 hours media was replaced with one of four media, (1 mL per well) each without added FCS: 1)DME (Sigma D5796), 2) DME without glucose and glutamine (Sigma D5030) with lactose added (9g/L), 3) DME without glucose and glutamine (Sigma D5030) with glutamine added (.58g/L), or 4) Media 2 with 83ml of a 16mg/ml solution of M1160 (Glutamine galactoside methyl ester) added. Cells were incubated for 72 hours. Cell growth was measured using the MTT assay.

Table 1: Media Compositions.

#	Media	GLUCOSE	LACTOSE	GLUTAMINE	ARGININE	PHENYLALANINE	LEUCINE
1	D5796	X		X	X	X	X
2	D5030		X		X	X	X
3	D5030 + Glut		X	X	X	X	X
4	Lot 69		X				
5	Lot 69 + ArgGal		X				
6	Lot 69 + LeuGal		X				
7	PAL - Arg		X	X		X	X
8	PAL-Arg + ArgGal		X	X		X	X
9	PAL-Leu		X	X	X	X	
10	PAL-Leu + LeuGal		X	X	X	X	
11	PAL-Phe		X	X	X		X
12	PAL-Phe + PheGal		X	X	X		X

Table 1: Media Compositions containing added sugars, glutamine or amino acids. Note: All media were FCS free. Conjugates were added at 2X AA concentrations used in standard media (D5796). PAL = Phe, Arg, Leu. D5796 = DME with Glc and Glutamine added. D5030 is minimal DME media prepared without Glc or Gln. Lot 69 = media comparable to M5030 but without Arg, Iso, Leu, Phe.

Figure 8: Effect of Amino Acid Conjugates on Selection.

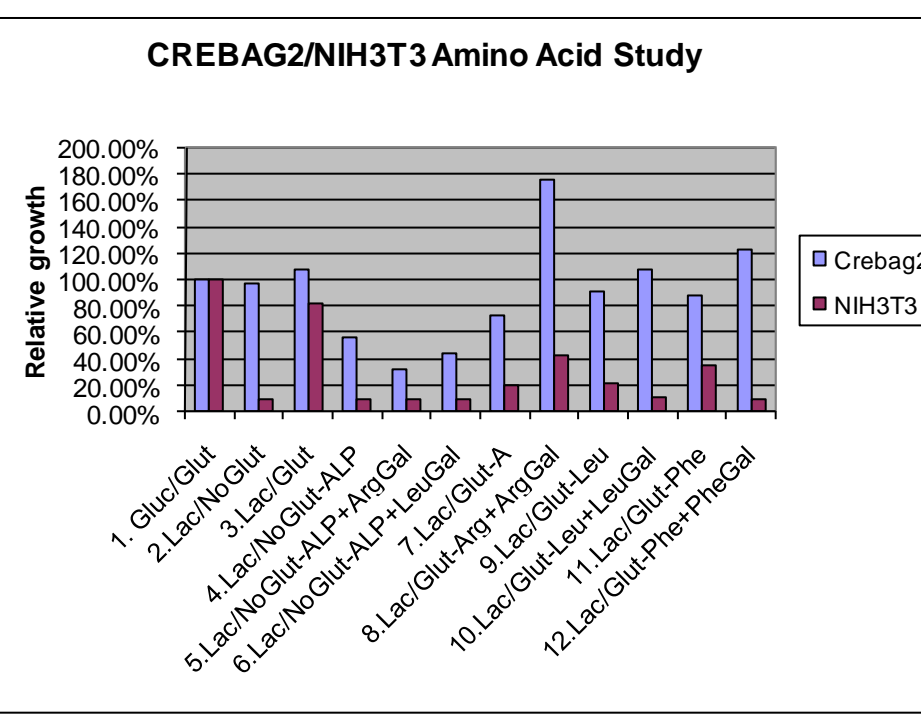


Figure 7. NIH3T3 and CREBAG2 cells were grown in 12 well plates with 1ml DME containing 9% FCS at 37°C with 10% CO₂ overnight. Media was removed from each well and replaced with 1ml of one of twelve serum free media prepared. (see Table 1) All media was glucose deficient except Media 1. ArgGal (M1125), LeuGal (M1146) and PheGal (M1143) were added at 2X concentration of corresponding amino acid in standard media (Sigma D5796). The plates were incubated three nights at 37° C with 10% CO₂. Cell growth was measured by MTT assay.

Figure 9: X-Gal Analysis of NIH3T3:CREBAG2 Coculture Selection.

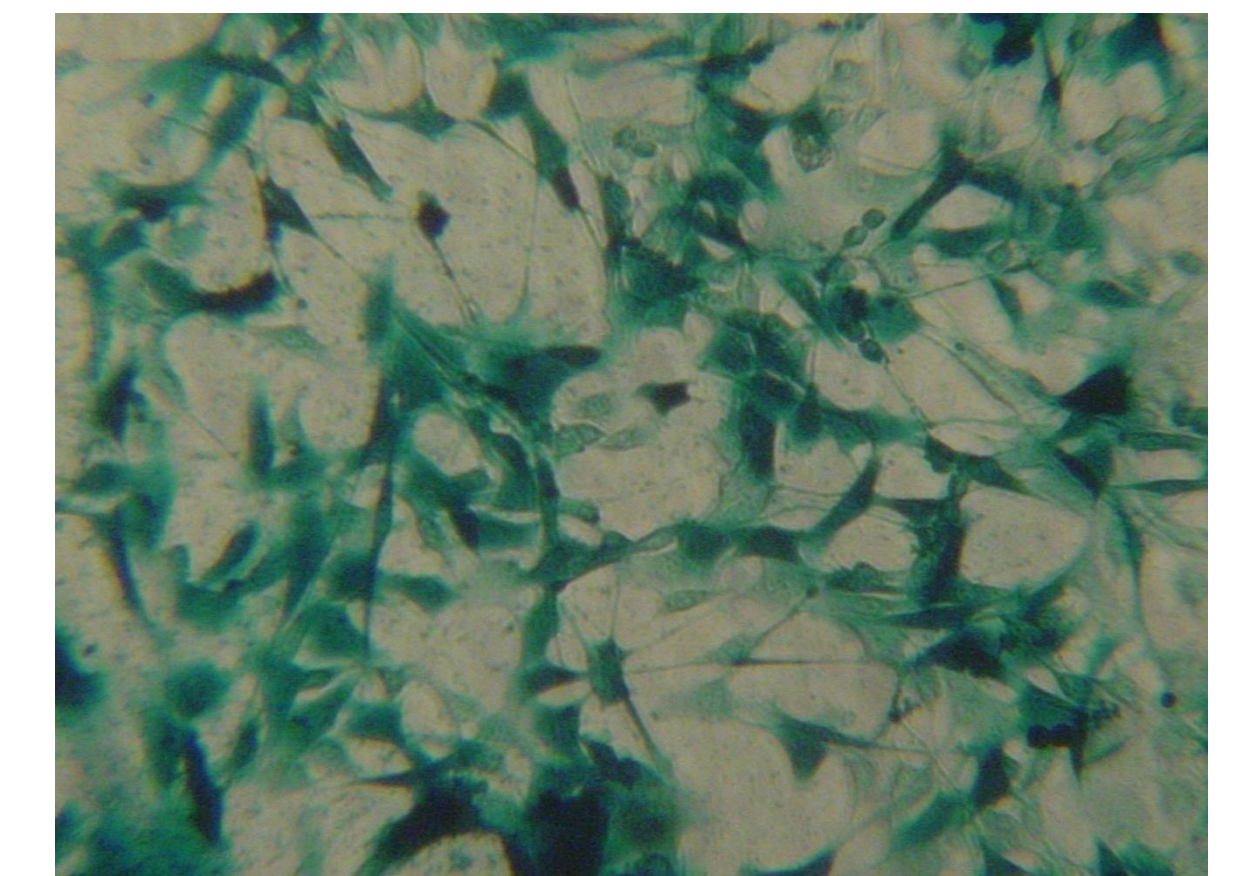


Figure 8. X-Gal staining of mixed cell co-culture after selection using Media 8, according to method described. 100% selection of LacZ+ CREBAG2 Cells was obtained.

Discussion and Conclusions

- A series of eight galactoside conjugates were prepared (see Structures) from seven amino acids: L-arginine, L-citrulline, L-phenylalanine, L-leucine, L-isoleucine, L-valine and L-glutamine as well as from guanosine and glucose (lactose).
- Enzymatic turnover studies for the synthesized conjugates were performed using purified β -galactosidase (*E. coli*) with analysis using HPLC (for M1143, M1067) or TLC (others) to monitor the turnover. In general, all of the conjugates showed good turnover activities and released the representative intact bioactive compounds.
- Control experiments with NIH3T3 cells and cell lysate samples demonstrated stability of the conjugates for up to 3 days under standard cell culture conditions.
- The effect of inhibitors mycophenolic acid (MPA) (a guanosine monophosphate) synthetase inhibitor) and methotrexate (a dihydrofolate reductase inhibitor) as well as their rescue agents guanosine (for MPA inhibition) and hypoxanthine (a dihydrofolate reductase rescue reagent) were evaluated. These inhibitor/rescue reagents however were not able to produce significant selection.
- The effect of glucose-free, lactose supplemented and L-glutamine deficient media were found to significantly affect cell growth
- Using a glucose, amino acid and L-glutamine deficient media in combination with amino acid conjugates and lactose was found to produce between 80-100% selection of *lacZ*-positive versus *lacZ*-negative cell lines in one round of selection (3 day culture).
- Work is underway to test these media systems for isolation of transgenic human stem cells in a non-destructive manner.
- Selection of E.coli bacteria has thusfar been unsuccessful due to prototrophic growth.
- Selection media utilizing these metabolic substrates will be available commercially from Marker Gene. These reagents and methods are protected by one or more US and International Patents.

References

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