Supplemental Data

Title:

CRISPR/Cas9 genetic modification of *CYP3A5 *3* in HuH-7 human hepatocyte cell line leads to cell lines with increased midazolam and tacrolimus metabolism

Authors:

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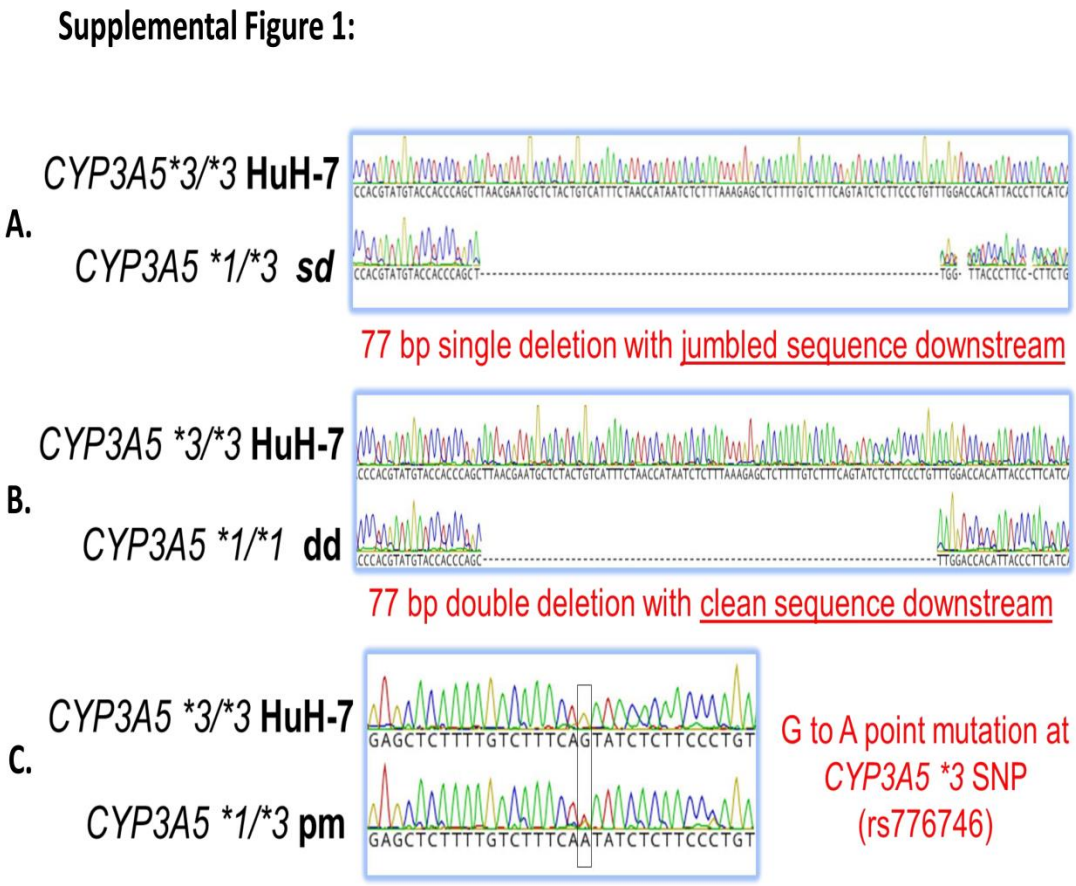
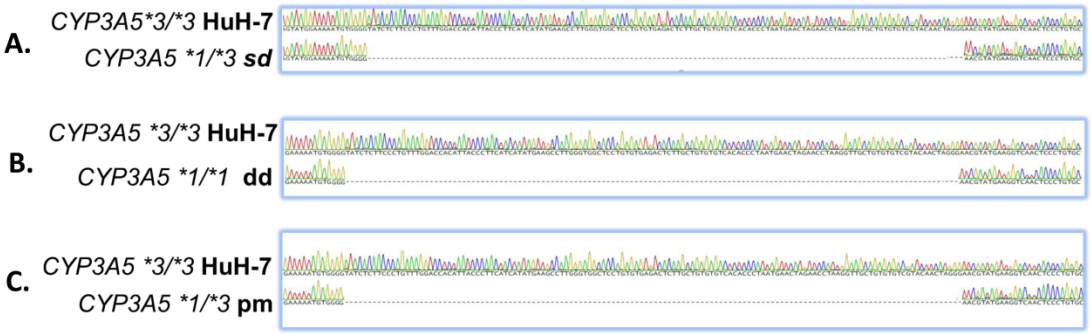
Supplemental Figure 1: DNA Sequence Chromatograms of *CYP3A5 *3* locus in Cell Lines
The sequences were aligned to reference sequence from the HuH-7 cell line that had genotype *CYP3A5 *3/*3*. **A.)** Heterozygous deletion of splice junction in cell line *CYP3A5 *1/*3* sd showed jumbled sequence downstream of deletion indicating heterozygosity. **B.)** Homozygous deletion of splice junction in cell line *CYP3A5 *1/*1* dd showed clean sequence downstream of deletion indicating homozygosity. **C.)** Heterozygous point mutation seen in cell line *CYP3A5 1*/*3* pm.



Supplemental Figure 2: Sequence Chromatograms of cDNA from CYP3A5 *3 locus mRNA in Cell Lines. Total RNA was isolated from the cell lines, reverse transcribed with oligo dT primer and the *CYP3A5* mRNA cDNA was sequenced with primers that flank the 131 base pair exon 3B. All cell sequences were aligned to HuH-7 reference control that was *CYP3A5 *3/*3* genotype. **A.)** Absence of exon 3B in cell line *CYP3A5 *1/*3* sd. It is possible we only saw the **1 CYP3A5* in this sequence because previous reports show that *CYP3A5 *3* mRNA is targeted for non-sense mediated decay and would be more difficult to identify in a heterozygote. **B.)** Absence of exon 3B in cell line *CYP3A5 *1/*1* dd. **C.)** Absence of exon 3B in cell line *CYP3A5 1*/*3* pm.

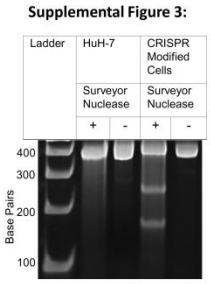
Supplemental Figure 2:



Supplemental Table 1: Primers used in this study for PCR, RT-PCR, and Sequencing.

Gene/Locus	Primer	forward Primer Sequence 5'->3'	Purpose
*CYP3A4 *22*	**22F*	AATTCTGCTGTCAGGGCAAC	PCR Amplification and Sequencing
	**22R*	TTGAGAGAAAGAATGGATCCAAAAA	PCR Amplification and Sequencing
	**22-1F*	GGCATAGAGTCTGCAGTCAGG	Sequencing
	**22-1R*	TCACCTTCTATCACACTCCATCA	Sequencing
	**22-2F*	TCAGTGTCTCCATCACACCC	Sequencing
	**22-2R*	GGATTGTTGAGAGAGTCGATGTT	Sequencing
*CYP3A5 *3*	*8F*	CTGTCAGAGGGGCTAGAGGT	PCR Amplification and Sequencing
	8R	CCTCCCAGGTTCAAGCGATT	PCR Amplification and Sequencing
	7853 F	GCATTTAGTCCTTGTGAGCACTTG	PCR Amplification and Sequencing
	8303 R	CATACGTTCTGTGTGGGGACAAC	PCR Amplification and Sequencing
	7884F	ACCTGCCTTCAATTTTTCACTG	Sequencing
	8267R	CTTCACTAGCCCGATTCTGC	Sequencing
	3A5 ex2F	GTCACAATCCCTGTGACCTGAT	PCR Amplification and Sequencing
	3A5 ex5R	TTGGAGACAGCAATGACCGT	PCR Amplification and Sequencing
	3A5 ex2F Seq	CTGTTTCACTTTGTAGATATGGGAC	Sequencing
	3A5 ex5R Seq	AATCCCACTGGGCCTAAAGAC	Sequencing
*CYP3A5*6*	*4F*	TCTGCCCATCTGTCACCAAT	PCR Amplification and Sequencing
	4R	TTGGCCACATGTCCAGTACT	PCR Amplification and Sequencing
	15488F	GGCACCAGATAACCACCTTC	Sequencing
	15989R	GGGCTCTAGATTGACAAAAACA	Sequencing
*CYP3A5*7*	*12F*	TCCTCCACACATCTCAGTAGGT	PCR Amplification and Sequencing
	12R	TAAGGCCTGACCTTGTCCCT	PCR Amplification and Sequencing
	28064F	ACTTCACGAATACTATGATCATTTACC	Sequencing
	28351R	CATTGACCCTTTGGGAATGA	Sequencing
	28448R	CATTGACCCTTTGGGAATGA	Sequencing
GAPDH	*GAPDHF*	GCATCCTGCACCACCA	qRT-PCR
	GAPDHR	GGATGACCTTGCCCACA	qRT-PCR

Supplemental Figure 3: Surveyor Assay used to select guide RNA. Surveyor nuclease assay indicated HuH-7 parental cells transfected with hCas9, guide RNA 2, and HDR template ssODN successfully mutated the *CYP3A5 *3* locus. Parental and CRISPR modified genomic DNA was used for PCR and surveyor nuclease detection of genome modification at **3* locus. Parental cell PCR products treated with surveyor nuclease had a 397 bp band, while the CRISPR modified cells' PCR products had bands of 397, 236 and 161 bp indicating genetic modification at the **3* locus in bulk transfected cells. These cells were then single-cell cloned to isolate cell line *CYP3A5 *1/*3* pm.



Supplemental Protocols:

Assay - Midazolam and metabolites in cell culture media

Detection and quantification of midazolam, 1-OH midazolam and 4-OH midazolam in cell culture media was performed using a high-performance liquid chromatograph (Agilent 1200 Series, Santa Clara CA) coupled with a TSQ Quantum triple stage quadrupole mass spectrometer (Thermo-Electron, San Jose, CA). The chromatographic separation was performed with a Waters UPLC HSS T3, 2.1 x 50 mm, reversed phase column with a 1.8-micron particle size (Waters, Milford, MA). The mobile phase used for gradient elution consisted of (A) 0.1% formic acid in water, (B) 0.1% formic acid in acetonitrile. The gradient was linear from 20-28% (B) from 0.0- 4.0 min, 20% (B) from 4.25-8.0 min, at a flow rate of 0.3 mL/min, for a total run time of 8.0 minutes. The column temperature was maintained at 45°C. The detector settings of the TSQ Quantum were: ESI with the stainless steel spray needle, positive polarity ionization, selective reaction monitoring mode (SRM); spray voltage, 4500 V; capillary temperature, 400 °C; argon collision gas pressure, 1.5 mTorr; unit resolution for Q1 and Q3, 0.7 u (FWHM).

Analyte Name	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	Retention Time (min)
1,4-dihydroxymidazolam	358	290	27	3.04
4-hydroxymidazolam	342	324	2	3.41
1-hydroxymidazolam-d4	346	328	2	4.62
1-hydroxymidazolam	342	324	2	4.69
Midazolam-d4	330	295	8	5.09
Midazolam	326	291	7	5.16

Following the addition of internal standard (120 ng of 1-hydroxymidazolam-d4 and Midazolam-d4) and 5% NH_4OH (0.15 mL), cell culture samples (0.15 mL) were extracted with 1.25 mL of 50:50 Methyl tert-butyl ether:Hexane using a multi-tube vortexer for 10 minutes. Following centrifugation at 15,000 x g for 5 minutes, the supernatant was removed and evaporated to dryness using a nitrogen evaporator (Zymark Turbo Vap LV, Hopkinton, MA) set at 37°C. The residue was reconstituted with 100 µL of mobile phase (A:B, 80:20). Midazolam, metabolites and the deuterated internal standards were obtained from Cerilliant Corporation (Round Rock, Texas). The assay was linear in the range of 1.0 – 1000 ng/mL for midazolam and the metabolites.

Tacrolimus in cell culture media using LC-MS/MS

Sample Extraction

For the calibration standards, an aliquot of 25 uL of each spiking solution is added to 0.50 mL of control blank matrix (cell culture media) in a 13 x 100 mm glass screw-cap culture tube. For the study samples an aliquot of 0.50 mL cell culture media is added to the respective tubes. A 20 uL aliquot of the internal standard (ISTD) working solution (Ascomycin - 1000 ng/mL) is added to each tube and all the samples are vortex-mixed briefly. A volume of 0.50 mL 0.01M ammonium acetate buffer (pH 7.5, adjusted by 1.5% ammonium hydroxide) is added to each sample tube. After a brief mixing, 4 mL of methyl-t-butyl ether is added to extract the desired compounds by mixing for 20 minutes followed by centrifugation for 5 min at 2000 rpm. The aqueous portion is frozen in a freezer at −80 ◦C and the organic portion is transferred to a clean 13 x 100 mm glass culture tube. The organic solvent is evaporated to complete dryness at 37 ◦C under a stream of nitrogen at 15 psi in a Turbo-Vap water bath evaporator. The residue is reconstituted in 0.15 mL of mobile phase. The resulting sample extract is transferred to a glass insert and is placed into an HPLC injection vial and a 7.5uL aliquot of each extracted sample is injected into the LC–MS/MS system.

Chromatographic Separation (Agilent 1100 - High Performance Liquid Chromatography Agilent Inc., Santa Clara, CA) conditions

Chromatographic separation is achieved with an isocratic elution (A: 20, B: 80) using an Acquity UPLC BEH C18, 1.7 um column (2.1 x 50 mm) with mobile phase A: 2 mmole/L ammonium acetate, 0.1% formic acid in DI water and mobile phase B: 2 mmole/L ammonium acetate, 0.1% formic acid in methanol. The flow rate is 0.2 mL/min and the column temperature is set to 65 ◦C. Tacrolimus elutes at 1.23 min (k'=1.23) and ascomycin elutes at 1.19 min (k'=1.16) for a total run time of 4 minutes.

Mass Spectrometer (API 4000, Sciex Inc., Redwood City, CA) conditions

Electrospray ionization (ESI) operating in positive ion mode is used to generate ammonium-adduct ions for mass spectrometric detection. Multiple-reaction mode (MRM) is used to acquire ion counts at different time points. The ESI ion spray voltage is 5000 V and the turbo gas temperature is 400 °C. Nitrogen is used for ion source gas (GS1), ion source gas (GS2), curtain gas (CUR), and collision gas (CAD) and set to flows of 50, 20, 10, and 4 psi, respectively. The declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) are optimized at instrument settings 95, 30, 10, and 30V respectively. The transitions (precursor to product) monitored are m/z 821.5→ 768.3 for tacrolimus and 809.2→ 756.3 for the internal standard, ascomycin. The dwell time is 50 msec for each transition and both quadruples are maintained at unit resolution.

Calibration Standards

A total of seven standards are used to establish the calibration range (0.125, 0.25, 0.5, 2, 5, 15 and 50 ng/mL).