# Anti-retroviral therapy is toxic to iPSC-derived NPCs in vitro and alters neuronal calcium transients in differentiated NPCs.

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## Abstract

HIV-positive (HIV+) pregnant women are given combination anti-retroviral therapy (cART) throughout pregnancy for maternal health and prevention of perinatal transmission of HIV. While treatment with cART has improved the life expectancy of many HIV+ individuals, there is growing concern that these drugs are neurotoxic and contribute to the development of HIV-associated neurocognitive disorders (HAND). Furthermore, little is known about the effect of cART on the developing brain. Previous research has linked the HIV integrase inhibitor Dolutegravir to fetal neural tube defects, and there may be other anti-retrovirals (ARVs) that impact fetal neurodevelopment. It is critical to optimize assays to test for ARV effects on neurodevelopment.

We describe a suite of 384-well screening assays that we have developed for this purpose. The first assay tests ARV treatment on human induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (NPCs) for effects on cell viability and self-renewal. Using this assay, we have demonstrated toxicity from Elvitegravir and Dolutegravir alone or in combination with Emtricitabine. Additionally, we investigated Biktarvy, a combination drug consisting of Bictegravir, Tenofovir Alafenamide and Emtricitabine given to HIV+ pregnant women. We found that a 3-day Cmax treatment of Biktarvy was toxic to two separate NPC lines (made in-house and Elixirgen Scientific).These results suggest that cART can affect neural progenitor survival in vitro and may also affect pathways associated with neurogenesis.

The second assay investigates how ARV treatment impacts the differentiation and fate of NPCs testing for changes in calcium activity and neuron to glia cell ratios. Elixirgen NPCs were differentiated in the presence of Biktarvy for 3 days (acute treatment) or for the entirety of the 6-week differentiation (continuous treatment). Using Vala Sciences' IC200 imaging platform, we identified differences in neuronal calcium transients between acute and continuously treated cultures. We also found that both acute and continuous treatment of Biktarvy at Cmax were toxic to the differentiating NPCs as they did not survive the differentiation and maturation protocol.

Finally, our third assay seeks to determine the effect of ARV treatment on matured tricultures containing hiPSC-derived neurons, astrocytes, and microglia. Elixirgen NPCs and iPSC-derived microglia (made in house) were differentiated for 5 weeks and received a 3-day or 7-day treatment of Biktarvy at Cmax. In contrast with what we observed in the NPC differentiation assay, the treatment did not cause alarming toxicity to the morphology and quantity of cells in culture.

## Methods

**Cell culture.** Quick-Neuron™ Precursor - Human iPSC-derived Neural Precursor Cells (Elixirgen Scientific, CW50065) were thawed and grown in NPC medium according to manufacturer's instructions. ACS1023-NPCs were made in-house using dual SMAD inhibitors to drive rapid differentiation of hiPSCs (ACS-1023; ATCC) into NPCs (based on the protocol described in Vessoni et. al, Human Molecular Genetics, 2016, Vol. 25, No. 7).

**Anti-retroviral (ARV) Treatment**. NPCs were seeded in 384-well plates (Greiner Bio-One) at a density of 2000 or 3000 cells per well. On Day 3, NPCs were exposed to the anti-retroviral Biktarvy in 0.2% DMSO, or 0.2% DMSO alone, and maintained until Day 6. Biktarvy is a fixed dose combination of Tenofovir Alafenamide, Emtricitabine and Bictegravir. All ARVs used in this study were purchased from Toronto Research Chemicals.

| Biktarvy ® (Gilead) | | | |
|---|---|---|---|
| Drug Names | 1/10 Cmax | Cmax | 3x Cmax |
| Tenofovir Alafenamide (TAF) | 60 nM | 600 nM | 1.7 µM |
| Emtricitabine (FTC) | 700 nM | 7 µM | 20 µM |
| Bictegravir (BIC) | 1.35 µM | 13.5 µM | 41 µM |

**Click-iT EdU assay**
On Day 6, Click-iT EdU Alexa Fluor 555 assay (Thermo Fisher Scientific) was performed for 2 hours to detect and quantify newly synthesized DNA, according to manufacturer's protocol. Hoechst 33342 were from Thermo Fisher Scientific.

**Click-iT EdU Imaging** IC200 automated HCS Structured Illumination Microscopy (IC200-SIM™), CyteSeer Reader automated microscope control software, and CyteSeer® High Content Image Analysis Software were all from Vala Sciences, Inc. (San Diego, CA, USA). Live cells were obtained by subtracting nuclei with an Average Pixel Intensity (API) above a certain threshold (to exclude dead, bright nuclei) from the total nuclei count.

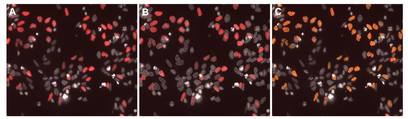
**NPC Differentiation with ARV Treatment**
Human iPSC-derived Neural Precursor Cells (Elixirgen Scientific) were seeded in T25 flasks. Differentiation was started when the cells reached 80% confluency in the flask (D0). Differentiation media is based on Vadodaria et al., Molecular Psychiatry, 2019. The NPCs were differentiated in flasks for 2 weeks before they were lifted and replated into 384 well plates. Cells were maintained in differentiation media until they were fixed or assayed for calcium Transients.

**Immunofluorescence of Differentiated NPCs**
3 weeks post-differentiation the mixed neuron/glial cultures were fixed with 2% PFA and 1.7% sucrose, permeabilized with 0.3% Triton-X, blocked with 2% BSA and labeled with antibodies for neurons (MAP2) and glial cells (GFAP). The nuclei were stained with Hoechst.
MAP2 antibody (Synaptic Systems), GFAP antibody (Cell Signaling), Hoechst and Alexa Fluor-labeled secondary antibodies (Thermo Fisher Scientific).

**Calcium Imaging on Differentiated NPCs**
At 3- or 6-weeks post-differentiation, the cells were loaded with Calbryte 590 (AAT Bioquest) and Hoechst (Thermo Fisher) in Phenol Free Brainphys Imaging Media. Images were acquired at 4 Hz, 120 seconds using Vala Sciences kinetic image cytometer (IC-200-KIC®) with a 20X/0.75 objective.

## Image analysis

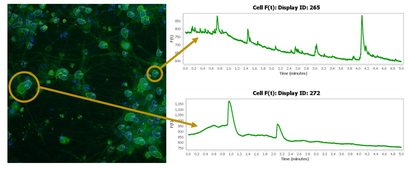
### NPC Click-iT Edu DNA replication assay




**Nucleus and Click-iT masks generated by CyteSeer®.** Vala Sciences' automated image analysis software CyteSeer® identified, segmented, and indexed each cell (based on nuclei staining). **A)** hiPSC-NPCs were exposed to Click-iT EdU for 2 hours. Nuclei were stained with Hoechst (gray). EdU incorporated into DNA is visualized with Alexa Fluor555 (red). **B)** Nuclear masks (white outlines) derived from the image in (A) by CyteSeer®. **C)** EdU positive cells (yellow masks) identified by CyteSeer®.

## Image analysis

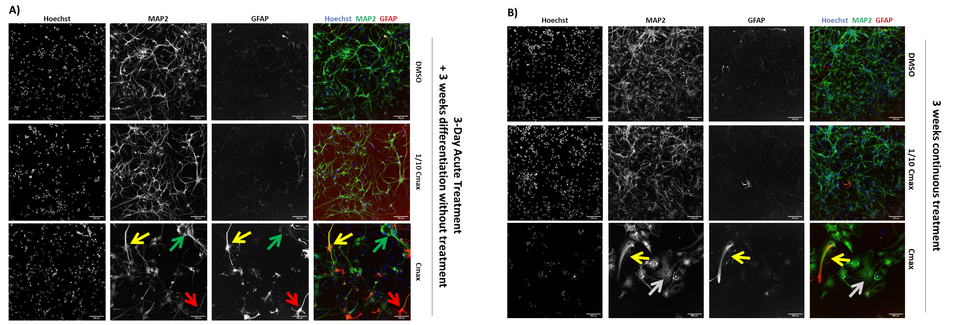
### Neuron calcium transient assay




**Neuron soma masks and calcium traces generated by CyteSeer®.** Vala Sciences' automated image analysis software CyteSeer® identified, segmented, and indexed each neuron based on nucleus and calcium staining. **A)** hiPSC-neurons loaded with Calbryte 590 (green) and Hoechst (blue). Neuron soma masks generated by Cyteseer® are outlined in white. **B)** CyteSeer®-derived functions of Calbryte 590 calcium fluorescence over time from single neurons.

## Results

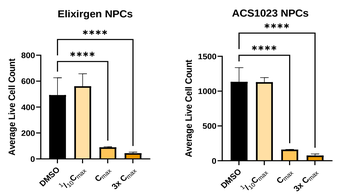
### 3 Days Acute or Continuous treatment with Cmax dose of BIKTARVY change the fate of differentiating NPCs




**Treatment with Cmax of Biktarvy impairs NPC differentiation into MAP2-positive neurons and GFAP-positive astrocytes.**
**A) Acute treatment:** Elixirgen NPCs treated with DMSO or with Biktarvy at 1/10 Cmax or Cmax for 3 days, followed by 3 weeks of differentiation without treatment. Treatment with DMSO or with 1/10 Cmax produced a majority of MAP2-positive neuron-like cells (green) and small numbers of GFAP-positive astrocyte-like cells (red). Treatment with Biktarvy at Cmax resulted in differentiated cells with mixed lineages: cells that predominantly express MAP2 (green arrows), cells that predominantly express GFAP (green arrows), and cells that express MAP2 and GFAP at similar levels (yellow arrows). GFAP-positive cells are relatively more common in Cmax-treated wells. Cmax-treated MAP2-positive cells have fewer and thicker neurites with less branching than in the DMSO controls. **B) Continuous treatment:** Elixirgen NPCs treated with DMSO or with Biktarvy at 1/10 Cmax or Cmax throughout 3 weeks of differentiation. Cmax Biktarvy produces MAP2-positive cells with enlarged cell bodies, amoeboid morphology (grey arrows), and very few neurites. In addition, it produced cells that express both MAP2 and GFAP (yellow arrows).

## Results

### BIKTARVY reduces live cell count and changes EdU incorporation rate in two different NPC lines



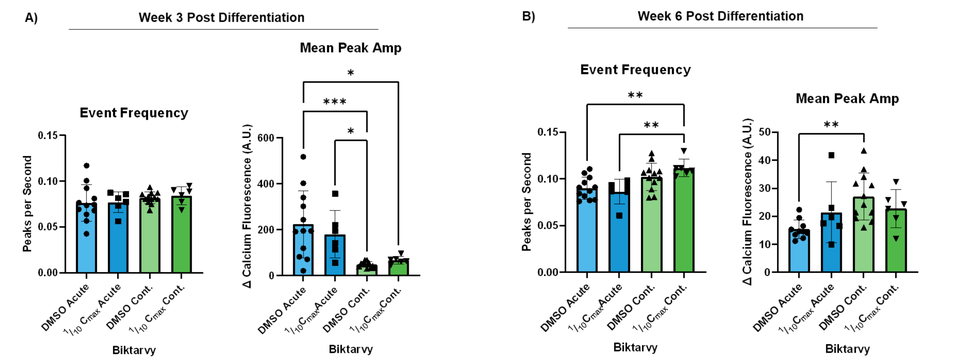

**A) Biktarvy treatment reduces live cell count in NPCs.** Three-day exposure with either Cmax or 3x Cmax dose of Biktarvy significantly reduced live cell count per well compared to DMSO control, in both lines.

**B) Biktarvy treatment changes proliferation rate in NPCs.** Three-day exposure with Cmax dose of Biktarvy significantly decreased the percent of NPCs replicating DNA and incorporating Click-iT EdU compared to DMSO controls, in both NPC lines. However, 3x Cmax dose of Biktarvy increased the Click-iT incorporation into DNA for both cell lines.

Each bar represents the mean ± SD for n=5 wells (n=27 for DMSO control). All statistical analysis were performed in Prism 9.
*P<0.05; ****P<0.0001 for difference from control by Dunnett's procedure.

## Results

### BIKTARVY alters calcium transient peak in neurons differentiated from NPCs.




**A)** At 3-weeks post-differentiation, neither Acute nor Continuous Biktarvy treatment of differentiating NPCs altered the Calcium Event Frequency. In the Continuous Biktarvy Treatment, Calcium Mean Peak Amplitude was reduced for both DMSO controls and Biktarvy-treated cells. **B)** At 6-weeks post differentiation, the calcium Event Frequency was unaltered compared to 3 weeks, while the calcium Mean Peak Amplitude was very low across the treatments and controls.

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