Figures and figure supplements

Mucosal effects of tenofovir 1% gel

Florian Hladik, et al.
Figure 1. Tenofovir-induced gene expression changes in the human rectum. (A) Heat map of differentially expressed genes in eight participants after a single (I) and after seven consecutive once-daily (VII) rectal applications of tenofovir 1% gel compared to baseline in biopsies taken at 9 cm and 15 cm proximal to the anal margin. Red and green bars signify strength of gene induction and suppression, respectively. 642 genes are shown, all of which exhibited an estimated FDR $\leq 0.05$ and a log$_2$ fold expression change of $\geq 0.5$ (induction) or $\leq -0.5$ (suppression) when evaluated jointly for all eight participants at time point VII in the 9 cm biopsies. (B) Numbers of significantly suppressed (green borders and numbers) and induced (red borders and numbers) genes after reduced glycerin tenofovir 1% gel (TFV) treatment and their overlap with nonoxynol-9 (N-9), hydroxyethyl cellulose (HEC) and no treatment (No tx). Circle area symbolizes the number of affected genes, overlap the number of genes independently affected by two or three conditions. (C) Correlation of log$_2$ fold gene suppression from baseline to Day VII between 9 and 15 cm biopsies. All 505 genes significantly suppressed at 9 cm are included. Genes depicted as blue dots were significantly suppressed in both 9 and 15 cm biopsies (15 cm FDR $< 0.05$), genes depicted as black dots were only significant in 9 cm biopsies (15 cm FDR $\geq 0.05$). Spearman rho correlation between the 9 and 15 cm biopsies expression and the corresponding p-value of a Spearman rank correlation test are indicated on the plot. Genes tested in Figure 2B by RT-ddPCR are specifically indicated.

DOI: 10.7554/eLife.04525.003
Figure 1—figure supplement 1. Correlation of log2 fold gene induction by tenofovir 1% gel from baseline to Day VII between 9 and 15 cm biopsies. All 137 genes at 9 cm significantly induced (FDR < 0.05) and with log2 fold change >0.5 are included. Genes depicted as blue dots were significantly induced in both 9 and 15 cm biopsies (15 cm FDR < 0.05), genes depicted as black dots were only significant in 9 cm biopsies (15 cm FDR ≥0.05).

DOI: 10.7554/eLife.04525.004
Figure 1—figure supplement 2. Log₂ fold gene suppression (A) and induction (B) from baseline to Day VII caused by N-9 and tenofovir in 9 cm biopsies. (A) All genes with an estimated FDR ≤ 0.05 and a log₂ fold expression change of ≤0.5 are included (N-9: 56 genes; tenofovir: 505 genes). (B) All genes with an estimated FDR ≤ 0.05 and a log₂ fold expression change of ≥0.5 are included (N-9: 60 genes; tenofovir: 137 genes). Differences in magnitude of gene expression changes were statistically compared between N-9 and tenofovir by unpaired Mann–Whitney tests. The box plots indicate medians and interquartile ranges and the whiskers indicate 10th–90th percentiles.

DOI: 10.7554/eLife.04525.005
Figure 2. Confirmation of microarray data. (A and B) Quantification of mRNA copy numbers measured in 9 cm biopsies by reverse transcription (RT) droplet digital PCR (ddPCR) relative to the housekeeping gene hemoglobin beta (HBB) copy numbers in seven additional study participants. (A) Nine selected genes induced in the microarrays: CCL19, CCL21, CCL23, CXCL9, CCR7, CD7, CD19, matrix metalloproteinase 12 (MMP12), and serine protease inhibitor of the Kazal type 4 (SPINK4). Copy numbers at baseline (0), after a single tenofovir gel application (I) and after seven consecutive once-daily applications (VII) are shown. Line colors signify each of the seven study participants. (B) Six selected suppressed genes: p21-activated kinase (PAK2), nuclear factor of activated T cells 5 (NFAT5), desmoplakin (DSP), TGF-β receptor associated protein (TGFBRAP), interleukin 10 (IL-10), and tripartite motif-containing protein 5 (TRIM5). (C) Normalized fold changes of gene expression at Day VII over baseline in all 15 individuals treated with tenofovir 1% gel. Red dots depict fold changes measured by microarray, blue dots depict fold changes measured by RT-ddPCR. The boxes indicate median and 25th–75th percentiles and the whiskers indicate 10th–90th percentile. Asterisks indicate statistical significance level relative to baseline (one asterisk $p < 0.05$; two asterisks $p < 0.01$, by one-sided Wilcoxon tests, adjusted for multiple testing). (D) Immunostaining of formalin-fixed 9 cm rectal biopsies from 10 participants for the proteins CD7 (immunohistochemistry [IHC]), CD3, UBD, and IL-10. The boxes indicate median and 25th–75th percentiles and the whiskers indicate 10th–90th percentiles. Asterisks indicate statistical significance level relative to baseline (one asterisk $p < 0.05$, two asterisks $p < 0.01$, by one-sided Wilcoxon tests, adjusted for multiple testing).
Figure 2. Continued
(immunofluorescence) and ubiquitin D (UBD; IHC), predicted to be induced by the microarrays, and for IL-10 (IHC), predicted to be suppressed. For CD7 and CD3, tissue sections were evaluated in their entirety and positive cells per mm² are shown at baseline (0) and after seven consecutive once-daily applications (VII). Representative images are shown in Figure 2—figure supplement 1. For UBD and IL-10, only columnar epithelial cells were evaluated. For UBD, the average mean staining intensities (MSI) per cell are shown. Representative images are shown in Figure 2—figure supplement 2. Colors signify each of the 10 study participants. The boxes indicate median and 25th–75th percentiles and the whiskers indicate the range. Paired Wilcoxon signed-rank test p values for differences between 0 and VII are listed.
DOI: 10.7554/eLife.04525.006
Figure 2—figure supplement 1. Immunohistochemistry for CD7, and immunofluorescence for CD3, in rectal biopsies before (0) and after 7 days (VII) of daily tenofovir 1% gel use. Three representative study participants each are shown and indicated by letters. Blue indicates nuclei, red indicates CD3 or CD7.

DOI: 10.7554/eLife.04525.007
Figure 2—figure supplement 2. Immunohistochemistry for IL-10 and ubiquitin D (UBD) in rectal biopsies before (0) and after 7 days (VII) of daily tenofovir 1% gel use. Three representative study participants each are shown and indicated by letters. Blue indicates nuclei, brown indicates IL-10 or UBD.

DOI: 10.7554/eLife.04525.008
Figure 3. Expression pattern and functional pathway analysis. (A) Ingenuity pathways analysis of tenofovir-induced effects in rectal biopsies, showing cellular localizations of and relationships between individual gene products. Red symbols indicate induction and green symbols suppression at Day VII relative to baseline in 9 cm biopsies. The diagram includes all significant genes identified as primarily located in the extracellular space and the cell.
nucleus. A few selected significant genes with products localizing to the plasma membrane (PM) or cytoplasm (CP) are also shown based on their putative functional roles. Direct (solid lines) and indirect (dashed) interactions between gene products are indicated. Line color is arbitrary and meant to indicate relationships between groups of genes. Yellow-shaded areas indicate zinc finger transcription factors. (B) Pathways analysis of tenofovir-induced effects in primary vaginal epithelial cells. Only genes that were suppressed or induced by tenofovir both in 9 cm rectum in vivo and in vaginal epithelial cells in vitro are shown. Primary vaginal epithelial cells derived from three healthy women were cultured with 50 or 500 μM tenofovir for 14 days. Global gene expression microarrays at 4, 7, and 14 days of culture were evaluated in comparison to untreated epithelial cells. Pre-processed microarray expression data were extremely consistent between the three vaginal cell cultures (mean Pearson correlation coefficient 0.9912, Figure 3—source data 1).
DOI: 10.7554/eLife.04525.009
Figure 3—figure supplement 1. Average strength of gene induction by tenofovir 1% gel across all 8
Figure 3—figure supplement 1. Continued

microarray study participants. Heat map colors depict fold-change at Day I and Day VII over baseline. Baseline is depicted as the vertical bar labeled ‘0’ filled with the shade of blue corresponding to a fold-change of 1. Genes included in the list exhibited ≥1.6-fold average induction on Day VII or were ≥1.1-fold induced in at least six of eight study participants, and some knowledge about the gene products exists in the literature. The heat map divisions signify three different kinetic patterns of gene induction: flat at day I and then induced; induced at day I and then flat; or induced at day I and then more strongly induced at day VII.

DOI: 10.7554/eLife.04525.011

Figure 3—figure supplement 2. Selected biological processes defined in the InnateDB database with significant enrichment of genes suppressed or induced by tenofovir 1% gel at Day VII in 9 cm biopsies. Green bars depict the percentage of genes identified as suppressed in a particular process out of the total number of genes included by InnateDB in that process. Red bars depict corresponding percentages for gene induction. Numbers of suppressed and induced genes are indicated above the bars. Gene enrichment in each biological process was tested for statistical significance as described in the ‘Materials and methods’ and the computed p values are depicted by the stars. Not all processes with significant gene enrichment are shown.

DOI: 10.7554/eLife.04525.012
Figure 4. Effects of tenofovir on primary vaginal epithelial cells. (A) Number of suppressed (green) and induced (red) genes in response to treatment with 50 μM (open circles) or 500 μM (filled circles) tenofovir for 1, 4, 7, or 14 days (n = 3 cell lines from different women). (B) Quantification of mRNA copy numbers at days 1 and 7 of culture by RT-ddPCR assays for two selected genes identified as suppressed (DSP and IL-10) and one induced (KIAA0101) in the microarray data set (n = 4 cell lines). (C) Quantification of IL-10 protein concentrations in vaginal epithelial cells at days 1 and 7 of culture by ELISA. Mean (±standard deviation) IL-10 concentrations in the untreated cultures were 5.65 pg/ml (±0.25) at day 1 and 5.86 pg/ml (±0.32) at day 7 of culture. Boxes and error bars in (B) and (C) signify means and standard deviations with vaginal epithelial cell cultures derived from four healthy women. Asterisks indicate statistical significance level relative to untreated (*p < 0.05; **p < 0.01; ***p < 0.001). (D) Selected biological processes defined in the InnateDB and DAVID databases with significant enrichment of genes suppressed or induced by 50 μM tenofovir in vaginal epithelial cells after 7 days of culture. Green bars depict the percentage of genes identified as suppressed in a particular process out of the total number of genes included in that process. Red bars depict gene induction. Numbers of suppressed and induced genes are indicated above the bars. Gene enrichment in each biological process was tested for statistical significance as described in the ‘Materials and methods’ and the computed p values are depicted by the stars. Not all processes with significant gene enrichment are shown. (E) Proliferation of vaginal epithelial cells without or with various concentrations of tenofovir (n = 3 cell lines). Boxes depict mean percent reduction of the alamarBlue reagent in comparison to the maximum reduction. Error bars signify standard deviations.

DOI: 10.7554/eLife.04525.013
Figure 5. Quantification of mitochondria-associated parameters. (A) PNPT1 mRNA copy numbers measured in 9 and 15 cm biopsies at baseline (0), after a single tenofovir gel application (I) and after seven consecutive once-daily applications (VII) by RT-ddPCR assay. (B) Mitochondrial ATP6 mRNA copy numbers measured in 9 cm biopsies after tenofovir or N-9 treatment. Line colors in (A) and (B) signify the 15 participants in the tenofovir arm. Black lines signify the 15 participants in the N-9 arm. Baseline values were compared between 9 and 15 cm biopsies by paired t-test and between tenofovir and N-9 by unpaired t-test. Expression changes over time were tested for statistical significance by ANOVA with Bonferroni adjusted post-tests. (C) Assessment of mitochondrial density by electron microscopy of 9 cm biopsies in two study participants. Each dot indicates the mean number of mitochondria per μm² in a separate 2000 x image. (D) Assessment of mitochondrial sizes by electron microscopy in the same biopsies. Each dot depicts the size in μm² of an individual mitochondrion measured at 5000x. Dot colors in (C) and (D) correspond to the line colors of the same two study participants in (A) and (B). Density and size changes were tested for statistical significance by unpaired t-tests. Horizontal lines and error bars depict means and standard deviations. (E) Representative electron microscopy images of normal mitochondria at baseline, and of enlarged and dysmorphic mitochondria at time point VII, in 9 cm biopsies of Subject Y. Fine structural detail is limited due to formalin fixation of biopsies. (F) PNPT1 and ATP6 gene expression in vaginal epithelial cell cultures in response to 1 and 7 days of 50 μM (blue boxes) or 500 μM (green boxes) tenofovir exposure in vitro. Boxes and error bars signify means and standard deviations across four independent experiments with epithelial cell cultures derived from the vaginal mucosa of four healthy women. Statistical significance levels in all figure panels are indicated by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; 'ns', not significant). DOI: 10.7554/eLife.04525.014
Figure 6. Unbiased mass spectrometry proteomics in rectal secretions from five study participants each in the tenofovir and the no treatment arm. (A) 483 proteins were consistently detected in all 10 study participants. Of these, 382 proteins in the tenofovir arm had log$_2$ values $>0$ for fold change between baseline and after seven daily gel applications, and 112 had log$_2$ fold change values $>0$ in the no treatment arm. Log$_2$ fold changes (x axis) and p values signifying the likelihood of change (y axis) for these proteins are shown. Upper panel, tenofovir arm (382 proteins); lower panel, no treatment arm (112 proteins). Data for proteins with log$_2$ fold values $\leq 0$ were not interpretable and are not shown. (B) Log$_2$ fold changes of the top 100 proteins upregulated between baseline and after 7 days of daily gel application in each of the five study participants in the tenofovir arm. (C) Selected biofunctional processes defined in the Ingenuity database with significant enrichment of proteins induced in rectal secretions by 7 days of daily tenofovir 1% gel use. The red bars with arrow heads depict the z scores for these biofunctions, indicating the strength of the directionality of the effect. Protein enrichment in each biofunction was tested for statistical significance as described in the ‘Materials and methods’ and the computed p values are depicted by the stars. Numbers of induced proteins are indicated above/below the bars.

DOI: 10.7554/eLife.04525.015