



Original Article

HIV-Tat induces a decrease in I_{Kr} and I_{Ks} via reduction in phosphatidylinositol-(4,5)-bisphosphate availability



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ABSTRACT

Patients with HIV present with a higher prevalence of QT prolongation, of which molecular bases are still not clear. Among HIV proteins, Tat serves as a transactivator that stimulates viral genes expression and is required for efficient HIV replication. Tat is actively secreted into the blood by infected T-cells and affects organs such as the heart. Tat has been shown to alter cardiac repolarization in animal models but how this is mediated and whether this is also the case in human cells is unknown. In the present study, we show that Tat transfection in heterologous expression systems led to a decrease in hERG (underlying cardiac I_{Kr}) and human KCNE1-KCNQ1 (underlying cardiac I_{Ks}) currents and to an acceleration of their deactivation. This is consistent with a decrease in available phosphatidylinositol-(4,5)-bisphosphate (PIP₂). A mutant Tat, unable to bind PIP₂, did not reproduce the observed effects. In addition, WT-Tat had no effect on a mutant KCNQ1 which is PIP₂-insensitive, further confirming the hypothesis. Twenty-four-hour incubation of human induced pluripotent stem cells-derived cardiomyocytes with Wild-type Tat reduced I_{Kr} and accelerated its deactivation. Concordantly, this Tat incubation led to a prolongation of the action potential (AP) duration. Events of AP alternans were also recorded in the presence of Tat, and were exacerbated at a low pacing cycle length. Altogether, these data obtained on human K⁺ channels both in heterologous expression systems and in human cardiomyocytes suggest that Tat sequesters PIP₂, leading to a reduction of I_{Kr} and I_{Ks} , and provide a molecular mechanism for QT prolongation in HIV-infected patients.

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1. Introduction

As a consequence of the development of antiretroviral therapies (ART), life expectancy of HIV-positive individuals has greatly increased. For this population, excess mortality is moderate in industrialized countries and comparable with populations having other chronic conditions [1]. Life expectancy of HIV-positive individuals has also increased in middle income countries, gaining for instance 10 years between 2004 and 2011 in South Africa [2]. Worldwide, the increasing access of patients to ART, from 31% in 2008 to 36% in 2014 leads to further increase in aging HIV-positive individuals [3,4]. This aging population faces additional pathologies that are over-represented as compared to the general

population. This is pointed out by a study on sudden cardiac death (SCD) in HIV positive individuals, showing a mean SCD rate 4.5 fold higher than in the general population [5]. Furthermore, up to 20% of the HIV patients are presenting with a long QT syndrome (LQTS), a greater prevalence than in the general population; prolonged QT interval is a risk factor for malignant arrhythmia [5–8]. However, the mechanistic link between LQTS and HIV remains unidentified.

Many drugs and combinations of drugs may be potential triggers of LQTS through inhibition of a major cardiac repolarizing current, I_{Kr} , conducted by the voltage-gated potassium channel hERG [9]. Among them, numerous drugs currently prescribed to HIV-positive individuals have been associated with a QT prolongation, such as macrolides, pentamidine, azole antifungals, fluoroquinolones and methadone [10,11]. On the other hand, QT prolongation on ECGs of HIV-positive individuals is correlated to the duration of HIV infection rather than the anti-HIV treatment [12]. Moreover, QT prolongation is observed in HIV-positive individuals under no medication recognized to prolong QT, suggesting

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a role for the virus itself in inducing LQTS [13]. In addition, several studies using animal models suggest a rather direct implication of HIV in LQTS. For instance, prolonged cardiac repolarization and decreased repolarizing potassium currents were observed in mice expressing the whole HIV-1 genome [14,15].

Among the ~15 proteins encoded by the virus, Tat appears as a potential candidate for inducing ventricular arrhythmias, as it prolongs the action potential of guinea-pig ventricular cardiomyocytes through a reduction in the hERG-related current, I_{Kr} [16]. Noteworthy, serum

levels of Tat remain high in treated patients, which could explain the high prevalence in prolonged QT interval despite antiretroviral therapies [12,17]. Among the documented properties of Tat in cells, it was shown that its affinity for phosphatidylinositol-(4,5)-bisphosphate (PIP_2) is very high, the dissociation constant (K_d) being in the low nanomolar range [18]. QT prolongation is majorly associated with the inhibition of two major cardiac delayed rectifier potassium currents: I_{Ks} and I_{Kr} , respectively carried by KCNQ1 channels associated with the regulatory subunit KCNE1 and hERG channels [19]. Interestingly

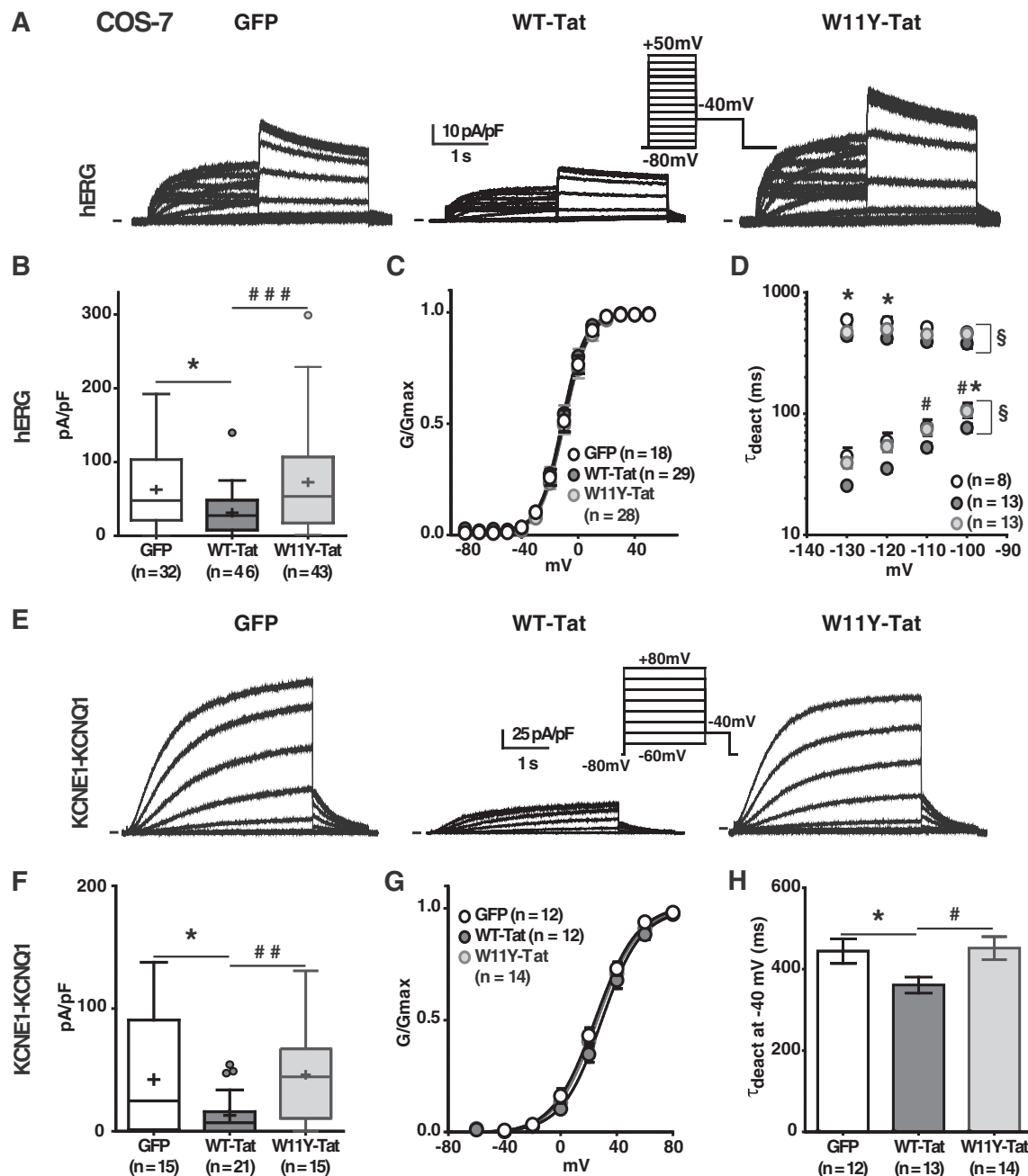


Fig. 1. Tat transfection alters hERG and KCNE1-KCNQ1 currents in COS-7 cells. (A) Representative superimposed current recordings in COS-7 cells co-transfected with hERG, and either GFP, WT-Tat/GFP or W11Y-Tat/GFP, a mutant with reduced PIP_2 affinity. (B) Tukey boxplots presenting hERG maximal tail current densities; (C) activation curves and (D) time constants of deactivation (fast and slow) *versus* voltage in the same conditions as in A. The fast component represents 80 to 90% of the deactivating current, depending on the potential, with no effect of Tat. (E) Representative current recordings in COS-7 cells co-transfected with KCNE1-KCNQ1, and either GFP, WT-Tat/GFP or W11Y-Tat/GFP. (F) Tukey boxplots presenting KCNE1-KCNQ1 maximal tail current densities; (G) activation curve and (H) time constants of deactivation at -40 mV in the same conditions as in E. Current densities differences were statistically evaluated using a Mann-Whitney test, hERG τ_{deact} using a two-way ANOVA for repeated measurements, and KCNE1-KCNQ1 τ_{deact} at -40 mV using Student *t*-test. * $p < 0.05$; for WT-Tat vs GFP. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ for W11Y-Tat vs WT-Tat. § $p < 0.05$.

Table 1

Tat transfection alters hERG and KCNE1-KCNQ1 currents in COS-7 cells: biophysical characteristics.

Tat transfected	COS-7 hERG			COS-7 KCNE1-KCNQ1		
	GFP	WT-Tat	W11Y-Tat	GFP	WT-Tat	W11Y-Tat
Current density	(32)	(46)	(43)	(15)	(21)	(15)
At 60 mV (pA/pF)	62.6 ± 9.5	31.2 ± 3.9*	72.6 ± 10.4###	42.2 ± 13.1	13.0 ± 3.9*	46 ± 10.3##
Activation	(18)	(29)	(28)	(12)	(12)	(14)
V _{1/2} (mV)	−10.2 ± 1.7	−11.1 ± 1.5	−8.98 ± 1.6	24.6 ± 2.3	29.9 ± 2.3	26.6 ± 1.9
K (mV)	7.6 ± 0.3	6.8 ± 0.1**	7.1 ± 0.1	13.4 ± 0.7	13.5 ± 0.6	13.9 ± 0.4

(n): Number of cells; Student *t*-test, *: $p < 0.05$, **: $p < 0.01$ vs GFP; ##: $p < 0.01$; ###: $p < 0.001$ vs WT-Tat; V_{1/2} and K: voltage for half-activation or -inactivation of the K⁺ currents and slope.

enough, both KCNQ1 and hERG are positively regulated by PIP₂ [20,21]. We thus hypothesized that Tat inhibits these channels by sequestering membrane PIP₂, leading to long QT syndrome in HIV patients.

In order to test this hypothesis, we evaluated the effect of Tat on cells expressing KCNQ1 or hERG channels. Tat over-expression led to a decrease in hERG and KCNE1-KCNQ1 currents and an acceleration of their deactivation. A mutant Tat with a profound decrease in PIP₂ affinity had no effect on these currents and WT-Tat had no effect on a PIP₂ insensitive mutant KCNQ1 channel, consistent with Tat acting through a reduction in available PIP₂. In human cardiomyocytes derived from induced pluripotent stem cells, 24-hour Tat incubation led to a decrease in cardiac I_{Kr} and acceleration of its deactivation, and these effects were associated with a prolongation of the action potential duration. Altogether, these observations suggest that in HIV-positive individuals, Tat sequesters PIP₂, leading to a decrease in I_{Kr} and I_{Ks} currents, providing a basis for QT prolongation.

2. Materials and methods

2.1. Tat protein preparation

Recombinant Tat was purified from *Escherichia coli* as previously described, resuspended as a 200 µg/ml stock solution in citrate buffer (110 mM sodium chloride, 30 mM sodium citrate, 20 mM sodium phosphate, pH 7.3), and stored at −80 °C. BCA protein assay (Pierce) was used to measure Tat protein concentration [22]. Tat transactivation activity was confirmed as previously described [23]. Briefly, COS-7 cells were cultured in 24-well plates and cotransfected using FuGENE 6 (Promega) with 0.22 µg/well pGL3-LTR, encoding firefly luciferase under the control of Tat-activated HIV-1-LTR promoter, and 1.78 µg/well pRL-TK (Promega), encoding Renilla luciferase under the control of Herpes simplex virus thymidine kinase promoter, to normalize the results. After 24 h, 2.3 µg/ml recombinant Tat (or the citrate buffer)

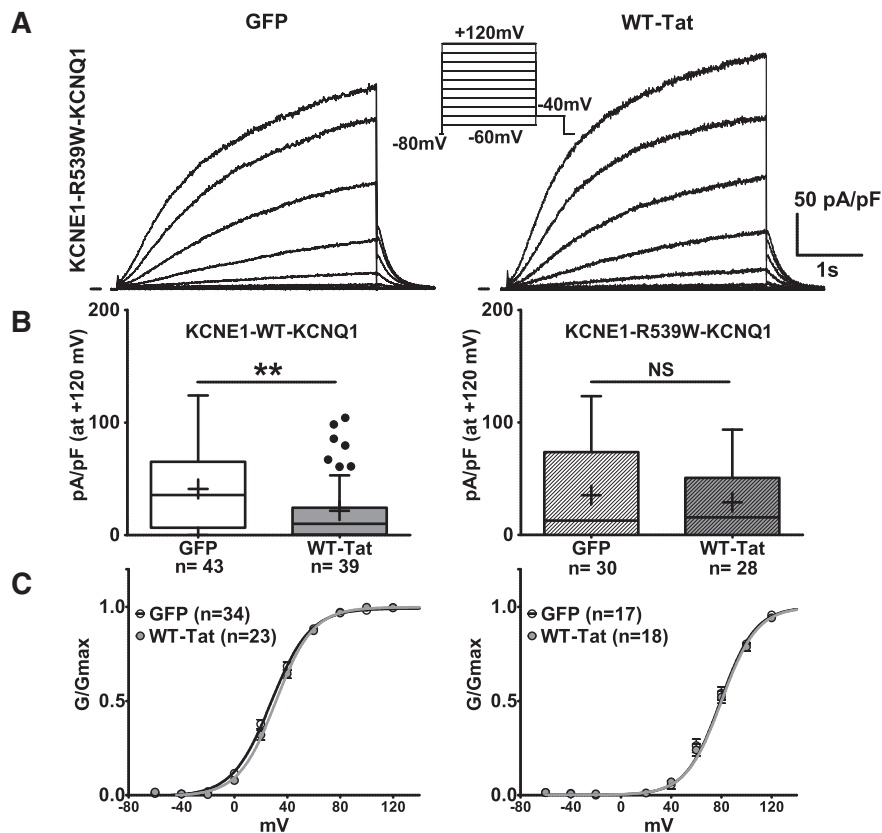


Fig. 2. Tat transfection does not alter the PIP₂ insensitive mutant KCNE1-R539W-KCNQ1 currents in COS-7 cells. (A) Representative superimposed current recordings in COS-7 cells co-transfected with KCNE1-R539W-KCNQ1, and either GFP (left) or WT-Tat/GFP (right). (B) Tukey boxplots presenting WT-Tat effect on WT (left) or KCNE1-R539W-KCNQ1 (right) maximal tail current densities, using the same stimulation protocol as in A (depolarization up to +120 mV); (C) activation curve in the same conditions as in A. Current densities differences were statistically evaluated using a Mann-Whitney test. ** $p < 0.01$.

and 100 μ M chloroquine (for Tat uptake) were added. One day later, cells were lysed for determination of dual firefly/Renilla luciferase activity ratio (Supplemental Fig. 1). Twenty-four hours before patch-clamp measurements and immunofluorescence assays, Tat was diluted and cells were incubated at concentrations of 200 ng/ml (~20 nmol/l) or 400 ng/ml (~40 nmol/l), consistent with patient serum levels [17].

2.2. Cell lines culture and transfection

HEK293 cells stably expressing hERG (HEK-hERG; CreaCell; protein accession number: NP_000229.1) and COS-7 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Eurobio) and antibiotics (100 IU/ml penicillin and 100 μ g/ml streptomycin; Gibco) at 5% CO₂ and 37 °C in a humidified incubator. For HEK-hERG cells, hERG expression was maintained by the application of the selection antibiotic G418 (1200 μ g/ml; Life Technologies).

When reaching 60–80% cell confluence, COS-7 cells were transiently transfected in 35-mm plates with 2 μ g of total DNA (as described below) complexed with 6 μ l of FuGENE 6. For Tat and KCNE1-KCNQ1 co-transfection experiments, the ionic currents were obtained from a fusion protein of the human KCNE1 (protein accession number: AAA58418.1) linked to the N-terminus of the human KCNQ1 (protein accession number: NP_000209.2), a kind gift from Dr. Robert S. Kass (Columbia University, New York, USA). DNA amounts were 200 ng of pCDNA3.1-WT-KCNE1-KCNQ1 or pCDNA3.1-KCNE1-R539W-KCNQ1 + 1.6 μ g of pBI-WT-Tat/GFP or pBI-W11Y-Tat/GFP bidirectional plasmid, or pEGFP-C1 (Clontech) + 200 ng of pUHD-15.1 (required for expression from pBI vector) [18]. For Tat and hERG co-transfection experiments, pSI-hERG (protein accession number: NP_000229.1) was used, a kind gift from Dr. Dan M. Roden (Vanderbilt University School of Medicine, Nashville, TN). DNA amounts were 600 ng of pSI-hERG + 1.2 μ g of pBI-WT-Tat/GFP or pBI-W11Y-Tat/GFP bidirectional plasmid, or pEGFP-C1 + 200 ng of pUHD-15.1. For Tat incubation experiments, DNA amounts were 600 ng of pSI-hERG + 1.4 μ g of pEGFP-C1 for experiments with hERG and 200 ng of pCDNA3.1-KCNE1-KCNQ1 + 1.8 μ g of pEGFP-C1 for experiments with KCNE1-KCNQ1. Cells were isolated, using trypsin, 24 h after transfection (of ion channels cDNA) for Tat incubation experiments or 48 h after transfection for Tat transfection experiments, and cultured in 35 mm plastic Petri dishes. In the latter case, medium was renewed 8 h before cells were split. For Tat incubation experiments, Tat or citrate buffer diluted in culture medium were applied 4–6 h after cells splitting and treated cells were incubated at 37 °C. All experiments were performed the day after cell splitting.

2.3. Culture of human induced pluripotent stem cells

The previously characterized foreskin fibroblast-derived human induced pluripotent stem (hiPS) cell clone C2a was used [24]. The hiPS cell line was maintained on mitotically-inactivated MEFs in DMEM/F12 medium supplemented with 2 mM L-glutamine, 0.07% β -mercaptoethanol, 20% knockout serum replacement, 5 ng/ml bFGF and 1% NEAA under low oxygen atmosphere (4% O₂). Cells were passaged by manual dissection of cell clusters every 6 to 7 days. Before cardiac differentiation, hiPS cells were manually transferred from MEFs to hESC-qualified matrigel-coated plates (0.05 mg/ml, BD Biosciences) and cultured in StemMACS (iPS-Brew XF) medium (Miltenyi Biotec). Passages were performed using Gentle Cell Dissociation Buffer (StemCell Technologies).

2.4. Differentiation of hiPS cells into cardiomyocytes and dissociation

Human iPS cells were differentiated into cardiomyocytes using the Matrix sandwich method, as previously described [25,26]. Cardiomyocytes derived from hiPS cells (hiPS-CMs) were dissociated around day 20 of differentiation, for 20 min in collagenase II (200 U/

ml; Gibco) at 37 °C. Isolated cells were then incubated at room temperature for 30 min in Kraft-Bruhe solution containing (in mmol/l): KCl 85, K₂HPO₄ 30, MgSO₄ 5, EGTA 1, Na₂-ATP 2, Na-pyruvate 5, creatine 5, taurine 20, and glucose 20, pH 7.2 [27]. For Tat immunofluorescence studies, dissociated hiPS-CMs were plated on IBIDI plates (Biovalley). Beating hiPS-CMs were cultured in RPMI1640 medium supplemented with B27 complete (Life Technologies) and 1% NEAA, changed every other day and used between day 10 and day 14 post-dissociation.

2.5. Single-cell electrophysiology in COS-7 and HEK-hERG cells

In HEK-hERG and transfected COS-7 cells, currents were recorded at room temperature using the patch-clamp technique. The cells were continuously superfused with a HEPES buffered Tyrode solution containing (in mmol/l): NaCl 145, KCl 4, MgCl₂ 1, CaCl₂ 1, HEPES 5, glucose 5, pH adjusted to 7.4 with NaOH. For K⁺ current recordings, pipettes (Kimble Chase; tip resistance: 1.8 to 2.5 M Ω) were filled with an intracellular medium containing (in mmol/l): KCl 100, Kgluconate 45, MgCl₂ 1, EGTA 5, HEPES 10, pH adjusted to 7.2 with KOH. All products were

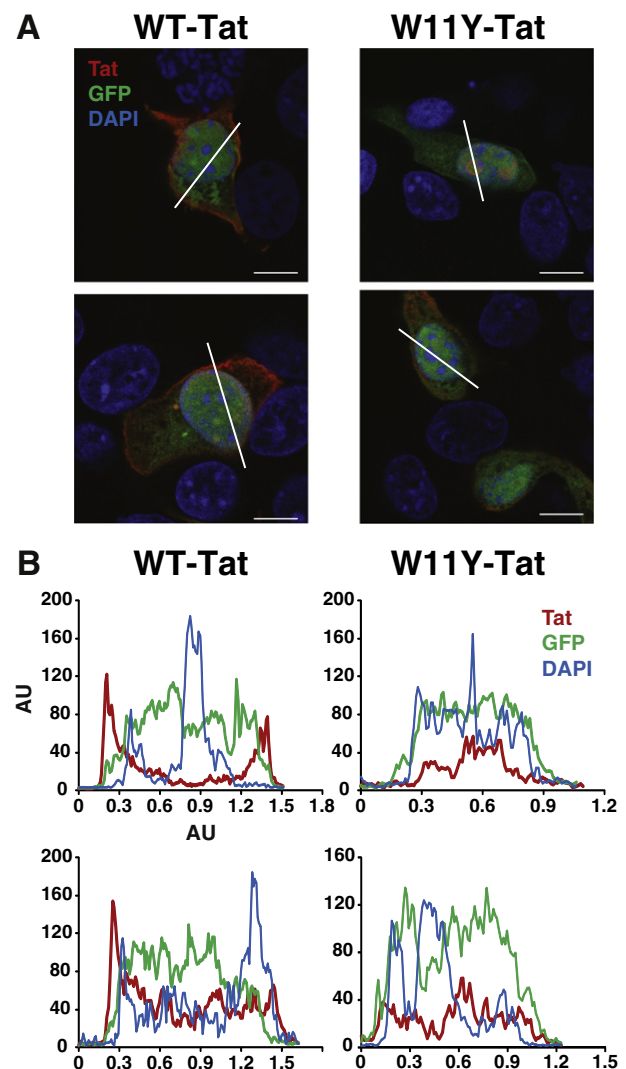


Fig. 3. WT-Tat but not W11Y-Tat localizes at the plasma membrane in COS-7 cells. (A) Representative confocal immunostainings of, in red, WT-Tat or W11Y-Tat in transfected COS-7 cells. GFP is used as a transfection reporter. Nuclei are stained with DAPI. Scale = 10 μ m. (B) Surface plots of Tat, GFP and DAPI fluorescence intensities in each cell at the level of the drawn lines in A. Higher WT-Tat densities are observed in the region of the plasma membrane, as opposed to W11Y-Tat.

purchased from Sigma. Stimulation and data recording were performed with Axon pClamp 10 through an A/D converter (Digidata 1440A) using an Axopatch 200B (all Molecular Devices). hERG current (I_{hERG}) density was measured using a depolarization from -80 mV to $+60$ mV for 2 s and repolarization to -40 mV for 2 s where the tail current was measured (stimulation frequency: 0.125 Hz). Activation curve was obtained by a series of 2-s depolarizations from -80 to $+50$ mV (same holding potential and frequency as above, increment: 10 mV) followed by a 2-s repolarization to -40 mV. Inactivation was studied with a three-pulse protocol: a 1-s depolarization from -80 mV to $+40$ mV was followed

by a 15-ms repolarization to potentials ranging from $+30$ to -130 mV and a 500-ms depolarization to $+40$ mV where the remaining current was measured (0.2 Hz). Kinetics of deactivation were evaluated by a two-exponential regression of the decaying current recorded at various potentials from -70 to -130 mV (3-s duration), after a 2-s depolarization from -80 mV to $+40$ mV (0.125 Hz). The same protocol was used to evaluate the recovery from inactivation kinetics by fitting the rising phase of the current, before deactivation, with a single exponential function. Finally, inactivation kinetics were studied with another three-pulse protocol: a 1-s depolarization from -80 mV to $+60$ mV

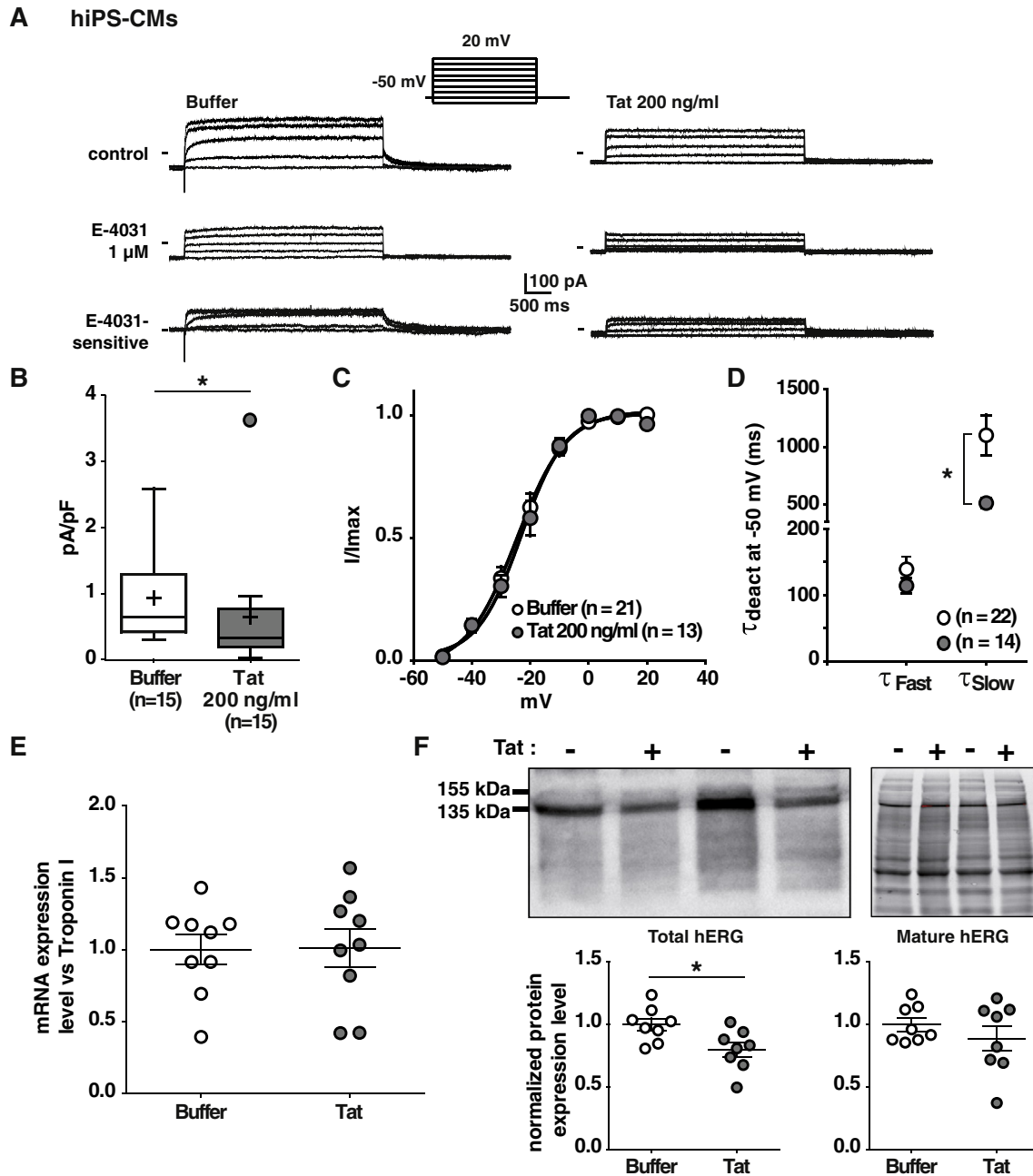


Fig. 4. Tat reduces I_{K_r} recorded from hiPS-CMs. (A) Representative superimposed currents recorded from hiPS-CMs incubated with buffer (left) or 200 ng/ml WT-Tat (Tat; right), before (control) and after 1 μ M E-4031 application. The E-4031-sensitive current, I_{K_r} , was obtained by digital subtraction of the current recorded after E-4031 application to the one recorded before application (inset: voltage-clamp protocol). (B) Tukey boxplots presenting I_{K_r} tail current density measured at -50 mV after depolarization at $+60$ mV. Mann-Whitney test: $*p < 0.05$. (C) I_{K_r} activation curves measured from the tail currents. (D) Averaged time constants of fast and slow deactivation upon repolarization at -50 mV. Student t -test: $*p < 0.05$. The fast component represents around 50% of the deactivating current in both conditions. (E) hERG mRNA expression level versus Troponin I mRNA. (F) Top left: Western blot analysis of hERG protein expression in hiPS-CMs. Two hERG specific bands were revealed (mature, glycosylated membrane subunit, 155 kDa, and immature, non-glycosylated cytoplasmic subunit, 135 kDa). Top right: stain-free expression served as internal control of gel loading. Bottom: normalized intensity quantification of the total and mature hERG bands in hiPS-CMs. Student t -test: $*p < 0.05$.

was followed by a 25-ms repolarization to -110 mV and 500-ms-depolarizations to potentials ranging from $+60$ to -60 mV where the inactivating current was fitted with a single exponential function (0.2 Hz). KCNE1-KCNQ1 current density was measured using depolarizations from a holding potential of -80 mV to various potentials from -60 mV to $+80$ mV for 4 s (or from -60 mV to $+120$ mV when comparing WT-Tat effect on WT KCNE1-KCNQ1 and KCNE1-R539W-KCNQ1) and repolarization to -40 mV for 1.2 s where the tail current was measured (increment: 20 mV, stimulation frequency: 0.125 Hz). Fit of the non-normalized activation curves was used to estimate full-activated KCNE1-KCNQ1 current density for each cell.

2.6. Action potential, I_{K_r} , and $I_{Ca,L}$ recordings in cardiomyocytes derived from hiPS cells

Before recording, cells were incubated for 24 h either with citrate buffer or with 200 ng/ml (~ 20 nmol/l) of Tat protein. Cells were continuously superfused with a cardiomyocyte Tyrode solution containing (in mmol/l): NaCl 140, KCl 4, CaCl_2 1, MgCl_2 0.5, glucose 10, HEPES 10; pH 7.4 (NaOH) at 35 ± 2 °C (room temperature for $I_{Ca,L}$) as previously described [26].

For action potential (AP) recording the permeabilized-patch configuration was used. The pipette (Sutter; tip resistance: 2 to 3 M Ω) solution contained (in mmol/l): K-gluconate 125, KCl 20, NaCl 5, amphotericin B 0.25, HEPES 5; pH 7.2 (KOH). APs were recorded at spontaneous rate or stimulated by 1.3–1.5 ms current pulses of 300–1500 pA at various pacing cycle lengths. APs were classified as nodal-, atrial- and ventricular-like, based on their duration, maximum upstroke velocity (dV/dt_{max}), peak to peak duration, amplitude (mV) and maximum diastolic potential, as previously defined [26].

For currents recording, the ruptured-patch configuration was used.

For I_{K_r} recording, the pipette was filled with a solution containing (in mmol/l): K-gluconate 125, KCl 20, HEPES 10, EGTA 10; pH 7.2 (KOH). A local gravity microperfusion system allowed application of 4 $\mu\text{mol/l}$ nifedipine to block $I_{Ca,L}$ and 1 $\mu\text{mol/l}$ E-4031 to block I_{K_r} , dissolved in cardiomyocyte Tyrode solution plus mannitol 30 mmol/l [28].

I_{K_r} tail current density was measured as the E-4031-sensitive current using a depolarization from -50 mV to $+60$ mV for 4 s and repolarization to -50 mV where the tail current was measured (stimulation frequency: 0.08 Hz). Activation was evaluated by a series of 4-s depolarizations from -60 to $+20$ mV (holding potential: -50 mV, increment: 10 mV, same frequency as above) followed by a repolarization to -50 mV. Deactivating currents were fitted by a standard bi-exponential function.

$I_{Ca,L}$ was recorded at room temperature. Wax-coated pipettes were filled with an intracellular medium containing (in mmol/l): CsCl_2 145, NaCl 5, CaCl_2 2, MgATP 5, EGTA 5, HEPES 10, pH adjusted to 7.2 with CsOH. During recording, the microperfusion system allowed application of an extracellular solution containing (in mmol/l): TEA-Cl 160, CaCl_2 5, MgCl_2 1, glucose 10, HEPES 10, TTX 0.01, mannitol 20; pH 7.4 (CsOH).

$I_{Ca,L}$ I/V curve was evaluated by a series of 200-ms depolarizations from a 3-s pre-pulse to -50 mV to potentials ranging from -60 to $+50$ mV (increment: 5 mV, stimulation frequency: 0.125 Hz) followed by a repolarization to the holding potential at -80 mV. Inactivation was studied with a two-pulse protocol: a 3-s depolarization to potentials ranging from -65 to -5 mV was followed by a 400-ms depolarization to 0 mV where the peak current was measured (holding potential: -50 mV, 0.125 Hz).

2.7. I_{KATP} recordings in mouse neonatal cardiomyocytes

Single cell suspensions from 1 to 2 day-old mouse neonatal hearts were prepared in a semi-automated procedure by using the Neonatal Heart Dissociation kit and the gentleMACS™ Dissociator. Hearts were first excised from the mice then ventricles were separated from the atrium. After gentleMACS™ dissociation, the cell supernatant was removed

Table 2

Tat reduces I_{K_r} recorded from hiPS-CMs: biophysical characteristics.

Tat incubated	iPS-CMs- I_{K_r}	
	Buffer	Tat 200 ng/ml
Current density	(15)	(15)
At 60 mV (pA/pF)	0.93 ± 0.18	$0.64 \pm 0.23^*$
Activation	(21)	(13)
$V_{1/2}$ (mV)	-23.7 ± 1.6	-22.7 ± 1.7
K (mV)	5.7 ± 0.5	5.7 ± 0.4

(n): Number of cells; Student *t*-test, *: $p < 0.05$; $V_{1/2}$ and K: voltage for half-activation of the K^+ currents and slope.

by centrifugation and the pellet was resuspended in culture medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 $\mu\text{g/ml}$), then the cells were plated on 65 mm diameter Petri dish (Nunc™). After 2 h of initial plating, myocytes were resuspended in the same culture medium and plated on 35 mm

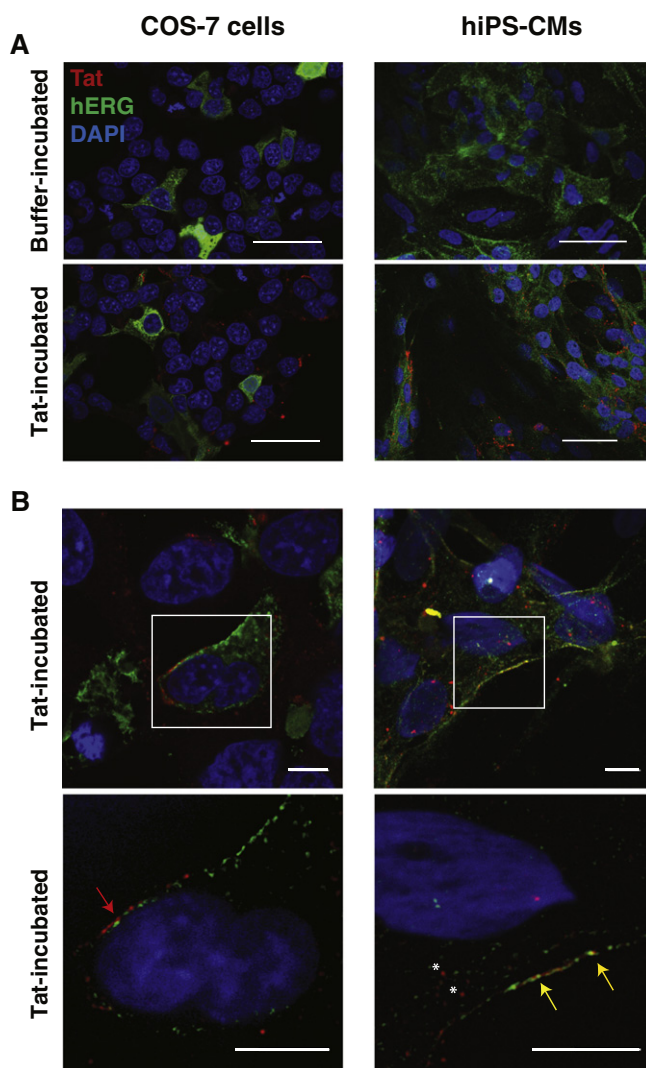


Fig. 5. Tat enters hiPS-CMs but not COS-7 cells. (A) Representative confocal immunostainings of Tat (red) and hERG (green). Left: COS-7 cells expressing hERG. Right: hiPS-CMs. Top: cells incubated with control buffer. Bottom: cells incubated with Tat. Scale = 50 μm . (B) Top: representative confocal immunostainings of Tat-incubated COS-7 cells and hiPS-CMs. Bottom: representative SIM immunostainings, showing that Tat remains in the extracellular compartment of COS-7 cells (red arrow) while in hiPS-CMs, Tat is located inside the cytoplasm (asterisks) and colocalizes with hERG at the plasma membrane (yellow arrows). Scale = 5 μm .

diameter Petri dishes at a density of 6×10^4 cells per plate. Myocytes were maintained in culture for 48 h.

Cells were continuously superfused with a cardiomyocyte Tyrode solution, as above, at 35 ± 2 °C. The pipette was filled with a solution containing (in mmol/l): K-gluconate 85, KCl 44, Na-pyruvate 5, HEPES 10, EGTA 0.001; MgCl_2 3; pH 7.2 (KOH). During recording, a local gravity microperfusion system allowed application of 20 $\mu\text{mol/l}$ glibenclamide, dissolved in an extracellular solution containing (in mmol/l): NaCl 140, KCl 4, CoCl_2 1, MgCl_2 0.5, mannitol 30, HEPES 10; pH 7.4 (NaOH) [28]. From a holding potential of -70 mV, followed by a 20-ms step to -20 mV, current was measured at the end of a 350 ms pulse to $+40$ mV (0.2 Hz). After patch rupture, the outward current increased gradually due to diffusion of cytosolic ATP into the pipette. When the current reached steady state, glibenclamide was added to the external solution to block the I_{KATP} current. A ramp protocol was used to monitor the current decrease upon glibenclamide application. From a holding potential of -70 mV, a 20 ms step to -110 mV was applied followed by a 500 ms ramp from -110 to $+40$ mV (stimulation frequency, 0.2 Hz). A step protocol (same as above) was applied before and after glibenclamide application to measure the glibenclamide-sensitive current.

2.8. Tat immunofluorescence

Two types of histological analyses were performed, (1) one to study localization of transfected Tat in COS-7 cells, (2) another to study localization of incubated Tat in both COS-7 and hiPS-CMs. (1) COS-7 cells were transfected with hERG and either WT- or W11Y-Tat/GFP bidirectional plasmids as for patch-clamp experiments. Forty-eight hours after transfection, cells were plated on IBIDI plates for 24 h. (2) COS-7 cells were transfected with hERG plasmid only, and 48 h after transfection, cells were plated on IBIDI plates. Both COS-7 cells and dissociated hiPS-CMs were then incubated with Tat protein at 200 ng/ml for 24 h.

Cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% saponin and blocked with 1% PBS-BSA. All cells were then stained with mouse monoclonal antibody directed against HIV-1 Tat (SC-65912, Santa Cruz) and cells incubated with Tat were co-stained with

goat polyclonal antibody directed against hERG (SC-15968, Santa Cruz) diluted in PBS. For all immunostainings, secondary antibody staining was performed using respectively Alexa 568 conjugated anti-mouse antibody and Alexa 488 conjugated anti-goat antibody (Molecular Probes). DAPI was used for nuclear staining. For Tat-transfected COS-7 cells, GFP was directly observed. Conventional imaging was performed using Nikon Confocal A1RSi and Super-resolution imaging using N-SIM (Structured Illumination Microscopy) microscope system (Nikon) equipped with a SR Apo 100 \times 1.49 N.A objective.

2.9. mRNA and protein expression analysis

For gene expression analysis, total RNA was extracted and reverse transcribed, from hiPS-CMs and HEK-hERG cells, as previously described [26]. PCR amplification was performed using FAM-labeled TaqMan probes: ACTB (Hs99999903_m1), KCNH2 (Hs04234270-g1) and Troponin I (Hs00165957-m1).

For Western blot analysis, 60 μg of protein lysate from hiPS-CMs or 10 μg of protein lysate from HEK-hERG, both treated for 24 h with 200 ng/ml Tat or buffer were incubated with mouse antisera against hERG protein (Santa Cruz). Secondary antibody staining was performed using goat anti-mouse IgG-HRP antibody (Santa Cruz). Stain Free gel technology (Bio-Rad) was used as loading control for protein normalization: the amount of total proteins in each lane of the blot was calculated and used for normalization [29,30].

2.10. Data analysis

Steady-state activation and inactivation curves were fitted with a single Boltzmann function using GraphPad Prism 5.02. Data are presented as mean \pm SEM when normally distributed or Tukey boxplots (Q1–Q3 boxes) when not. Statistical significance was estimated using Student's *t*-test, Mann-Whitney test, or two-way ANOVA for repeated measures when appropriate. Post-hoc test for multiple comparison (Holm-Sidak method) was performed when needed. A value of $p < 0.05$ was considered as the threshold for statistical significance.

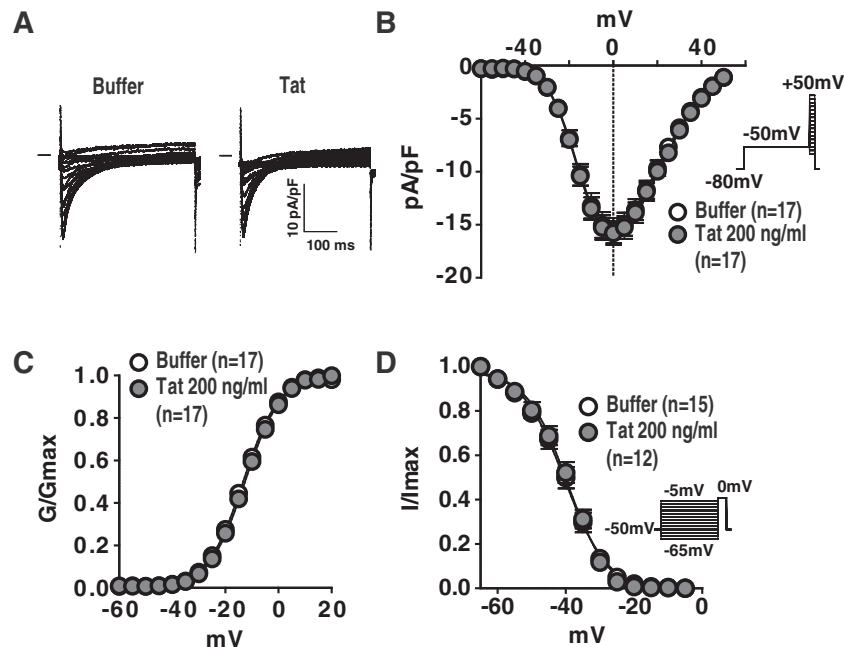


Fig. 6. Tat has no effect on I_{CaL} recorded in hiPS-CMs. (A) Representative superimposed currents recorded from hiPS-CMs incubated with buffer or 200 ng/ml WT-Tat (Tat). (B) Current density-voltage relationship. Inset: protocol used (C) I_{CaL} activation curves obtained from current density-voltage curves. (D) Voltage-dependence of inactivation. Inset: protocol used.

Table 3

Quantitative parameters used for classification of action potentials obtained from patch-clamp experiments on control cells.

hiPS-CM APs	AP amplitude (mV)	MDP (mV)	Peak to peak duration (ms)	dV/dt _{max} (V/s)	APD ₉₀ (ms)	(APD ₃₀ –APD ₄₀)/(APD ₇₀ –APD ₈₀)	APD ₅₀ /APD ₉₀
Nodal-like (n = 4)	63.0 ± 4.4	–41.7 ± 3.7	650.1 ± 147	2.1 ± 0.4	174.4 ± 2.5	2.3 ± 0.2	0.6 ± 0.002
Atrial-like (n = 7)	77.3 ± 2.2	–47.2 ± 1.5	901.2 ± 188.5	4.4 ± 0.5	215.9 ± 22.9	1.0 ± 0.1	0.6 ± 0.001
Ventricular-like (n = 9)	102.6 ± 3.1	–58.6 ± 2.1	1301.2 ± 310.8	10.9 ± 1.2	269.4 ± 30.1	4.9 ± 0.7	0.8 ± 0.02

MDP: Maximum diastolic potential. dV/dt_{max}: maximum upstroke velocity.

3. Results

3.1. Tat transfection leads to a decrease in hERG and KCNE1-KCNQ1 currents and acceleration of deactivation, consistent with a decrease in PIP₂ availability

We first evaluated the effects of Tat co-expression with hERG or KCNQ1 fused to KCNE1 (KCNE1-KCNQ1) in COS-7 cells. This set of experiments allowed us to compare the effects of WT-Tat with those of W11Y-Tat, which presents a profoundly reduced PIP₂ affinity, although having a normal transactivation activity [18]. In contrast with WT-Tat, this mutant cannot enter the cells and thus, testing the effects of its intracellular application requires its transfection. Fig. 1A shows representative hERG current recordings in cells co-transfected with GFP, WT-Tat, or W11Y-Tat. I_{hERG} density was reduced in presence of WT-Tat as compared to GFP. In order to test whether this is due to a reduction in PIP₂ availability, we evaluated the effects of the W11Y-Tat. Consistent with the hypothesis, we did not observe any alteration of the hERG current.

It was shown that a decrease in PIP₂ leads to a destabilization of hERG open state, provoking a reduction in the current amplitude and an acceleration in channel deactivation without any shift of the activation curve to positive potentials [21]. In accordance with a PIP₂-reduction effect, WT-Tat and not W11Y-Tat led to the same changes in current density and deactivation without any shift in the activation curve (Fig. 1B–D, Table 1). These data suggest that Tat inhibits hERG by sequestering PIP₂.

For KCNE1-KCNQ1 also, a decrease in PIP₂ availability leads to a decrease in current density and an acceleration of deactivation [20]. Again, WT-Tat but not W11Y-Tat led to the same effects as when the channel interaction with PIP₂ is altered (Fig. 1E–H, Table 1). To confirm the hypothesis that Tat reduces the current amplitude of delayed rectifier currents through a decrease in available PIP₂, we tested the effect of WT-Tat on the mutant KCNE1-R539W-KCNQ1 channel that is insensitive to PIP₂ depletion, when triggered by wortmannin application or by CiVSP phosphatase activation [31]. We compared current amplitude in cells expressing KCNE1-R539W-KCNQ1 in the absence and presence of WT-Tat (Fig. 2). As opposed to WT KCNE1-KCNQ1, WT-Tat expression did

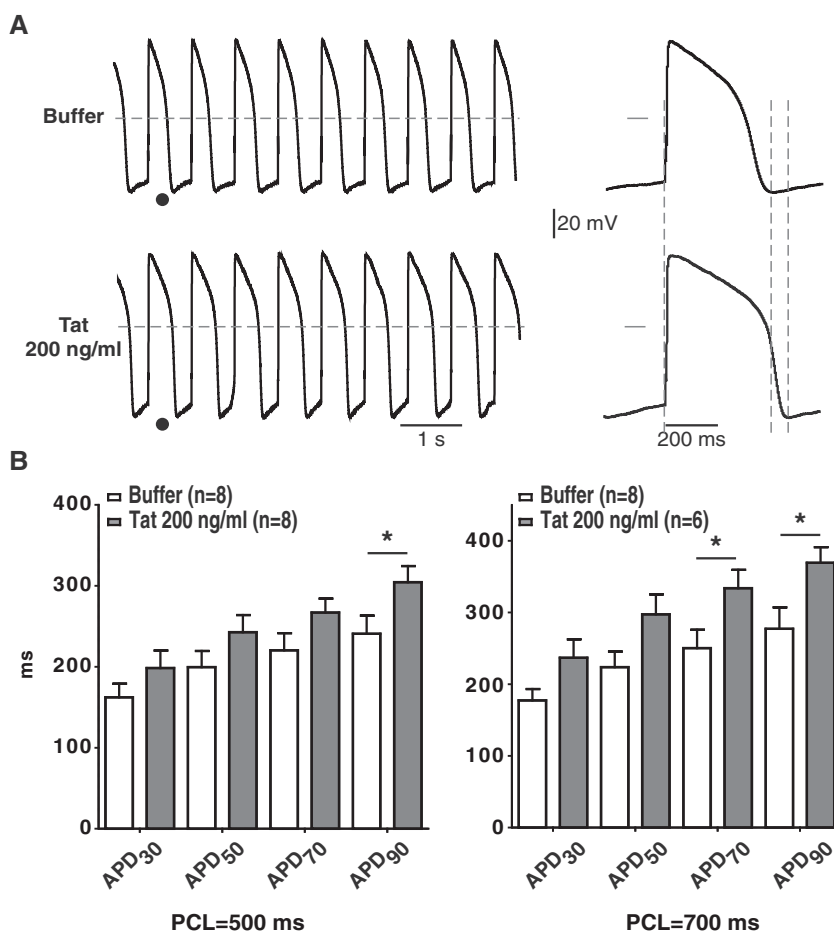


Fig. 7. Tat significantly increases ventricular-like action potential durations in hiPS-CMs. (A) Left, representative ventricular-like APs recorded from hiPS-CMs incubated with buffer or 200 ng/ml WT-Tat (Tat), stimulated at a pacing cycle length (PCL) of 700 ms. Right, expanded APs tagged ● in left panel. Horizontal dashed lines and bars: 0 mV. Vertical dashed lines stress the duration differences. (B) Averaged AP duration at 30 (APD₃₀), 50 (APD₅₀), 70 (APD₇₀) and 90% (APD₉₀) of full repolarization measured in ventricular-like cells incubated with buffer or WT-Tat at 2 different PCLs. *: $p < 0.05$ (Holm-Sidak post-hoc test).

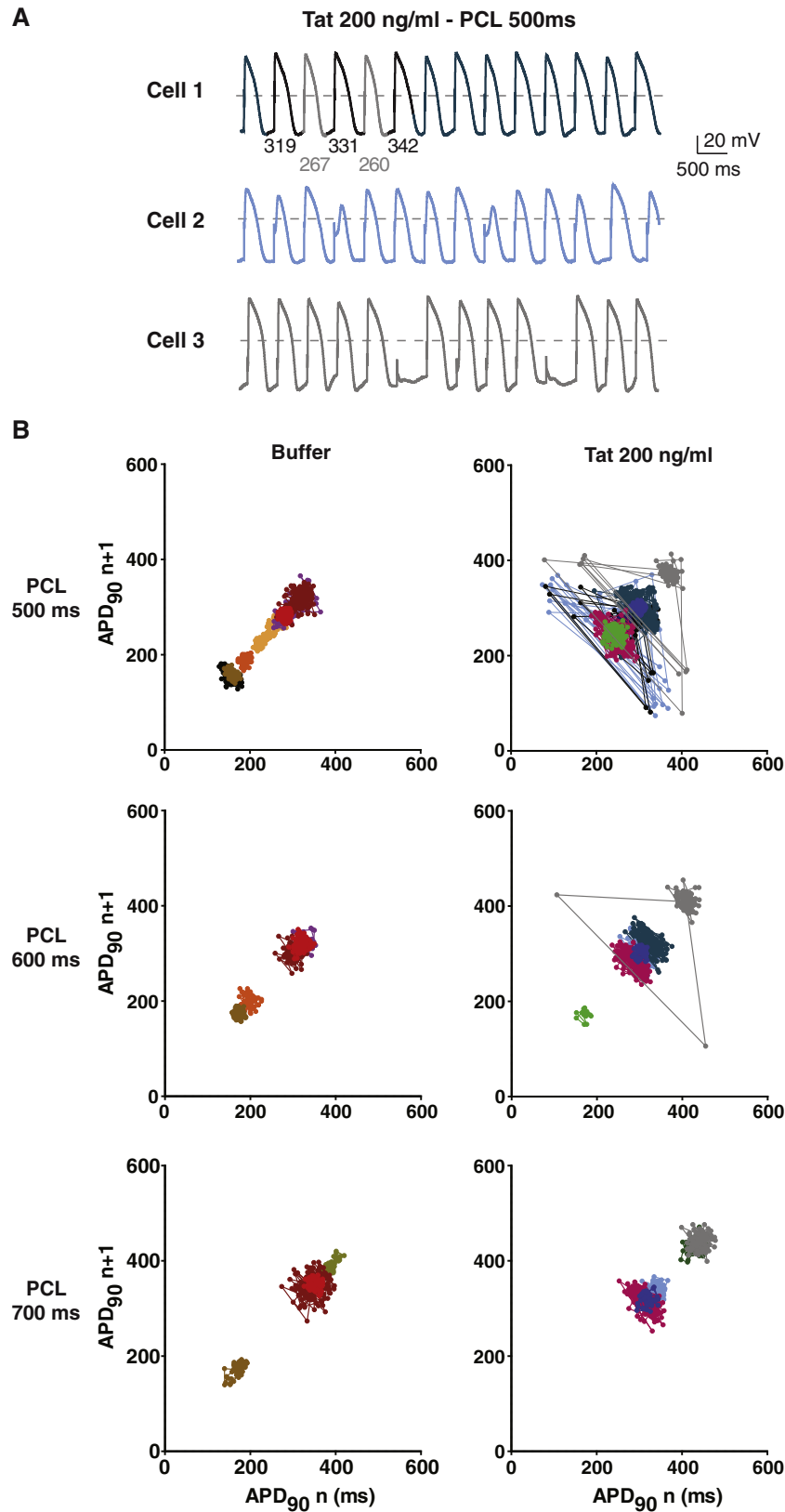


Fig. 8. Tat incubation leads to alternans events in ventricular-like hiPS-CMs. (A) Representative ventricular-like action potentials recorded from three different hiPS-CMs incubated with 200 ng/ml WT-Tat (Tat). Cell 1: APD₉₀ of five consecutive APs are indicated below. (B) Poincaré plots of APD₉₀ (one color per cell) recorded at three different PCLs. The graphs show APD₉₀ of each AP (number $n + 1$) versus the APD₉₀ of its preceding one (n). Same color code used in A.

not affect KCNE1-R539W-KCNQ1 current amplitude measured after channel full activation, reinforcing the interpretation that Tat decreases KCNE1-KCNQ1 channel activity through a decrease in available PIP₂.

Altogether, these data suggest that Tat inhibits hERG and KCNE1-KCNQ1 currents by sequestering PIP₂.

To localize Tat, immunofluorescence assays were performed using the same conditions as for patch-clamp experiments, i.e. cotransfection of hERG with either WT- or W11Y-Tat/GFP bidirectional plasmids. They confirmed that WT-Tat localizes at the COS-7 plasma membrane, through its interaction with PIP₂ [32]. Unlike WT-Tat, W11Y-Tat localized diffusely in the cytosol, its lack of affinity for PIP₂ preventing its plasma membrane localization (Fig. 3A & B). This is consistent with the absence of W11Y-Tat effect on PIP₂-dependent channels activity.

3.2. Tat incubation does not alter hERG and KCNE1-KCNQ1 currents in heterologous expression systems

In HIV patients, T-cells-secreted Tat can enter multiple cell types, through the interaction with various ubiquitous receptors [18]. We thus tested the effect of Tat applied externally on hERG and KCNE1-KCNQ1 channels activity. We first studied the effect of Tat 24-hour incubation in HEK293 cells stably expressing hERG channels. We chose a 24-hour incubation since in the different biological assays in which Tat had been tested (neurosecretion [32] and phagocytosis [33]), it was found to require some time (3–4 h) for Tat, after initial internalization, to accumulate on PIP₂ and to perturb processes relying on this phosphoinositide. Moreover such a 24-hour incubation, and not acute application, had already been associated with a decrease in hERG current density in this model [16]. Surprisingly and unlike the previous observations, Tat incubation (200 or 400 ng/ml) did not induce any change (Supplemental Fig. 2A; Supplemental Table 1). Neither activation nor inactivation curves were modified by Tat incubation (Supplemental Fig. 2B & D; Supplemental Table 1), nor the kinetics of deactivation, inactivation and recovery from inactivation (Supplemental Fig. 2C, E & F). In addition, neither mRNA nor total hERG protein levels were affected (Supplemental Fig. 2G & H). Tat entry into cells occurs through receptors of which expression vary from one cell type to another [34]. The observed absence of Tat effect may then be due to insufficient penetration into HEK cells. In order to evaluate whether the cell type could explain the absence of incubated Tat effect on I_{hERG}, this analysis was repeated in COS-7 cells transiently expressing hERG (Supplemental Fig. 3). Again 200 ng/ml Tat incubation did not lead to any change in either hERG current density or biophysical parameters. In addition, we did not observe any effect of Tat incubation on KCNE1-KCNQ1 channel activity in COS-7 cells (Supplemental Fig. 4).

3.3. Tat incubation decreases I_{Kr} in cardiomyocytes derived from human induced pluripotent stem cells

Cardiomyocytes differentiated from hiPS cells provided us with the opportunity to evaluate the effect of Tat incubation on a more relevant cellular model than non-cardiac cells. As shown in Fig. 4A & B, I_{Kr}, isolated as the E-4031-sensitive current, was significantly reduced in hiPS-CMs incubated for 24 h with 200 ng/ml Tat. After full activation, the maximum tail E-4031-sensitive current density measured at –50 mV was reduced by 31% (Fig. 4B; Table 2; $p < 0.05$). In addition, no detectable I_{Ks} was observed after E-4031-application. Thus, activation curve and deactivation kinetics were measured using the total K⁺ tail current. The activation curve was not modified but the current deactivation was accelerated (Fig. 4C & D; Table 2, $p < 0.05$). Therefore, we observed similar effect of Tat on the activation gate as compared to the experiments in Tat-transfected COS-7 cells, reminiscent of the effect of a decrease in PIP₂. This suggests that hiPS-CMs express receptors allowing Tat internalization in sufficient quantity to alter the currents, as opposed to COS-7 and HEK293 cellular models. We directly tested this hypothesis using confocal and Structured Illumination Microscopy (SIM) imaging on

Tat-incubated COS-7 cells expressing hERG, and hiPS-CMs. Confocal images showed that Tat protein was comparably present in both cell types after 24-hour incubation (Fig. 5A). Higher magnification analyses highlighted that in COS-7 cells expressing hERG, Tat protein did not enter the cells. In marked contrast, in hiPS-CMs, Tat was found both in the cytoplasm and/or vesicular structures that are presumably endocytic elements as well as colocalized with hERG proteins, at the plasma membrane (Fig. 5B). Altogether, these experiments in hiPS-CMs suggest that Tat, when applied at biologically relevant concentrations is able to affect PIP₂ availability, thereby affecting I_{Kr} current.

Since Tat also affects transfected KCNE1-KCNQ1, which is related to the cardiac I_{Ks} current, we set out to study the effect of Tat on I_{Ks} in native tissue. However, in our model of cardiomyocytes differentiated from hiPS cells, we did not observe any tail current in presence of 1 μ M E-4031 (Fig. 4A), suggesting that KCNE1 and/or KCNQ1 subunits are not expressed/functional in this model. We also applied 1 μ M epinephrine to enhance this current (as in [35,36]) but again, no tail current was observed in 1 μ M E-4031.

In addition to hERG and KCNQ1, PIP₂ has been shown to modulate the activity of a plethora of ion channels, yet it is possible that many of these channels are not affected by PIP₂ if variations are limited to its physio/pathophysiological range [37,38]. To address this issue, we tested the effect of Tat on several channels suggested to be regulated by PIP₂. PIP₂ has previously been shown to modulate the L-type Ca²⁺ current, I_{Ca,L}, in neurons and in natural killer cells and Ca_v1.2 and Ca_v1.3 in heterologous expression system [39–41]. We investigated Tat effects on the L-type Ca²⁺ current in hiPS-CMs. Tat incubation did not modify I_{Ca,L} density or biophysical properties (Fig. 6), showing that endogenous Ca²⁺ channels activity is not affected by Tat in hiPS-CMs. This observation suggests that hiPS-CM Ca²⁺ channels are not as sensitive as hERG to a decrease in available PIP₂.

In order to evaluate if Tat also alters the activity of an inward rectifier channel, we chose a channel which has a low apparent affinity to PIP₂. Such channel, which is less stabilized by PIP₂ is more prone to a decrease in activity when available PIP₂ is decreased [42]. Thus we selected the K_{ATP} channel, since the alpha-subunit of this channel, Kir6.2, has a low affinity to PIP₂ and is more prone to be sensitive to Tat [42]. As no K_{ATP} current was detected in the hiPS-CMs as a glibenclamide-sensitive outward current measured at +40 mV, we used neonatal mouse cardiomyocytes, in which a 24-hour incubation with 200 ng/ml Tat led to a decrease in the K_{ATP} current (Supplemental Fig. 5). This suggests that Tat may lead to a remodeling of the electrical activity of cardiomyocytes through an alteration of the activity of several PIP₂-sensitive channels.

3.4. Tat related reduction of I_{Kr} is not due to a reduction in membrane hERG

Since Tat has been shown to interfere with protein expression in host cells, it may also act on hERG through a reduction in its expression [43]. To test whether Tat directly alters expression of native hERG in hiPS-CMs, we performed qRT-PCR and Western blot analyses. While no reduction of hERG mRNA was observed, total hERG protein amount was reduced by about 20% only (Fig. 4 E & F). Moreover, the mature (i.e. glycosylated) fraction responsible for the K⁺ current [44], was not significantly reduced (Fig. 4F), suggesting that inhibition of hERG protein expression is not responsible for hERG current decrease and hence that in cardiomyocytes, Tat incubation mostly affects hERG gating.

3.5. Tat modifies the electrical activity of cardiomyocytes derived from human induced pluripotent stem cells

Action potentials (APs) were then recorded using the permeabilized patch configuration in hiPS-CMs. Three types of AP shapes were obtained and categorized as nodal-, atrial-, and ventricular-like APs (Table 3). Since LQTS is associated with ventricular AP prolongation, we focused

on ventricular-like APs paced at two cycle lengths. As illustrated in Fig. 7A, APs were prolonged in hiPS-CMs incubated for 24 h with 200 ng/ml Tat. Only APD₇₀ and APD₉₀ were prolonged as expected with hERG inhibition ($p < 0.05$; Fig. 7B). In addition, most Tat-incubated cells showed a higher APD₉₀ dispersion compared to cells incubated with buffer (Fig. 8). This dispersion resulted from AP duration and amplitude alternans (Fig. 8A). APD₉₀ alternans were exacerbated at faster stimulation rates (shorter PCLs; Fig. 8B).

4. Discussion

HIV-Tat binds PIP₂ with a very high affinity, the K_d being 20 to 50 times lower than the K_d of the phospholipase C pleckstrin homology domain, one of the best cellular ligands for PIP₂ [18]. Knowing that hERG and KCNQ1 currents are modulated by membrane PIP₂ levels, we tested whether Tat can affect those currents by sequestering PIP₂, decreasing its availability for hERG and KCNQ1 channels. Co-transfecting Tat with either channel led to modification of the biophysical parameters in accordance with a decrease in available PIP₂. Moreover, the W11Y-Tat mutant, which is 300 times less affine for PIP₂ [18], has no effect on the activity of both channels. Importantly, W11Y-Tat mutation does not alter its native transactivation activity [23], suggesting that loss of effect of W11Y-Tat on hERG and KCNE1-KCNQ1 channels activity is rather due to the disrupted interaction with PIP₂ than through another mechanism, such as the regulation of gene expression. Since W11Y-Tat is not localized to the membrane, one might argue that WT-Tat effects may be mediated through alternative mechanisms independent of PIP₂, such as a direct interaction with the ion channels of interest. However, a KCNE1-KCNQ1 mutant which is not sensitive to PIP₂ is not affected by WT-Tat, confirming that Tat acts by sequestering PIP₂. Incubation of hiPS-derived cardiomyocytes with WT-Tat also leads to a decrease in I_{Kr} and acceleration of deactivation, consistent with a decrease in available PIP₂. Finally, the cells electrical activity is affected through delayed repolarization and AP alternans. Altogether, these results suggest that Tat affects cardiomyocytes repolarization through an interaction with PIP₂, reducing the major repolarizing currents I_{Kr} and I_{Ks} . Those results are particularly significant as, despite the proven effect of PIP₂ on the gating of many channels, the relevance of such a regulation in physiological or pathophysiological conditions is still questioned. It has been suggested that in cardiac cells, PIP₂ levels are not affected enough by PLC-coupled receptors to alter I_{Kr} and I_{Ks} [45]. This work enlightens a pathophysiological condition where I_{Kr} is indeed reduced through a decrease in available PIP₂. Even if there is no direct measurement of available PIP₂, all the observations made in the present work on I_{Kr} , I_{Ks} and I_{KATP} measurements together with the observations made in another study on neurosecretory cells (annexin A2 recruitment and peripheral actin depolymerization) are consistent with a decrease in available PIP₂ [32].

We show that other PIP₂-sensitive channels, such as the K_{ATP} channel [46] may also be affected. Since this channel is implicated in ischemic preconditioning, this could explain at least partly the increase in acute myocardial infarction in HIV-patients adjusted for hypertension, diabetes, and dyslipidemia [47]. In human cardiomyocytes, we did not observe any effect of Tat incubation on the L-type calcium current I_{CaL} , which may be explained by a lower sensitivity to PIP₂ variation. The exact sensitivity of cardiac I_{CaL} has not been precisely evaluated, however, only a fraction of the calcium current is PIP₂-sensitive, as shown by Dr-VSP-induced PIP₂ depletion. Only 35% of I_{CaL} is supported by PIP₂ as a cofactor in tsA201 cells, and only 28% in SCG neurons [48]. On the other hand, 100% of KCNE1-KCNQ1 and 70% of hERG currents are supported by PIP₂ as a cofactor, as shown in excised patch in COS-7 cells and 100% of I_{KATP} using also excised patch in cardiomyocytes [20,21,49]. To further validate the hypothesis of a lower sensitivity to PIP₂ variation of I_{CaL} , a comparison performed in the same model, and especially in cardiomyocytes would be necessary to quantify both the fraction of PIP₂-sensitive current, and the apparent PIP₂ affinity of this fraction, in

all these channels. Such impact of a channel affinity for PIP₂ on its regulation by a PIP₂-dependent process has already been described for KCNQ channels [50].

Of note, PIP₂ affecting the activity of many channels, Tat effects may not be restricted to I_{Kr} , I_{Ks} and I_{KATP} [51]. Consequently, potential impact on other PIP₂-sensitive currents, such as an increased persistent sodium current may also be involved in the observed action potential prolongation. Nevertheless, cumulative effect of about 30% reduction of I_{Kr} and I_{Ks} has been shown to be sufficient to prolong QT interval, and a mutation that reduces I_{Kr} by 30–40%, similar to this study, is associated with Long QT syndrome, suggesting that Tat effect on I_{Kr} is sufficient to provoke Long QT syndrome [52,53].

Tat incubation affected I_{Kr} in human cardiomyocytes, but not in COS-7 and HEK293 models. This highlights the usefulness of hiPS-derived cardiomyocytes as a more reliable model than heterologous systems. Most importantly, it validates the use of hiPS-CMs to study cardiac non-genetic diseases such as infectious diseases like AIDS. One could consider the absence of detectable I_{Ks} in non-stimulated condition as a limit of this model. But this characteristic makes this model closer to hardly available human cardiomyocytes as compared to guinea pig cardiomyocytes in which I_{Ks} presents a much larger contribution to the repolarizing currents without any adrenergic stimulus [54–56].

Bai and collaborators did observe an effect of Tat incubation on hERG current amplitude in a similar HEK293 model as we used [16]. However, these authors used a short isoform of Tat encoded by the first exon of the protein only (72 residues; Tat-72). The Tat protein used in our study is a 86 residues long (Tat-86), two-exon form of Tat that is present in viral isolates [34]. Although both Tat-72 and Tat-86 contain the transactivation domain, and are frequently used for *in vitro* studies, Tat-86 is more readily taken up than Tat-72 by various cell types [57]. In addition, the second exon endows Tat with additional activities and is critical for both *in vitro* and *in vivo* HIV replication [58]. Therefore, the use of Tat-86 appears to be more relevant to evaluate HIV impact on human cardiomyocyte electrical activity.

In LQT patients, T-wave alternans mirroring ventricular AP alternans are associated with delayed repolarization and are a known risk factor for sudden death [59]. In hiPS-derived cardiomyocytes, Tat incubation resulted in a significant reduction in I_{Kr} current, associated with ventricular AP prolongation, as would be expected, and alternans occurrence. Up to now, such human AP alterations could be addressed with *in silico* studies only. For example, similar AP prolongation at low rate and APD alternans at elevated rate were observed when I_{Kr} alterations due to a human LQT-related hERG mutation were implemented in a mathematical model of the human ventricular action potential [60]. Similarly, mild reduction in repolarization reserve due to alteration of I_{Kr} parameters led to APD alternans using another *in silico* model [61]. These results are also in agreement with the observations in canine cardiomyocytes of AP alternans suppression by overexpression of hERG [62]. However, *in silico* or animal models are limited to investigate the potential multiple effects of actors such as Tat on their various mechanistic targets, in a situation far more complex than a single mutation.

In conclusion, this study suggests that PIP₂ is the link between HIV-Tat and cardiac arrhythmias observed in HIV-positive individuals. In neurosecretory cells, it has been proposed that Tat perturbs the recruitment of several PIP₂-dependent key players of the exocytotic machinery [32]. Combined with this previous investigation, our work suggests a general remodeling of PIP₂-dependent processes by HIV *via* Tat. It would be interesting to test whether other processes, immune or not immune, are also affected by Tat through PIP₂.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jymcc.2016.08.022>.

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Disclosures

None.

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