Roscovitine inhibits CaV3.1 (T-type) channels by preferentially affecting close-state inactivation.

Viktor Yarotskyy and Keith S. Elmslie

Department of Anesthesiology (V.Y., K.S.E), Penn State College of Medicine, Hershey, PA, 17033 and Department of Pharmacology (K.S.E), Kirksville College of Osteopathic Medicine, AT Still University, Kirksville, MO 63501
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Corresponding author: Viktor Yarotskyy, Department of Pharmacology and Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642, Tel: 585-276-3070, Fax: 585-273-2652, E-mail: Viktor_Yarotskyy@URMC.Rochester.edu.

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Abbreviations: AP, action potential waveform; CaV, voltage-dependent calcium channel; CDK, cyclin-dependent kinase; Cntl, control; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; HERG, Human Ether-a-go-go Related Gene; Iss, steady state current; ITail, tail current amplitude; k, Boltzmann slope factor; NMG, N-methyl-d-glucamine; Rosc, R-roscovitine; \( \tau_{\text{Act}} \), activation time constant; \( \tau_{\text{CSI}} \), close-state inactivation time constant; \( \tau_{\text{Deact}} \), deactivation time constant; \( \tau_{\text{fast}} \), fast component recovery from inactivation time constant; \( \tau_{\text{Inact}} \), inactivation time constant; \( \tau_{\text{slow}} \), slow component recovery from inactivation time constant; \( V_{0.5} \), half maximal voltage; WO, washout

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Abstract

T-type calcium channels (CaV3) play an important role in many physiological and pathological processes, including cancerogenesis. CaV3 channel blockers have been proposed as potential cancer treatments. Roscovitine, a tri-substituted purine, is a cyclin-dependent kinase (CDK) inhibitor that is currently undergoing phase II clinical trials as anti-cancer drug and has been shown to affect calcium and potassium channel activity. Here we investigate the effect of roscovitine on CaV3.1 channels. CaV3.1 channels were transiently expressed in HEK293 cells and currents were recorded using the whole-cell patch clamp technique. Roscovitine blocks CaV3.1 channels with higher affinity for depolarized cells (EC$_{50}$ = 10 µM), which is associated with a negative shift in the voltage-dependence of closed-state inactivation. Enhanced inactivation is mediated by roscovitine-induced acceleration of closed-state inactivation and slowed recovery from inactivation. Small effects of roscovitine were also observed on T-channel deactivation and open-state inactivation, but neither could explain the inhibitory effect. Roscovitine inhibits CaV3.1 channels within the therapeutic range (10-50 µM) in part by stabilizing the closed-inactivated state. The ability of roscovitine to block multiple mediators of proliferation, including CDKs and CaV3.1 channels, may facilitate its anticancer properties.
Introduction

T-type calcium channels (CaV3) are low voltage-activated channels with fast inactivation and slow deactivation kinetics that consist of three family members CaV3.1 ($\alpha_{1G}$), CaV3.2 ($\alpha_{1H}$), and CaV3.3 ($\alpha_{1I}$) (Perez-Reyes, 2003). CaV3 channels are widely distributed among different cell types including neurons, cardiomyocytes, smooth and skeletal muscles (Perez-Reyes, 2003). In spite of establishing many aspects of function and cell-specificity, nicely reviewed by Edward Perez-Reyes (Perez-Reyes, 2003), their role in many physiological and pathophysiological processes remains unclear. However, an emerging body of evidence suggests that CaV3 channels can participate in pathological processes such as chronic pain (Jagodic et al., 2008) and cancer cell proliferation (Gray and Macdonald, 2006; Lee et al., 2006; Heo et al., 2008; Lu et al., 2008; Taylor et al., 2008a; Taylor et al., 2008b). These findings, in particular the role in cancer, have made CaV3 channels an attractive clinical target (Gray and Macdonald, 2006).

Roscovitine is a tri-substituted purine, which initially was proposed as an anticancer therapy because of its blocking effect on cyclin-dependent kinases (CDK) (Meijer et al., 1997; Fischer and Gianella-Borradori, 2003; Wesierska-Gadek et al., 2007). This drug is also known as CYC202 and Seliciclib and is currently undergoing phase II clinical trials as a treatment for non-small cell lung cancer and nasopharyngeal cancer. Emerging evidence suggests that roscovitine may have additional targets involved with tumor development. For example, we have recently shown that roscovitine can also inhibit Human Ether-a-go-go Related Gene (HERG) potassium channel activity (Ganapathi et al., 2009), and HERG channel block can reduce growth of certain cancer types (Pardo et al., 2005). CaV3 channels are potential targets for anti-cancer therapy (Gray and Macdonald, 2006). CaV3.1 channels are expressed in many human cancer cell types including liver, ovarian and breast cancers, and proliferation is reduced by inhibition of these
channels by either down regulation (siRNA) or drug application (Lu et al., 2008; Taylor et al., 2008a; Li et al., 2009; Li et al., 2011). Through its unique effects on ion channels, roscovitine has provided critical insights into gating mechanisms and the treatment of disease (Buraei et al., 2005; Cho and Meriney, 2006; Buraei et al., 2007; Yarotskyy and Elmslie, 2007; Buraei and Elmslie, 2008; Ganapathi et al., 2009; Yarotskyy et al., 2009; Yazawa et al., 2011), which motivated us to test the effect of roscovitine on CaV3.1 channels. We find that CaV3.1 channels are inhibited by roscovitine. This inhibition is potentiated by depolarized voltages so that at a membrane potential of -70 mV the EC$_{50}$ = 10 μM, which is at the low end of the therapeutic range (10-50 μM) for roscovitine block of cancer cell proliferation (Meijer et al., 1997; Fischer and Gianella-Borradori, 2003; Wesierska-Gadek et al., 2007). We conclude that the inhibition of CaV3.1 channels could provide a third anti-cancer mechanism for roscovitine in addition to CDK and HERG block, which will likely enhance the therapeutic efficacy of roscovitine as an anti-cancer drug.
Methods

**HEK cell Transfection** - We utilized either calcium phosphate precipitation (Yarotskyy and Elmslie, 2007) or lipofectamine 2000 (Yarotskyy et al., 2010) to transfet HEK293 cells with CaV3.1 channels (cloned from rat pancreatic β cells, a generous gift from Dr. Ming Li, Tulane University Medical School, New Orleans, LA; Genbank number AF125161) (Zhuang et al., 2000), which provided highly reproducible expression 24 – 48 h after transfection. HEK293 cells were maintained in standard Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixtures at 37°C in a 5% CO₂ incubator. HEK293 cells were transfected by adding cDNA plasmids as follow (in µg): 11.5 α₁G (CaV3.1), 2.15 TAG (SV40 large T-antigen, to increase expression efficiency) and 1 green fluorescent protein (to visualize transfected cells), and incubated at 5% CO₂ for 8 h after which the transfecting medium was replaced by the standard DMEM medium. The transfected cells were split next day into 35 mm dishes that served as the recording chamber. Recordings were performed 24-48 h after transfection.

**Measurement of ionic currents** - Cells were voltage-clamped using the whole-cell configuration of the patch clamp technique. Pipettes were pulled from Schott 8250 glass (Garner Glass, Claremont, CA) on a Sutter P-97 puller (Sutter Instruments Co., Novato, CA). Currents were recorded using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and digitized with an ITC-18 data acquisition interface (Instrutech Corporation, Port Washington, NY). Experiments were controled by a Power Macintosh G3 computer (Apple Computer, Cupertino, CA) running S5 data acquisition software written by Dr. Stephen Ikeda (NIH, NIAAA, Bethesda, MD). Leak current was subtracted online using a -P/4 protocol. Recordings were carried out at room temperature and the holding potential was -120 mV, unless otherwise stated. Some studies
examined CaV3.1 current activated by an action potential waveform (AP) that was generated using a series of voltage steps and ramps with the following values (HP = -120 mV): -80 mV for 1 ms, -80 to 37 mV in 0.5 ms, 37 to 40 mV in 0.1 ms, 40 to 37 mV in 0.1 ms, 37 to -80 mV in 1.5 ms, and 11.5 ms at -80 mV. A 20 Hz 10-AP train was generated using the same voltage changes except that the 1 ms step to -80 mV was removed and voltage between APs within the train was -120 mV. Whole-cell currents were digitized depending on voltage step duration at 50 (up to 100 ms), 10 (200 ms), and 4 (2000 ms) kHz after analog filtering at 1 - 10 kHz.

Data analysis - Data were analyzed using IgorPro versions 5 and 6 (WaveMetrics, Lake Oswego, OR) running on a Macintosh computer. Percentage inhibition was measured by comparing the steady state drug effect to the average of current measured before (control) and following full recovery (washout). Activation $\tau$ ($\tau_{\text{Act}}$) was determined by fitting a single exponential function to the step current after a 0.3 ms delay (Buraei et al., 2007). The effect of roscovitine on T-channel inactivation was measured by using either 100 or 1000 ms inactivating voltage steps followed by a 20 ms test step to -20 mV. The $I/I_{\text{max}}$ ratio for the current measured from the test step was plotted against inactivating voltage (either 100 or 1000 ms steps) and fitted by a single Boltzmann equation to yield half maximal voltage ($V_{0.5}$), slope factor (k) and the magnitude of inactivation. Inactivation $\tau$ ($\tau_{\text{Inact}}$) was determined by fitting a single exponential function from peak step current to the end of the step. The development of closed-state inactivation was determined by measuring the effect of increasing duration of voltage steps to -70 mV on a 20 ms step to -20 mV (Serrano et al., 1999) (see Fig. 6A). The recovery from inactivation protocol utilized a 1000 ms inactivating step to either -20 or -70 mV followed by a 20 ms test step to -20 mV after an increasing recovery time at -120 mV. Group data were calculated as mean $\pm$ S.D. throughout the paper. A paired t-test was used for within-cell comparisons. One-way ANOVA
with Tukey HSD posthoc test was used to test for differences among three or more independent groups.

Solutions - The internal pipette solution contained (in mM) 104 N-methyl-d-glucamine (NMG)-Cl, 14 Creatine-PO$_4$, 6 MgCl$_2$, 10 NMG-HEPES, 5 Tris-ATP, 0.3 Tris-GTP and 10 NMG-EGTA with osmolarity $= 280$ mOsM and pH $= 7.3$. The external recording solution contained (in mM) 30 BaCl$_2$, 100 NMG-Cl, 10 NMG-HEPES, osmolarity $= 300$ mOsM and pH $= 7.3$. R-roscovitine was prepared as a 50 mM stock solution in dimethylsulphoxide (DMSO) and stored at $-30^\circ$C. All external solutions contained the same DMSO concentration so that the roscovitine concentration was sole variable when changing solutions. Test solutions were applied from a gravity-fed perfusion system that provided complete solution exchange within 1-2 sec.

Chemicals - All experiments utilized R-roscovitine (de Azevedo et al., 1997) from LC Labs (Woburn, MA). DMEM, FBS, 100x antibiotic/antimycotic (penicillin, streptomycin, and amphotericin B) were from Invitrogen (Carlsbad, CA). Other chemicals were obtained from Sigma (St. Louis, MO).
Results

Roscovitine blocks T-type channels.

Roscovitine has provided surprising insights into ion channel gating mechanisms and disease treatment (Buraei et al., 2005; Cho and Meriney, 2006; Buraei et al., 2007; Yarotskyy and Elmslie, 2007; Buraei and Elmslie, 2008; Ganapathi et al., 2009; Yarotskyy et al., 2009; Yazawa et al., 2011). Thus, we were interested in determining if T-channels would be affected. CaV3.1 channels expressed in HEK293 cells were activated using 15 ms depolarizing steps ranging from -100 mV to +20 mV, followed by repolarization to -100 mV (Fig. 1). Roscovitine (45 µM) strongly blocked the current in a voltage-independent manner (Fig. 1A, B, C). Figure 1C shows no significant difference in the percentage inhibition across all current-generating voltages, which suggests that open channel block is not a mechanism for inhibition. The activation vs. voltage relationship was examined in more detail by plotting tail current amplitude (ITail) vs. step voltage (Fig. 1D), which showed a very small right shift induced by 45 µM roscovitine. Single Boltzmann function fitting yielded half-maximal activation voltage (V_0.5) for control = -38.0 ± 4.5 mV, for roscovitine = -36.7 ± 4.4 mV, and for washout = -37.6 ± 4.6 mV (n = 9) (Fig. 1D); and slope factor (k) 5.41 ± 0.56, 6.08 ± 0.64, and 6.01 ± 0.82 (n = 9) for control, roscovitine, and washout, respectively (Fig. 1D). The roscovitine-induced changes were very small (ΔV_0.5 = 0.9 ± 0.9 mV, and Δk = 0.38 ± 0.35), but significantly different from control (n = 9, p < 0.05 for both). It is very unlikely that these small changes in activation voltage parameters would have physiological significance.
Roscovitine does not affect activation but slows deactivation of CaV3.1 channels.

We have reported previously that roscovitine slowed CaV1.2 channel activation, which contributes to the inhibitory effect (Yarotskyy and Elmslie, 2007; Yarotskyy et al., 2009). For that reason, we examined the effect of roscovitine on CaV3.1 channel activation speed by comparing activation time constants ($\tau_{\text{Act}}$) determined by fitting step current onset with a single exponential function. We found no effect of roscovitine on $\tau_{\text{Act}}$ at any CaV3.1 current-generating voltage. Indeed, normalized step currents were superimposed in control vs. 45 µM roscovitine (Supplemental Data Figure 1).

The absence of an effect on CaV3.1 current activation suggests that the small shift in activation $V_{0.5}$ observed in figure 1D is induced by a different mechanism. Since there is precedent for roscovitine to slow calcium channel deactivation (Buraei et al., 2005; Buraei et al., 2007), we measured the speed of CaV3.1 channel closing over voltages ranging from -50 to -160 mV. CaV3.1 current was activated by 10 ms steps to -10 mV followed by a 72 ms repolarizing (tail) step (Fig. 2). The tail current was fitted by a single exponential function to yield the deactivation time constant ($\tau_{\text{Deact}}$), which was plotted against tail voltage (Fig. 2C). Roscovitine significantly increased $\tau_{\text{Deact}}$ at voltages ranging from -140 mV to -50 mV ($p < 0.05$). The effect was small and can be better demonstrated by the roscovitine-induced change in $\tau_{\text{Deact}}$ (% $\Delta\tau_{\text{Deact}}$) (Fig. 2D). Qualitatively, this effect is similar to that of roscovitine on CaV2.1 and CaV2.2 channels (~7-fold increase in $\tau_{\text{Deact}}$) (Buraei et al., 2005; Buraei et al., 2007), but the increase of CaV3.1 current $\tau_{\text{Deact}}$ was small and did not exceed 20%. This small effect on $\tau_{\text{Deact}}$ is likely to mediate the minor roscovitine-induced right shift in activation $V_{0.5}$ (Fig. 1D), and possibly could reduce the overall CaV3.1 current inhibition by a small amount.
Roscovitine blocks CaV3.1 channels in dose-dependent manner.

To determine the dose-dependent effect of roscovitine, we tested the effect of 1, 10, 30, 45, and 100 µM roscovitine on CaV3.1 current (Fig. 3). Fractional block was plotted against roscovitine concentration and fitted by Hill’s equation yielding EC$_{50}$ = 40.5 ± 7.6 µM and Hill’s coefficient = 1.55 ± 0.11 (n = 5). This EC$_{50}$ was in the range obtained for CaV1.2 channels (Yarotskyy and Elmslie, 2007). Some CaV3 channel blockers, such as mibefradil (McDonough and Bean, 1998), octanol (Eckle and Todorovic, 2010), and T-type antagonist-A2 (TTA-A2) (Uebele et al., 2009) can preferentially affect inactivated channels. We determined if roscovitine had apparent higher affinity at a more depolarized holding potential at which CaV3.1 channels inactivate. We found that a -70 mV holding potential significantly decreased the roscovitine EC$_{50}$ = 10.0 ± 1.0 µM, and Hill’s coefficient = 1.22 ± 0.15 compared with the results from HP -120 mV (Fig. 3D). This result supports the idea that roscovitine preferentially affects inactivated channels.

Roscovitine slows open-state inactivation

Based on the effect of holding potential on the dose-response relationship, we expected to observe a significant enhancement of inactivation by roscovitine. Using 100 ms voltage steps to inactivate CaV3.1 channels, we found that roscovitine had the opposite effect with a significant decrease of inactivation at voltages ≥ -40 mV (Fig. 4). There was a small roscovitine-induced enhancement of inactivation, but only at -80 and -70 mV. The more widespread effect was a ~20% decrease of inactivation (Fig. 4A) that corresponded with a significant slowing of inactivation at voltages > -40 mV (Fig. 4B, C). While decreased inactivation was unexpected, we realized that the reduced inactivation corresponded to current-generating voltages, while a small
but significant enhancement of inactivation was observed at voltages hyperpolarized to channel activation. This suggested that roscovitine may enhance only closed-state inactivation, while slightly decreasing open-state inactivation.

**Roscovitine affects closed-state inactivation.**

The four-fold reduction in roscovitine EC\textsubscript{50} with depolarized holding potential (-120 to -70 mV) supports the idea that the inactivated state is stabilized by roscovitine (Fig. 3). Since holding potential changes generally affect closed-state (also called steady-state) inactivation, we tested the effect of longer voltage steps (1000 ms) (Fig 5), which hyperpolarized the inactivation V\textsubscript{0.5} by 20 mV from -52 mV for 100 ms to -73 mV for 1000 ms (Fig. 4A vs. 5A) so that much of the inactivation was now observed from closed CaV3.1 channels. Using this paradigm, 45 µM roscovitine significantly left-shifted the inactivation vs. voltage relationship ~10 mV to enhance inactivation at voltages around the resting potential (e.g. -70 mV) (Jones, 1989). Boltzmann equation fits of the inactivation –voltage relationship yielded V\textsubscript{0.5} = -73.4 ± 2.8 mV, -82.5 ± 2.5 mV, and -72.6 ± 2.8 mV (n = 6) for control, roscovitine and washout, respectively (Fig. 5B). The Boltzmann slope factor was also significantly increased, while maximum inactivation was significantly decreased for current generating voltages as observed for inactivation measured from 100 ms steps (Fig. 4A). The enhancement of closed-state inactivation is reminiscent of the inhibitory effect of roscovitine on N-type channels (Buraei and Elmslie, 2008). The decrease in open state inactivation could have a potentiating effect on CaV3.1 current, but the enhancement of closed-state inactivation appears to dominate and increase roscovitine-induced inhibition of CaV3.1 channel activity.

**Roscovitine affects inactivation kinetics.**
The enhancement of closed-state inactivation by roscovitine could result from either an increased speed of inactivation, decreased recovery from inactivation or both. We examined the time course for the development of closed-state inactivation by examining the effect of increasing -70 mV step of duration (10 ms to 1900 ms) on current elicited at -20 mV (Fig. 6A). The decrease of CaV3.1 current with step duration was fitted by single exponential function to yield the close-state inactivation time constant ($\tau_{CSI}$) at -70 mV (Fig. 6B, C) and fractional current remaining at the end of the 1900 ms step (steady state current, $I_{SS}$) (Fig. 6D). 45 µM roscovitine significantly decreased $\tau_{CSI}$ by 184 ms (24%, $p < 0.05$, n = 5, Fig. 6C) and $I_{SS}$ by 0.14 (52%, $p < 0.05$, Fig 6D) relative to the averaged control and washout values ($\tau_{CSI} = 751$ ms, $I_{SS} = 0.26$). These results show that roscovitine enhances close-state inactivation by speeding entry into the inactivated state.

The recovery from inactivation was investigated using 1000 ms inactivating steps (-20 mV) to inactivate CaV3.1 channels and short test steps to -20 mV of increasing time from the inactivating step to measure the recovery of current (Fig. 7A). The recovery voltage was -120 mV. We found that 45 µM roscovitine significantly slowed recovery from inactivation (Fig. 7A, B). The recovery time course was best fit using a double exponential function, which yielded $\tau_{fast}$ and $\tau_{slow}$ (Fig. 7B). Roscovitine (45 µM) doubled $\tau_{fast}$ from 121 ± 36 ms and 131 ± 21 ms for control and washout to 257 ± 89 ms (p < 0.05, n = 5), and $\tau_{slow}$ was nearly doubled to 1181 ± 227 ms from 652 ± 111 and 695 ± 100 ms (n = 5, p < 0.05) in control and washout. The relative amplitude of the slow recovery component was increased from 56% (average control & washout) to 71% of the total (ns, p = 0.08). Thus, the enhancement of closed-state inactivation could result from slowed recovery from inactivation as well as faster inactivation. This was further investigated by measuring the recovery from inactivation following 1000 ms steps to -70 mV.
As observed following -20 mV steps, 45 µM roscovitine significantly slowed the recovery from inactivation generated by -70 mV steps (Fig. 7D). Thus, recovery from closed-state inactivation is slowed by roscovitine. One difference was that the recovery was well described by a single exponential equation with the recovery \( \tau \) in between the \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) measured following the -20 mV step. The expectation was that the \( \tau \)'s would be similar since the recovery voltage was -120 mV for both data sets. However, it is clear that a slow component of recovery exists following the -70 mV step since the amplitude of the single exponential function reaches only 93% (p < 0.05) of the maximum recovery observed at 15 sec (Fig. 7C). The relatively small magnitude of the slow component (7% for -70 mV vs. 56% for -20 mV steps) is the likely reason it was not well described by our exponential fitting. Most importantly our data clearly show that the enhancement of roscovitine-induced inhibition at -70 mV results from accelerated entry into and slowed recovery from closed-state inactivation.

**Physiological impact of roscovitine.**

Based on our voltage step data, we speculated that the dominant effect of roscovitine would be inhibition of physiologically-activated current. However, deactivation kinetics critically shape Ca\(^{2+}\) influx through action potential-activated CaV channels (Llinas et al., 1981; Llinas et al., 1982; Buraei et al., 2005), and roscovitine significantly slows CaV3.1 channel deactivation, which could potentially offset inhibition. Thus, we determined the effect of 45 µM roscovitine on CaV3.1 current activated by a 2-ms action potential waveform (AP) (Fig. 8). As predicted, roscovitine-induced inhibition dominated with a 53 ± 4% inhibition of charge influx via CaV3.1 channels (Fig. 8 C). Compared with the 53 ± 6% inhibition of step current by 45 µM roscovitine (HP -120 mV, Fig. 3D), it appears that slowed deactivation has little or no effect on the roscovitine-induced inhibition of AP-activated CaV3.1 current.
A separate issue involves the effect of roscovitine to reduce open-state inactivation. We wanted to determine if this reduction would have an impact on AP-activated current, which was accomplished by examining the effect of 45 µM roscovitine on CaV3.1 currents generated during a 10-AP 20-Hz train (Fig. 9). Under control conditions, CaV3.1 current decreased with each AP within the train as expected for accumulated inactivation (termed accommodation, Fig. 9A, B, D), and roscovitine significantly enhanced accommodation over this 10-AP train (Fig. 9D). As a result, roscovitine-induced inhibition was significantly increased from the first to the tenth AP within the train (Fig. 9C, E). Thus, the roscovitine-induced reduction of open state inactivation has little or no effect and is dominated by slowed recovery from inactivation to increase inhibition during the AP train.
Discussion

We have found that roscovitine blocks CaV3.1 channels in a dose-dependent and holding potential-dependent manner. Depolarizing the holding potential from -120 mV to -70 mV decreased the EC₅₀ by four-fold. Given that more than 50% of the channels are inactivated at the -70 mV holding potential, we tested the idea that roscovitine enhanced CaV3.1 channel inactivation. Surprisingly, inactivation measured from current-generating voltages was slowed by roscovitine to yield a small, but significant, decrease of inactivation. Thus, open-state inactivation was not enhanced by roscovitine. However, longer voltage steps (1000 ms) increased inactivation from closed states, which was significantly enhanced by roscovitine. The development of closed-state inactivation was accelerated by roscovitine, while recovery from inactivation was slowed. Thus, increased occupancy of closed-state inactivation is a major mechanism by which roscovitine inhibits T-channel activity.

Roscovitine inhibits CaV3.1 current by preferentially affecting inactivated channels.

We have previously shown that roscovitine-induced inhibition of CaV2.2 and CaV1.2 calcium channels was associated with enhanced voltage-dependent inactivation (Yarotskyy and Elmslie, 2007; Buraei and Elmslie, 2008). For CaV2.2 channels, the roscovitine effect resulted from enhanced closed-state inactivation (Buraei and Elmslie, 2008), while CaV1.2 channel open-state inactivation was selectively affected by roscovitine (Yarotskyy and Elmslie, 2007). Like the CaV2.2 channel, roscovitine-induced CaV3.1 channel inhibition was enhanced at depolarized holding potentials that were associated with a 10 mV left shift in the CaV3.1 channel closed-state inactivation vs. voltage relationship. This enhancement results from roscovitine speeding the development of closed-state inactivation and slowing the recovery from inactivation. The acceleration of closed-state inactivation is in stark contrast to the significant slowing of open-
state inactivation, which mediated a small, but significant, decrease of open-state inactivation. Thus, roscovitine demonstrates that open and closed-state inactivation can be differentially modulated in CaV3.1 channel, which may have clinical benefits. For CaV1.2 channels, the preferential enhancement of closed-state inactivation by dihydropyridine antagonists (e.g. nifedipine or amlodipine) makes them potent antihypertensive drugs without negative cardiac effects (e.g. negative ionotropy or bradycardia) (Elmslie, 2004).

Our data shows that the recovery from inactivation following 1 sec steps to -20 mV was best fit by a double exponential equation in both control and roscovitine supporting multiple components. It is tempting to relate the two recovery components to open-state (fast recovery) and closed-state (slow recovery) inactivation. However, previous work using CaV3.2 channels demonstrated that recovery was well described by a single exponential process and the time constant was similar if inactivation occurred from either the open or closed state (Serrano et al., 1999), which supports a common recovery pathway for these inactivation processes. Serrano et al (1999) used short (60 ms) steps to inactivate CaV3.2 channels from the open state, while our step duration was 1 s for studying recovery kinetics. Our measurement of recovery from closed-state inactivation (1 sec at -70 mV) clearly had multiple components with the majority of current (93%) recovering by 2.2 sec at -120 mV, but full recovery was only achieved within the 15 sec interval between sweeps at -120 mV. Unfortunately, this slow component (7%) was too small for an accurate determination of the time course. It has long been recognized that CaV3 channels can recover from inactivation with multiple components and that the slower recovery component can be increased with longer step durations (Bossu and Feltz, 1986; Herrington and Lingle, 1992). Thus, the slow recovery component may correspond to a slow inactivation state for which occupancy requires longer and stronger depolarization. Importantly, roscovitine
significantly slowed recovery to contribute to the enhancement of closed-state inactivation, and, thus, inhibition.

One concern was how the multiple effects of roscovitine on CaV3.1 channels would impact physiologically-activated current. While our data strongly support the dominance of inhibition for CaV3.1 current activated by voltage steps, deactivation kinetics play an important role in determining Ca\(^{2+}\) influx via action potential-activated CaV channels (Llinas et al., 1981; Llinas et al., 1982; Buraei et al., 2005). Since roscovitine significantly slowed CaV3.1 channel deactivation, we wanted to determine if this had a measureable impact. However, there was no difference between the roscovitine-induced inhibition of CaV3.1 step current vs. AP-activated charge influx. Thus, slowed deactivation was likely too small to significantly impact inhibition.

The differential effect of roscovitine on open-state vs. closed-state inactivation could also have a physiological impact. While the decrease of open-state inactivation is small, it is possible that this effect would be measureable under conditions where open-state inactivation dominates. One such condition could be an AP train, where open-state inactivation is expected to accumulate with each pulse. In our test, we used -120 mV as the interpulse potential to limit the potential impact of closed-state inactivation. However, roscovitine still increased inhibition during the AP train so that current during the tenth pulse was significantly smaller than that of the 1 pulse. This increase is likely the result of the slowed recovery from inactivation induced by roscovitine. Even though open-state inactivation is reduced, the recovery from open-state inactivation at -120 mV is slowed by roscovitine, and it is this slowed recovery that enhances inhibition during the AP train.
Does closed-state inactivation fully explain roscovitine-induced inhibition?

Many T-channel antagonists show enhanced block at depolarized holding potentials including mibebradil (McDonough and Bean, 1998; Martin et al., 2000), octanol (Eckle and Todorovic, 2010), and TTA-A2 (Ubele et al., 2009). However, significant block of CaV3 current at holding potentials that maximally recover CaV3 channel inactivation suggests that closed channel block can also occur, but with lower affinity (McDonough and Bean, 1998). This appears to be the case with roscovitine as well. We observed 50% inhibition at -120 mV induced by 45 µM roscovitine. While this concentration left shifted the inactivation –voltage relationship 10 mV (1000 ms steps), maximal recovery from inactivation was still achieved at -120 mV (Fig. 5A). Thus, it appears that closed CaV3.1 channels are sensitive to block by roscovitine, but this state has at least a four-fold lower affinity for the drug than the closed inactivation state.

One surprise was that the enhancement of closed-state inactivation by roscovitine is common between CaV2.2 and CaV3.1 channels, but open-state inactivation of CaV1.2 channels is selectively enhanced. Sequence comparisons between the three CaV gene families shows much closer homology between the CaV1 and CaV2 families (~52%) compared with the CaV2 and CaV3 families (~28%) (Catterall et al., 2005). Based on this, it is seems more likely that CaV2.2 channels would share a common mechanism with CaV1.2 channels than with CaV3.1 channels. Roscovitine reveals the potential for similar closed-state inactivation mechanisms between the distantly related CaV2.2 and CaV3.1 channels. However, block of closed channels distinguishes CaV3.1 channel, since no inhibition was observed at holding potentials from which CaV2.2 channels were fully recovered from closed-state inactivation (Buraei and Elmslie, 2008).
**Roscovitine is a promising anti-cancer drug that exhibits beneficial polypharmacy.**

Roscovitine is undergoing phase II clinical trials as an anti-cancer drug based on its CDK blocking effect (Meijer et al., 1997; Hahntow et al., 2004; Benson et al., 2007; Wesierska-Gadek et al., 2007). Until very recently, the anti-cancer properties were solely linked to CDK block with a therapeutic window of 10–50 µM (Meijer et al., 1997; Fischer and Gianella-Borradori, 2003; Hahntow et al., 2004). We recently showed that roscovitine’s anti-cancer effect may also involve potassium channel blockade (Ganapathi et al., 2009). HERG potassium channels can regulate cancer cell proliferation, and HERG blockers have been shown to reduce proliferation and invasiveness (Arcangeli, 2005; Masi et al., 2005; Pardo et al., 2005; Raschi et al., 2008). Thus, HERG blockers have been proposed as an adjuvant cancer therapy (Pillozzi et al., 2007; Raschi et al., 2008). Roscovitine blocked HERG channels with EC$_{50}$ = 27 µM, which could complement the CDK inhibition to more potently suppress the cancer cell development. Here we reveal a third potential anti-cancer activity of this drug, which is to inhibit calcium entry through CaV3.1 channels with EC$_{50}$ = 10 µM at the -70 mV holding potential (at the low end of the roscovitine therapeutic window). While polypharmacy was once thought to be an undesirable property for a drug, recent insights have revealed important benefits of multiple drug actions in treating disease, and polypharmacy has gained new importance in the pharmaceutical industry (Hopkins, 2008; Howitz and Sinclair, 2008; Yang et al., 2008).

CaV3 channels appear to support abnormal calcium entry to enhance proliferation of cancer cells and CaV3 channel blockers are promising anti-cancer drugs (Gray and Macdonald, 2006; Lee et al., 2006; Heo et al., 2008; Lu et al., 2008; Taylor et al., 2008a; Taylor et al., 2008b). Roscovitine more potently blocks CaV3.1 channels in the closed inactivated state, which increases its affinity at more depolarized holding potentials. Changes in membrane potential that
are controlled by potassium channel activity (e.g. HERG) appear to be critical in the control of cell proliferation by enhancing calcium entry and controlling cell volume (Pardo, 2004). Roscovitine block of potassium channel activity would depolarize the cancer cell to increase CaV3.1 channel closed-state inactivation and enhance roscovitine-inhibition of CaV3.1 channel activity. Thus, the polypharmacy action of roscovitine could have synergistic benefits.

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Authorship Contributions

Participated in research design: Yarotskyy and Elmslie

Conducted experiments: Yarotskyy and Elmslie

Contributed new reagents or analytic tools: None

Performed data analysis: Yarotskyy and Elmslie

Wrote or contributed to the writing of the manuscript: Yarotskyy and Elmslie
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Footnotes

1 Current address Department of Pharmacology and Physiology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642

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Legends for figures

**Figure 1.** Roscovitine blocks CaV3.1 channels.  
**A,** Typical currents recorded in control (gray, Cntl), 45 µM roscovitine (black, Rosc), and washout (gray, WO). The voltage protocol is shown below the currents.  **B,** Step current vs. voltage (I-V) relationship from the cell in panel A demonstrates the inhibitory effect of 45 µM roscovitine (black squares, Rosc) relative to control (gray circles, Cntl) and washout (gray triangle, WO) across all voltages that evoked current.  **C,** The mean percentage inhibition (±SD, n = 9) induced by 45 µM roscovitine was calculated from the average of control and washout currents for voltages that generated current. The flat relationship indicates no voltage-dependence of peak current inhibition, which was supported by statistical analysis (ANOVA) showing no statistical differences across the voltage range.  **D,** Normalized tail current amplitude measured 0.3 ms after repolarization to -100 mV is plotted against step voltage for data from the cell in panel A. Symbols have the same meaning as for panel B. The smooth lines are single Boltzmann function fits, which yielded \( V_{0.5} = -41.0 \text{ mV}, -39.4 \text{ mV}, \) and \(-40.4 \text{ mV},\) and slope factor = 5.54, 6.22, and 5.83 for control, 45 µM roscovitine, and washout, respectively. Horizontal dashed line shows maximal open state occupancy.

**Figure 2.** Roscovitine slowed deactivation of CaV3.1 channels.  **A, B,** Typical currents recorded in control (gray trace), 45 µM roscovitine (black trace, Rosc), and washout (smaller gray trace). The repolarizing step was -70 mV following a depolarizing 10 ms step to -10 mV. The currents in panel A were normalized to peak tail current (**B**) to highlight the slower deactivation induced by roscovitine.  **C,** The deactivation time constant (\( \tau_{\text{Deact}} \)) was determined by fitting each tail current with a single exponential function. \( \tau_{\text{Deact}} \) was plotted against repolarizing (tail) voltage to
gauge the speed of deactivation over voltages ranging from -50 to -160 mV. Roscovitine significantly increased $\tau_{\text{Deact}}$ at voltages depolarized to -140 mV (black line, $p < 0.05$, $n = 9$). Data are shown as mean ± SD for control (gray circles), 45 µM roscovitine (black squares), and washout (gray triangles). D. The percentage change in $\tau_{\text{Deact}}$ (%$\Delta\tau_{\text{Deact}}$) induced by roscovitine (from panel C) was calculated from the average of control and washout data. The mean ± SD are shown with the data significantly different from zero indicated by the line ($p < 0.05$).

**Figure 3.** Roscovitine block is more potent at depolarized holding potentials. A, C, Representative traces show the inhibitory effect of roscovitine at -120 mV (A) and -70 mV (C) holding potential. Current was elicited by 100 ms steps to -20 mV from either -120 mV (A) or -70 mV (C). The smooth line on the control current of panel A is a single exponential fit with $\tau = 19$ ms (see Fig. 4). (C). The numbers at the left of each trace indicate the applied roscovitine concentration (µM). B, The time course for roscovitine inhibition of peak current from the same cell shown in panel A. The interval between sweeps is 5 s. The horizontal lines indicate the duration of roscovitine application for the given concentration (in µM). D, Fractional block is plotted versus roscovitine concentration. Data collected at -120 mV ($n = 5$) and -70 mV ($n = 6$) holding potentials (HP) are shown as filled and open circles, respectively. Smooth lines are Hill’s equation fits, which yielded $EC_{50} = 40$ µM and 10 µM, and Hill’s coefficient 1.53 and 1.21 for HP = -120 mV and -70 mV, respectively.

**Figure 4.** Roscovitine slows open-state inactivation. A, Open-state inactivation was investigated using a voltage protocol consisting of 100 ms inactivating steps to voltages ($V_{\text{Inact}}$) ranging from -120 mV to 40 mV followed by 20 ms postpulse to -20 mV to assess channel availability. The
postpulse current was normalized to that at -120 mV (I/I_{Max}) and plotted versus V_{Inact} in control (Cntl, gray circle), 45 µM roscovitine (Rosc, black square) and upon washout (WO, gray triangle) (n = 6). The smooth lines are fits to the inactivation data using the Boltzmann equation, which yielded V_{0.5} = -53.8 mV, -53.7 mV, and -52.6 mV, slope factor = -7.4, -8.7, and -7.4 and maximum inactivation = 0.91, 0.85, and 0.90 for control, 45 µM roscovitine, and washout, respectively. Roscovitine significantly increased inactivation at -80 and -70 mV, but significantly decreased inactivation at voltages ≥ -40 mV as indicated by the lines and asterisks (p < 0.05). B, The inactivating component of the current was measured by fitting a single exponential equation to current elicited by V_{Inact}. The inactivation time constant (τ_{Inact}) is plotted versus V_{Inact} for control, 45 µM roscovitine and washout. See figure 3A for a representative single exponential fit to inactivation over a 100 ms voltage step. The symbols have the same meaning as in panel A. τ_{Inact} in roscovitine was significantly larger than control and washout for voltages ≥ -30 mV as indicated by the line and asterisk. C, τ_{Inact} in control and washout was averaged and used to determine the percentage change induced by 45 µM roscovitine (from the data in panel B). The percentage change in τ_{Inact} (%ΔV_{Inact}) is plotted versus V_{Inact}. The line and asterisk indicate data significantly different from zero (p < 0.05).

Figure 5. Roscovitine enhances closed-state inactivation. A, For this study the voltage protocol was similar to that described in figure 4 except that the V_{Inact} range was from -140 to 0 mV and the duration was 1000 ms. The postpulse current was normalized to that at -140 mV (I/I_{Max}) and plotted against V_{Inact} for control (Cntl, gray circles), 45 µM roscovitine (Rosc, black squares), and washout (WO, gray triangles). The smooth lines are single Boltzmann function fits, which yielded V_{0.5} = -73.5 mV, -82.7 mV, and -73.1 mV and slope factor = -7.6, -10.7, and -8.1 for
control, roscovitine, and washout, respectively. Roscovitine significantly increased inactivation at voltages from -120 mV to -40 mV, but significantly decreased inactivation at voltage > -50 mV (p < 0.05). B, Boltzmann fitting parameters were averaged from the 6 cells used to generate panel A and shows the significant roscovitine-induced decrease in $V_{0.5}$ (left panel), increase in slope factor (middle panel) and decrease in maximum inactivation (right panel) (*, p < 0.05). Roscovitine data are shown in the black bars (R), while control (C) and washout (W) are shown in gray bars.

Figure 6. Roscovitine speeds the development of close-state inactivation. A, The voltage protocol to investigate the development of inactivation at voltages hyperpolarized to channel activation consisted of two stimuli, prepulse (pre) and postpulse (post), to -20 mV that bracketed a -70 mV step with duration varying from 10 ms to 1900 ms. B, The postpulse to prepulse current ratio ($I_{Post}/I_{Pre}$) was plotted against the inactivating step duration and the data were fitted by single exponential function to yield the time constant of close-state inactivation ($\tau_{CSI}$), and residual steady-state current ($I_{SS}$). Symbols have the same meaning as for figure 5 (n = 5). C, Roscovitine significantly decreased $\tau_{CSI}$ (R, 45 µM, black), compared to control (C, white) and washout (WO, gray). D, $I_{SS}$ was significantly decreased in 45 µM roscovitine (black, R), compared to control (white, C) and washout (gray, WO) (n = 5, p < 0.05).

Figure 7. Roscovitine slows the recovery from inactivation. A, Inactivation was induced by a 1000 ms step to -20 mV followed by steps to -120 mV varying from 10 ms to 1000 ms and a 20 ms postpulse to -20 mV to assess channel availability. For clarity, tail currents following the postpulse were removed to more clearly illustrate the recovery from inactivation for control (top
traces) and in 45 µM roscovitine (bottom traces). 

**B,** Postpulse currents were normalized to the peak current from the inactivating step and plotted versus recovery time at -120 mV to show the time course for recovery from inactivation for control (gray circles), 45 µM roscovitine (black squares), and washout (gray triangles) (n = 5). The 15 s interval between sweeps provided full recovery from inactivation so that was included as the maximum recovery point. The smooth lines are double exponential function fits that yielded \( \tau_{\text{fast}} = 118 \text{ ms}, 246 \text{ ms}, \text{ and } 129 \text{ ms}, \text{ and } \tau_{\text{slow}} = 593 \text{ ms}, 1074 \text{ ms}, \text{ and } 691 \text{ ms} \) for control, 45 µM roscovitine, and washout, respectively. The amplitude of slow component relative to the fast recovery component \( (A_{\text{slow}}/A_{\text{fast}}) \) was 1.4, 3.3, 1.3 for control, roscovitine and washout, respectively. 

**C,** The recovery from closed-state inactivation was investigated using a triple pulse protocol where 15 ms steps to -20 mV bracketed a 1000 ms step to -70 mV. For the \( I/I_{\text{Max}} \) ratio, \( "I_{\text{Max}}" \) was measured during the prepulse (before the -70 mV step) and \( "I" \) was measured from the postpulse (following the -70 mV step). The interval between sweeps was 15 sec, which provided full recovery from inactivation. The smooth lines are single exponential fits to the data with \( \tau = 402, 609 \text{ and } 409 \text{ ms} \) for control, 45 µM roscovitine and washout, respectively. The fractional amplitude of the recovery component was 0.59, 0.72 and 0.58 for control, roscovitine and washout, respectively. The recovery at 2200 ms was 95%, 92% and 92% of the maximal value (at 15 sec) for control, roscovitine and washout, respectively. 

**D,** The recovery \( \tau \) following 1000 ms steps to -70 mV was averaged from 8 cells. The gray bars show control (C) and washout (W) data, while the black bar shows data in 45 µM roscovitine (R). Data are presented as mean ± SD (*, p < 0.05).

**Figure 8.** Roscovitine inhibits physiologically activated CaV3.1 current. 

**A,** CaV3.1 current was elicited by a 2-ms action potential waveform (AP) that ranged from -80 to +40 mV. The holding
potential was -120 mV and the voltage was -80 mV for 1 ms prior to the start of the AP, which was too short to inactivate CaV3.1 channels. 45 µM roscovitine (Rosc, black trace) inhibited the current relative to that of control (Cntl, gray trace) and washout (WO, gray trace). B, CaV3.1 current was integrated from the onset of inward current to the end of the record (12 ms) to determine the charge influx (pC). Roscovitine (45 µM) was applied as indicated by the black bar and the interval between sweeps was 5 sec. C, The mean ± SD percent inhibition induced by 45 µM roscovitine is shown (p < 0.05). The control value for calculating inhibition was the average of current before and upon recovery from roscovitine.

Figure 9. Increased action potential frequency enhances roscovitine-induced inhibition of CaV3.1 current. A, Currents were generated by a 20-Hz AP train (HP -120 mV) in control (Cntl, gray) and 45 µM roscovitine (Rosc, black). For clarity, currents generated upon washout of roscovitine are not shown, but see panel B. The smooth lines are single exponential fits to the data in panel B. B, Current induced by each AP was integrated over 15 ms and plotted vs. the AP number within each train. The smooth lines are single exponential fits to the data. Data are shown in control (upward triangle), roscovitine (black square) and upon washout (downward triangle) from the same cell in panel A. C, The roscovitine-induced inhibition was calculated (from panel B) for each action potential generated current in the train. Note the increase in roscovitine-induced inhibition over the AP train. D, Accommodation was calculated as the decrease in current from the first to the last AP in the train. The mean (± SD, n = 5) percent accommodation is shown for control (C), 45 µM roscovitine (R) and upon recovery (WO). The asterisk indicates significant difference from control (average of C and WO; p < 0.05). E, The roscovitine-induced inhibition of integrated current was calculated (as in panel C) for the first
and tenth sweep in the action potential train. The average percent inhibition (±SD, n = 5) induced by 45 μM roscovitine is shown. The asterisk indicates that the inhibition of the 10th sweep is significantly (p < 0.05) larger than that of the first sweep.
Figure 1

A. Diagram showing currents with labels Rosc, Cntl&WO, and a scale of 0.5 nA and 5 ms.

B. Graph showing voltage (mV) with points for Cntl, Rosc, and WO, with current values of 0.3 and 0.6 nA.

C. Graph showing percentage inhibition with voltage range from -60 to 20 mV.

D. Graph showing normalized tail current (I_Tail) with voltage range from -80 to 0 mV and a value of 0.5.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Figure 8
Figure 9
Roscovitine inhibits CaV3.1 (T-type) channels by preferentially affecting close-state inactivation.

Viktor Yarotskyy and Keith S. Elmslie

Journal of Pharmacology and Experimental Therapeutics

Supplemental Figure 1 – Roscovitine fails to affect CaV3.1 activation kinetics. A, CaV3.1 currents activated by a step to -20 mV are shown in control (Cntl, gray trace) and 45 µM roscovitine (Rosc, black trace). B, The traces in panel A were normalized to peak to highlight the absence of an effect of roscovitine on activation kinetics. C, CaV3.1 current activation was fit using a single exponential equation to determine the activation
The fit was constrained to the activating portion of the current that was 0.3 ms following the onset of the voltage step to the peak. Data are mean ± SD from control (gray circle), 45 µM roscovitine (black square) and washout (gray triangle). There were no significant differences between roscovitine and control (average of Cntl and WO data).