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ORIGINAL RESEARCH

Involvement of Rho-Associated Coiled-Coil Containing Kinase (ROCK) in BCR-ABL1 Tyrosine Kinase Inhibitor Cardiovascular Toxicity

Brian Yu, BA,[a,*] Afaf E.G. Osman, MD,[b,*] Nikola Sladojevic, MD, PhD,[c] Nicole Prabhu, MD,[c] Haw-Chih Tai, PhD,[c] Daiqing Chen, MS,[c] Gerardo Perla, BA,[c] Linus Park, BA,[c] Richard A. Larson, MD,[d] James K. Liao, MD[c]

ABSTRACT

BACKGROUND Second- and third-generation BCR-ABL1 tyrosine kinase inhibitors (TKIs) are associated with cardiovascular adverse events (CVAEs) in patients with Philadelphia chromosome-positive (Ph+) leukemia.

OBJECTIVES We hypothesized that second- and third-generation BCR-ABL1 TKIs may cause CVAEs through the activation of Rho-associated coiled-coil containing kinase (ROCK).

METHODS Peripheral blood mononuclear cells from 53 Ph+ patients on TKIs and 15 control patients without Ph+ leukemia were assessed for ROCK activity through capillary electrophoresis (median follow-up = 26 months [Q1-Q3: 5-37 months]). We also investigated the effects of TKIs and ROCK on endothelial dysfunction in vitro, which could contribute to CVAEs.

RESULTS Patients receiving second- and third-generation TKIs had 1.6-fold greater ROCK activity compared with patients receiving imatinib and control patients. Elevated ROCK activity was associated with an increased incidence of CVAEs in Ph+ leukemia patients. In endothelial cells in vitro, we found that dasatinib and ponatinib treatment led to changes in actin intensity and endothelial permeability, which can be reversed by pharmacologic inhibition of ROCK. Ponatinib led to decreased cell proliferation, but this was not accompanied by senescence. Dasatinib and ponatinib treatment led to phosphor-inhibition of endothelial nitric oxide synthase and decreased nitric oxide production. ROCK inhibition reversed endothelial permeability and endothelial nitric oxide synthase–related endothelial dysfunction. Imatinib and nilotinib induce phosphorylation of p190RhoGAP.

CONCLUSIONS Our findings suggest ROCK activity may be a prognostic indicator of CVAEs in patients receiving BCR-ABL1 TKIs. With further study, ROCK inhibition may be a promising approach to reduce the incidence of CVAEs associated with second- and third-generation BCR-ABL1 TKIs. (J Am Coll Cardiol CardioOnc 2022;4:371–383) © 2022 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

From the [a]Stanford Cardiovascular Institute, Stanford University, Stanford, California, USA; [b]Department of Hematology and Hematologic Malignancies, University of Utah, Salt Lake City, Utah, USA; [c]Section of Cardiology, Department of Medicine, University of Chicago, Chicago, Illinois, USA; and the [d]Section of Hematology/Oncology, Department of Medicine and the Comprehensive Cancer Center, University of Chicago, Chicago, Illinois, USA. *Drs Yu and Osman contributed equally to this work and are co-first authors.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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ABBREVIATIONS AND ACRONYMS

ALL = acute lymphoblastic leukemia

CVAE = cardiovascular adverse event

CML = chronic myeloid leukemia

eNOS = endothelial nitric oxide synthase

PBMC = peripheral blood mononuclear cell

Ph+ = Philadelphia chromosome-positive

ROCK = Rho-associated coiled-coil containing kinase

TKI = tyrosine kinase inhibitor

2G/3G = second/third generation

Chronic myeloid leukemia (CML) and Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) are neoplasms characterized by a reciprocal translocation (t[9;22][q34;q11.2]) that fuses the *ABL*1 gene with the *BCR* gene. The resulting Ph+ chromosome contains BCR-ABL1, a constitutively active tyrosine kinase that drives leukemic transformation and proliferation. The development of imatinib, the first BCR-ABL1-targeted tyrosine kinase inhibitor (TKI), dramatically increased patient survival. Subsequently, highly active second-generation (2G) TKIs (ie, dasatinib, nilotinib, and bosutinib) were developed to overcome acquired resistance or intolerance to imatinib. Ponatinib, a third-generation (3G) TKI, was developed with the goal of overcoming resistance to imatinib and 2G TKIs and was the first TKI approved for Ph+ leukemias with the BCR-ABL1^{T315I} gatekeeper mutation. The second TKI to be approved for the BCR-ABL1^{T315I} gatekeeper mutation was asciminib, which was approved in 2021 for Ph+ CML. Second-generation and 3G TKIs overcome resistance to imatinib and produce higher rates of major molecular responses.[1] This enhanced efficacy has been accompanied by vascular toxicity and cardiovascular adverse events (CVAEs).[2-6]

In clinical trials, ponatinib, and to a lesser extent dasatinib and nilotinib, have been associated with the development of coronary artery disease (CAD)[4] and arterial thrombotic events,[1] including cerebral vascular events and myocardial infarction.[3-5] Indeed, in 5-year follow-up analyses of the phase 2 PACE (Ponatinib for Chronic Myeloid Leukemia Evaluation and Ph+ Acute Lymphoblastic Leukemia) trial, the cumulative incidence of arterial occlusive events in CML and Ph+ ALL patients receiving ponatinib was 25%.[1,3] However, imatinib is the only BCR-ABL1 TKI without demonstrable cardiac toxicity.[1,4]

The mechanisms underlying the cardiovascular toxicity associated with TKIs remain incompletely understood. The ABL-related gene ARG (also known as ABL2), a tyrosine kinase closely related to ABL1, is a target for 2G and 3G TKI inhibition.[7,8] An important downstream target of ARG is p190RhoGAP,[9] which could be the mechanism by which ABL kinases regulate cell motility, cell-cell contact, and the actin cytoskeleton. One of the primary actions of p190RhoGAP is its ability to inhibit the Rho/Rho-associated coiled-coil containing kinase (ROCK) pathway.[9] Both isoforms of ROCK, ROCK1 and ROCK2, have been implicated in a wide range of cardiovascular[10] and cerebrovascular disorders[11] through diverse mechanisms, including endothelial dysfunction.[10] Consequently, in this study, we hypothesized that increased ROCK activity is involved in TKI-induced vascular adverse events.

TABLE 1 Ph+ Leukemia Patient Characteristics[a]

Ph+ CML and Ph+ ALL Patients (N = 53)	
Baseline characteristics	
Female sex	24 (45)
Mean age, y	54 ± 16
CML phase (n = 49)	
Chronic phase	15 (31)
Chronic phase (newly diagnosed, untreated)	8 (16)
Complete hematologic response	2 (4)
Complete cytogenetic response	4 (8)
Major molecular response (<0.1% detectable BCR/ABL1 transcripts)	14 (28)
Complete molecular response (undetectable BCR/ABL1 transcripts)	13 (27)
Accelerated phase	1 (2)
ALL phase (n = 4)	
Less than major molecular response	1 (25)
Major molecular response (<0.1% detectable BCR/ABL1 transcripts)	1 (25)
Complete remission (undetectable BCR/ABL1 transcripts)	2 (50)
Therapies and follow-up events	
Median duration of total TKI therapy, mo	58 (20-99)
Actively receiving statin therapy	15 (28)
Duration of follow-up, mo	26 (5-37)
Vascular adverse events (cumulative incidence at 3 years)[b]	7 (16.3)
Deaths (cumulative incidence at 3 years)[c]	2 (5.5)

Values are n (%), mean ± SD, or median (Q1-Q3). [a]Abstracted clinical characteristics are from initial blood draw. [b]Cumulative incidence calculated from Fine-Gray's model with competing risk of all-cause mortality. [c]Cumulative incidence calculated from the Kaplan-Meier model.

ALL = acute lymphoblastic leukemia; CML = chronic myeloid leukemia; Ph+ = Philadelphia chromosome-positive; TKI = tyrosine kinase inhibitor.

METHODS

For detailed methods and materials, including peripheral blood mononuclear cell (PBMC) isolation, in vitro and cellular assays, and catalog numbers, refer to the Supplemental Methods section.

PATIENT RECRUITMENT. A total of 53 patients with Ph+ CML and Ph+ ALL were recruited between May 2016 and April 2022 (**Table 1**). The inclusion criteria included any patient with Ph+ CML and Ph+ ALL as confirmed by cytogenetics and a polymerase chain reaction assay, patients receiving/soon to be receiving TKI or in treatment-free remission, and patients ≥18 years of age who were treated at the University of Chicago Medical Center. ROCK activity was evaluated at sequential clinic visits for a subset of

patients who were followed longitudinally at our outpatient clinic. We defined CVAEs as any cerebrovascular accident, transient ischemic attack, myocardial infarction, CAD, or peripheral arterial disease (PAD), including any of these requiring intervention. We considered new-onset CVAEs as the first event/intervention after starting a BCR-ABL1 TKI or, in the case of CAD and PAD, as patients without a diagnosis of CAD and/or PAD at baseline who were later diagnosed with CAD and/or PAD by noninvasive and invasive testing. A total of 15 patients were included as a control group and were recruited between January 2015 and August 2019. The inclusion criteria for the control group were as follows: no prior diagnosis of CML or Ph+ ALL, left ventricular ejection fraction ≥50%, and age ≥18 years. Events were abstracted from electronic medical records and phone follow-up. Two patients were lost to follow-up after blood draw and were not included in the Kaplan-Meier analyses.

In total, we studied the ROCK activity of 49 adult patients with Ph+ CML and 4 patients with Ph+ ALL. At baseline, 8 patients were sampled at the initial diagnosis before starting TKI therapy (7 of whom were later sampled on TKI), and 39 were initially sampled while actively receiving TKI therapy. Patients were classified as being on a particular TKI drug if they had been receiving that TKI for more than 1 month and off TKI if they had been off TKI for more than 1 month. Patients in treatment-free remission were sampled at least 1 month after stopping TKIs (median = 9 months [Q1-Q3: 3-45 months]).

All study participants provided written informed consent. The study protocol was approved by the Institutional Review board at the University of Chicago Medical Center. This study complies with the Declaration of Helsinki.

ASSESSMENT AND ANALYSIS OF PBMC ROCK ACTIVITY. ROCK activity evaluation was performed using an automated capillary electrophoresis system (Wes, ProteinSimple) with antibodies to phospho-Thr^{853} myosin phosphatase target subunit 1 (MYPT1; 1:250 [#36-003, Sigma-Aldrich]) and MYPT1 (1:250 [#925101, BioLegend]), respectively. Capillary electrophoresis was chosen to evaluate ROCK activity in patient samples because of the fast turnaround time (3 hours) and low amount of patient protein required per run (1 μg). If more than 1 blood sample was collected while on the same TKI, the ROCK activity values for that patient were averaged for the purposes of subsequent analyses. The exception was with Kaplan-Meier analyses, where only the first ROCK activity reading for patients on TKI was used to examine the use of ROCK activity as a prognostic indicator of future vascular adverse events.

STATISTICAL METHODS. Variables are presented as mean ± SD for normally distributed variables or median with 25th to 75th percentiles (Q1-Q3) for non-normally distributed variables. Comparisons between normally and non-normally distributed continuous variables were conducted with Welch's *t*-test (unequal variance) or the Mann-Whitney *U* test, respectively. Significant differences in multiple comparisons were calculated by Holm-Sidak's multiple comparisons test. Chi-square or Fisher exact tests were used to evaluate significance in categoric variables. Event-free survival was estimated with the Kaplan-Meier method and compared with the log-rank test or a Fine-Gray competing risk model. HRs with 95% CIs for patients above and below the third quartile of ROCK activity were calculated with the use of a Cox proportional hazards survival model. In order to adjust for potential confounding, we performed additional Cox analyses with adjustment for age and body mass index (BMI). A 2-sided P value < 0.05 was considered statistically significant. All statistical analyses were performed using R version 3.6.3 (R Foundation for Statistical Computing) or GraphPad Prism version 8.0 (GraphPad Software).

RESULTS

CLINICAL CHARACTERISTICS. Patients on 2G/3G TKIs were significantly younger than patients on imatinib or control patients without Ph+ leukemia (**Tables 1 and 2**). Otherwise, there were no major differences in cardiovascular risk factors. The duration of TKI therapy was not significantly different in patients receiving imatinib and patients receiving 2G/3G TKIs (**Table 2**). Several patients switched TKIs during the study (Supplemental Table 1).

ROCK ACTIVITY IN PH+ LEUKEMIA PATIENTS. To investigate how the activity of ROCK changes with BCR-ABL1 TKI therapy in patients with Ph+ leukemia, we isolated PBMCs from 53 patients with Ph+ CML or Ph+ ALL and 15 control patients and evaluated the ratio of phosphorylated Thr^{853} on MYPT1 to the total MYPT1 protein through capillary electrophoresis. ROCK is the only known kinase of Thr^{853} on MYPT1.[12] Patients actively receiving 2G and 3G TKIs had 1.6-fold greater ROCK activity relative to control patients ($P < 0.001$) and patients actively receiving imatinib ($P < 0.001$) (**Figure 1A**). Patients receiving nilotinib, dasatinib, or ponatinib have significantly greater ROCK activity compared with patients receiving imatinib (Supplemental Figure 1). Patients with newly diagnosed CML who had not yet started

TABLE 2 Cardiovascular Risk Factors by TKI Treatment/Generation[a]

	Control (n = 15)	Imatinib (n = 11)	2G/3G Generation TKI (n = 35)	Control Versus 2G/3G TKI *P* Value	Imatinib Versus 2G/3G TKI *P* Value
Age, y	59 ± 13	63 ± 15	49 ± 15	0.028	0.009
Female	8 (53)	7 (64)	14 (40)	0.76	0.53
BMI, kg/m^2	35.0 ± 11.9	28.7 ± 5.0	31.1 ± 8.3	0.18	0.35
SBP, mm Hg	133 ± 29	125 ± 16	132 ± 17	0.88	0.21
Duration on TKI therapy, mo	–	65 (47-116)	51 (17-99)	–	0.40
Medical condition					
Hypertension	7 (47)	6 (54)	15 (43)	>0.99	0.73
Type 2 diabetes	3 (20)	1 (9)	7 (20)	>0.99	0.66
Atrial fibrillation	1 (7)	1 (9)	1 (3)	0.51	0.43
CKD stage 4/5	0 (0)	1 (9)	1 (3)	>0.99	0.43
Laboratory values[b]					
Total cholesterol, mg/dL	171 ± 42	172 ± 35	174 ± 67	0.86	0.94
HDL, mg/dL	57 ± 20	61 ± 24	55 ± 24	0.71	0.53
LDL, mg/dL	92 ± 34	93 ± 28	95 ± 52	0.83	0.89
Triglycerides, mg/dL	111 ± 68	117 ± 69	127 ± 69	0.48	0.72
ESR, mm/h	55.5 ± 49	18.5 ± 7.8	23.2 ± 21.2	0.12	0.77
CRP, mg/L	5.1 ± 4.6	4.2 ± 1.4	10.3 ± 19.7	0.57	0.40
Framingham Risk Score, %[b]	17.1 (9.9-29.6)	14.1 (8.8-17.5)	12.4 (4.1-20.7)	0.080	0.79
QRISK3, %[c]	4.5 (2.6-7.0)	5.3 (1.7-17.6)	4.4 (1.2-11.0)	0.80	0.20

Values are mean ± SD, n (%), or median (Q1-Q3). [a]Clinical or laboratory values were abstracted from date of initial blood draw. For patients whose clinical or laboratory values were not assessed at initial blood draw, the most proximal value to their initial blood draw was recorded. [b]Total cholesterol, HDL, LDL, and triglyceride values were missing from 1 patient in the control group, 4 patients in the imatinib group, and 12 patients in the 2G/3G generation TKI group. This prevented Framingham Risk Score calculations from the same patients in each group. ESR values were available for 2 patients in the control group, 2 patients in the imatinib group, and 11 patients in the 2G/3G generation TKI group. CRP values were available for 5 patients in the control group, 8 patients in the imatinib group, and 18 patients in the 2G/3G generation TKI group. [c]QRISK3 scores could not be calculated for 6 patients in the control group, 3 patients in the imatinib group, and 3 patients in the 2G/3G TKI group.

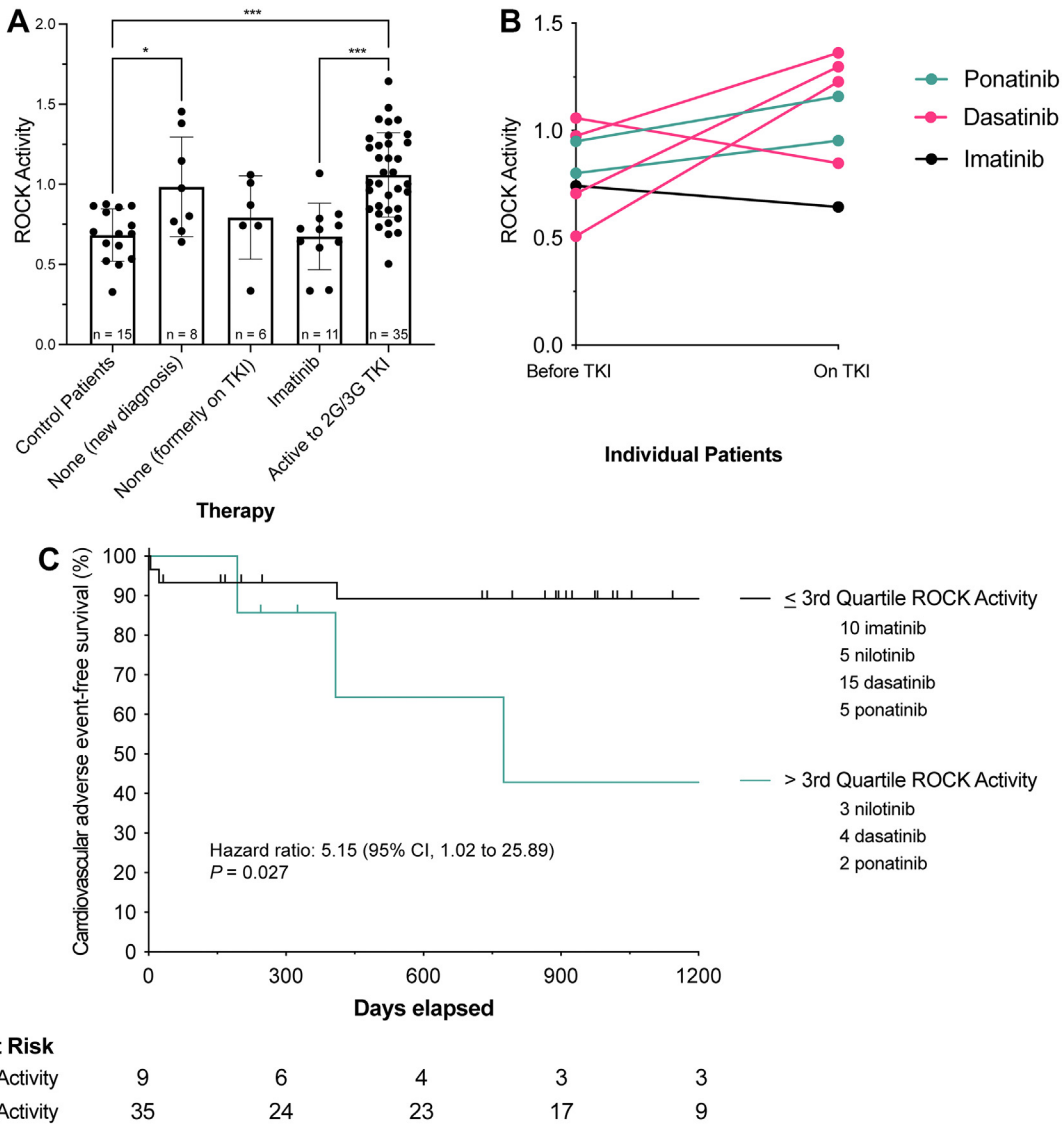
2G/3G = second or third generation; BMI = body mass index; CKD = chronic kidney disease; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; HDL = high-density lipoprotein; LDL = low-density lipoprotein; SBP = systolic blood pressure; TKI = tyrosine kinase inhibitor.

TKI therapy also had elevated ROCK activity. In patients whose ROCK activities before and during TKI treatment were obtained, TKI treatment resulted in robust increases in ROCK activity for 3 patients who were started on dasatinib and 2 patients who were started on ponatinib. However, 1 patient who was started on dasatinib and 1 patient who was started on imatinib showed slightly decreased ROCK activity (**Figure 1B**). Overall, these results suggest that patients treated with 2G and 3G TKIs have higher ROCK activity.

The cumulative incidence of vascular events was calculated from Fine-Gray's model with consideration for the competing risk of all-cause mortality (**Table 1**). Kaplan-Meier survival curves (median follow-up 26 months [Q1-Q3: 5-37 months]) were constructed to evaluate if the first ROCK activity reading in patients actively receiving TKIs was associated with subsequent vascular adverse events (**Figure 1C**). A total of 4 individuals above the third quartile (1.24) of ROCK activity had vascular adverse events, whereas 3 individuals at or below the third quartile ROCK activity had vascular adverse events (HR: 5.15; 95% CI: 1.02-25.89; $P = 0.027$ by the log-rank test) (**Figure 1C,** Supplemental Table 2). After adjustment for age[13] and BMI,[14] 2 confounders of ROCK activity, HR was 7.1 (95% CI: 1.10-46.08; $P = 0.039$). These results suggest that elevated ROCK activity may be an indicator of future vascular adverse events in patients receiving TKIs.

BCR-ABL1 TKIs INCREASE ROCK ACTIVITY AND ALTER F-ACTIN DISTRIBUTION IN ENDOTHELIAL CELLS. Given that patients receiving 2G and 3G TKIs had much greater levels of ROCK activity, we hypothesized that 2G and 3G TKIs may induce vascular adverse events[1,4] through their actions on human endothelial cells. Treatment of endothelial cells with nilotinib, dasatinib, and ponatinib caused dose-dependent increases in ROCK activity at 24 hours (**Figures 2B to 2D**), whereas ROCK activity did not change in endothelial cells treated with imatinib (**Figure 2A**). The maximal ROCK activity response was at 250 nmol/L for ponatinib (1.8-fold; $P < 0.001$), 1,000 nmol/L for dasatinib (2.1-fold; $P < 0.001$), and 1,000 nmol/L for nilotinib (2.0-fold; $P < 0.001$). Given that ponatinib is considered the most cardiotoxic TKI,[4] we sought to evaluate the equimolar effects of each TKI on endothelial function. Accordingly, we used 250 nmol/L as the TKI concentration for the remainder of our experiments. For imatinib, nilotinib,

FIGURE 1 ROCK Activity Association With 2G/3G BCR-ABL1 TKI Treatment and Cardiovascular Adverse Events

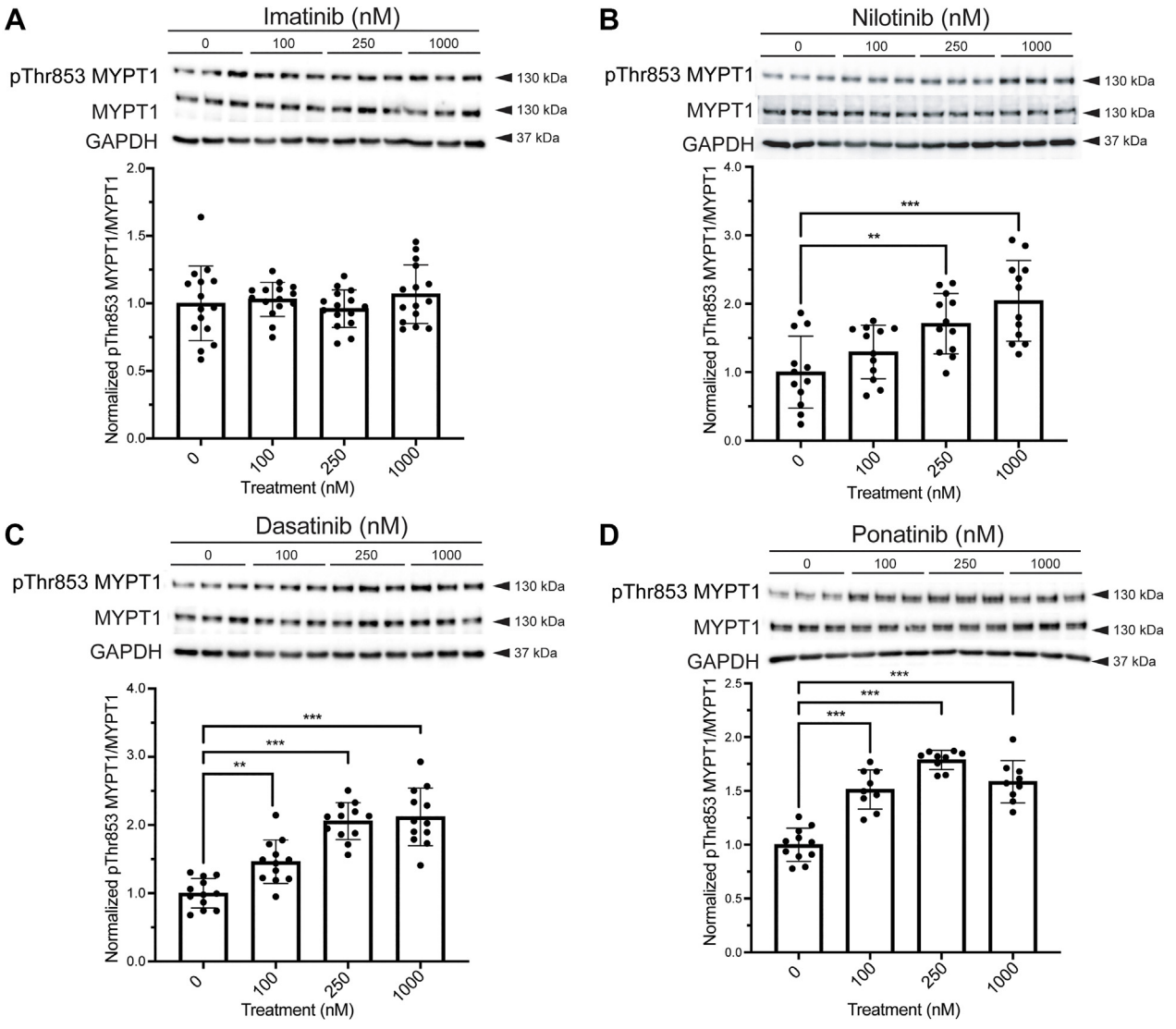


Rho-associated coiled-coil containing kinase (ROCK) activity was assessed from patient peripheral blood mononuclear cells by capillary electrophoresis by taking the ratio of phospho-Thr^{853} myosin phosphatase target subunit 1 (MYPT1) to MYPT1. **(A)** ROCK activity of patients by therapy. Patients who switched between tyrosine kinase inhibitors (TKIs) were included corresponding to the most recent TKI actively received. If a patient initially not on TKI was later active on TKI, they were included in both groups (n = 7). **(B)** ROCK activity change for patients before and during TKI initiation (n = 7). **(C)** Kaplan-Meier survival curves in TKI-receiving patients comparing the incidence of cardiovascular adverse events (CVAEs) between patients with ROCK activities above/below the third quartile (1.24). The *P* value is from the log-rank test. Error bars represented as SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

dasatinib, and ponatinib, a concentration of 250 nmol/L is sufficient to significantly inhibit most off-target tyrosine kinases in vitro, including *ARG* (*ABL2*).[7,8,15]

The activation of ROCK plays a critical role in actin cytoskeletal dynamics in endothelial cells, leading to prominent cytoplasmic F-actin stress fiber formation.[10] These changes in actin cytoskeletal dynamics contribute to endothelial dysfunction.[16] Consequently, we evaluated the actin in endothelial cells treated with TKIs. The treatment of endothelial cells with ponatinib and dasatinib led to a striking formation of F-actin stress fibers and increased actomyosin formation relative to control cells (**Figures 3A and 3B**). In contrast, neither nilotinib nor imatinib affected F-actin stress fiber intensity. The increase in

FIGURE 2 Second-/Third-Generation BCR-ABL1 TKIs Increase ROCK Activity in Endothelial Cells

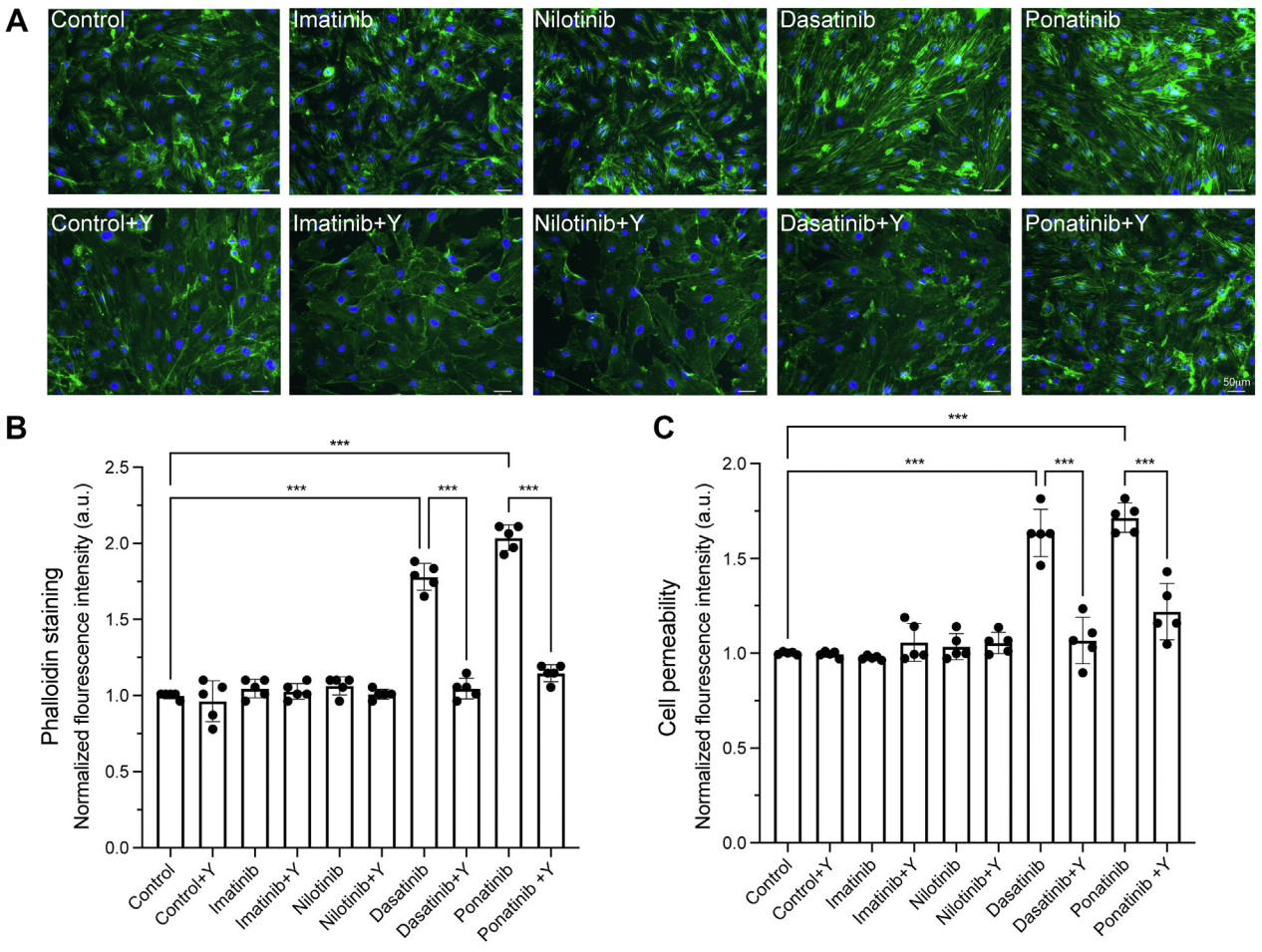


Immunoblots and quantification of ROCK activity (the ratio of phosphor-Thr853 MYPT1 to total MYPT1) of human aortic endothelial cells treated with 0 to 1,000 nmol/L of **(A)** imatinib, **(B)** nilotinib, **(C)** dasatinib, and **(D)** ponatinib for 24 hours. $n = 10$ to 15 per treatment concentration. Error bars represented as SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; other abbreviations as in Figure 1.

actomyosin formation with dasatinib and ponatinib was reversed with ROCK inhibitor Y-27632. To study the functional relevance of these changes in the actin cytoskeleton, we conducted transwell permeability assays with 40 kDA dextran. Both dasatinib and ponatinib, but not imatinib or nilotinib, increased endothelial cell permeability, which was reversed with Y-27632 (Figure 3C). These findings indicate that increased F-actin stress fiber formation and endothelial cell permeability with dasatinib and ponatinib were mediated by ROCK.

ROCK MEDIATES TKI-INDUCED ENDOTHELIAL DYSFUNCTION. Increased ROCK activation is associated with endothelial cell dysfunction through the negative regulation of endothelial nitric oxide synthase (eNOS).[17] eNOS is regulated post-translationally by phosphorylation at several sites by ROCK, including down-regulation of its activity by Thr^{495}

FIGURE 3 **Ponatinib and Dasatinib Alter Actin and Permeability of Endothelial Cells**



(A) Human aortic endothelial cells were cultured on coverslips coated with gelatin and treated or cotreated with 250 nmol/L imatinib, nilotinib, dasatinib, or ponatinib; 20 μmol/L Rho-associated coiled-coil containing kinase inhibitor Y-27632 (Y), or control (dimethyl sulfoxide) for 24 hours. F-actin and nuclei were stained with phalloidin and 4′,6-diamidino-2-phenylindole, respectively, to visualize the effect of treatments on cellular actin organization. Images are representative of 3 to 5 fields per condition. **(B)** Quantification of the F-actin intensity in endothelial cells. **(C)** Quantification of fluorescein isothiocyanate–labeled 40 kDA dextran in transwell permeability assays after 24 hours. Scale bars in **A** are 50 μm. For actin fluorescence intensity, n = 5 views per treatment. For transwell permeability assays, n = 5. Error bars represented as SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

phosphorylation and Ser^{1177} dephosphorylation.[18,19] Although treatment of endothelial cells with imatinib and nilotinib did not show any changes in Thr^{495} phosphorylation (**Figures 4A and 4B**), both dasatinib and ponatinib treatment led to dose-dependent phosphorylation of eNOS at Thr^{495} (**Figures 4C and 4D**). Dasatinib- and ponatinib-induced phosphorylation of Thr^{495} was completely reversed by Y-27632 administration (**Figures 4C and 4D**). In contrast, phosphorylation of Ser^{1177} was not altered with ponatinib administration (Supplemental Figure 2A), perhaps reflecting the more direct role of ROCK for Thr^{495} in contrast to Ser^{1177} because Ser^{1177} is phosphorylated by AKT via ROCK-mediated phosphatase and tensin homolog (PTEN) inhibition.[19] We confirmed that increased Thr^{495} phosphorylation is associated with decreased eNOS activity with 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM), a fluorescent probe that binds directly with nitric oxide to form a fluorescent benzotriazole. Treatment of endothelial cells with nilotinib, dasatinib, and ponatinib reduced DAF-FM fluorescence by 1.2-, 2.1-, and 2.2-fold, respectively (**Figures 4E and 4F**). Cotreatment of endothelial cells with Y-27632 reversed the effects on DAF-FM fluorescence by nilotinib, dasatinib, and ponatinib (**Figures 4E and 4F**). Cotreatment of ponatinib-treated cells with Y-27632 also led to increases in the levels of nitrite and

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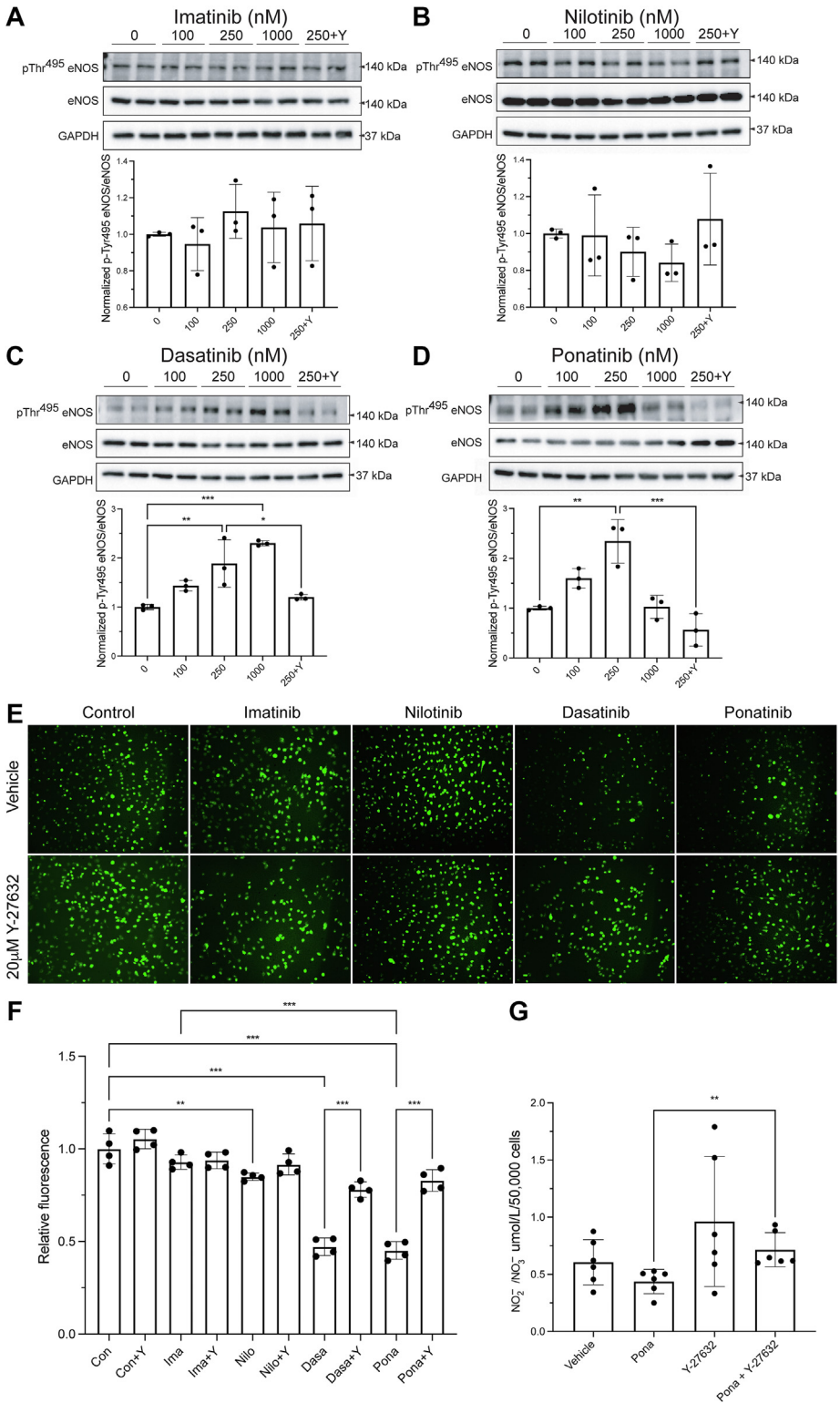
FIGURE 4 Second-/Third-Generation TKIs Lead to Endothelial Cell Dysfunction



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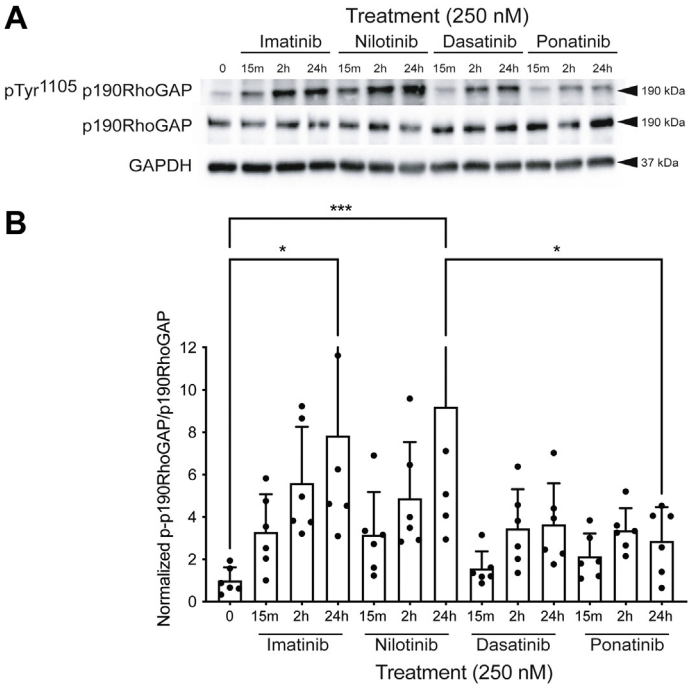
nitrate by the Griess reaction (Figure 4G). Furthermore, extended treatment of endothelial cells with ponatinib reduced the expression of eNOS by 1.3-fold at day 3 and by 1.9-fold at day 5 of treatment (Supplemental Figure 2B).

Treatment of endothelial cells with ponatinib led to decreased cell proliferation as measured by 5-ethynyl-2'-deoxyuridine (EdU) incorporation (Supplemental Figure 3A) but did not result in increased apoptosis or necrosis (Supplemental Figure 3B). Because of the decreased EdU incorporation in cells treated with ponatinib, we conducted further studies on cellular senescence with senescence-associated beta-galactosidase staining and protein expression of cell cycle markers p21 and p53. No differences were observed in senescence-associated beta-galactosidase staining upon treatment with TKIs. Furthermore, the expression of p21 and p53 was not changed by ponatinib treatment (Supplemental Figures 3C to 3F), suggesting that ponatinib likely induced endothelial cellular quiescence rather than senescence. Taken together, these results indicate that ROCK activation plays a central role in the endothelial dysfunction associated with ponatinib and dasatinib.

DIFFERENTIAL TKI-INDUCED p190RhoGAP PHOSPHORYLATION. An important negative upstream regulator of ROCK is p190RhoGAP. Phosphorylation of p190RhoGAP at Tyr^{1087} or Tyr^{1105} leads to recruitment of p190RhoGAP to the plasma membrane.[9] Because several kinases, including ARG (ABL2), directly phosphorylate p190RhoGAP at Tyr^{1105}, we investigated whether BCR-ABL1 TKIs could affect p190RhoGAP phosphorylation. We chose the time points 15 minutes, 2 hours, and 24 hours based on our finding that TKIs induce ROCK activation within 24 hours (Figure 2). We found that each TKI exerted different phosphorylation and dephosphorylation patterns at different time points in endothelial cells (Figures 5A and 5B). Compared with the vehicle control, imatinib and nilotinib increased Tyr^{1105} phosphorylation at 24 hours by 7.9-fold ($P = 0.014$) and 8.6-fold ($P < 0.001$), respectively (Figure 5B). However, ponatinib and dasatinib did not lead to an increase in Tyr^{1105} phosphorylated p190RhoGAP. Instead, ponatinib treatment showed 3.2-fold lower phosphorylated p190RhoGAP levels compared with nilotinib ($P = 0.037$) at 24 hours (Figure 5B). In line with the differential effects on endothelial cell structure and function, changes in p190RhoGAP phosphorylation may also partially explain why imatinib and 2G/3G TKIs exhibit different ROCK activity levels in patient samples and in vitro.

FIGURE 5 Tyrosine Kinase Inhibitors Exert Differential Phosphorylation Patterns on p190RhoGAP

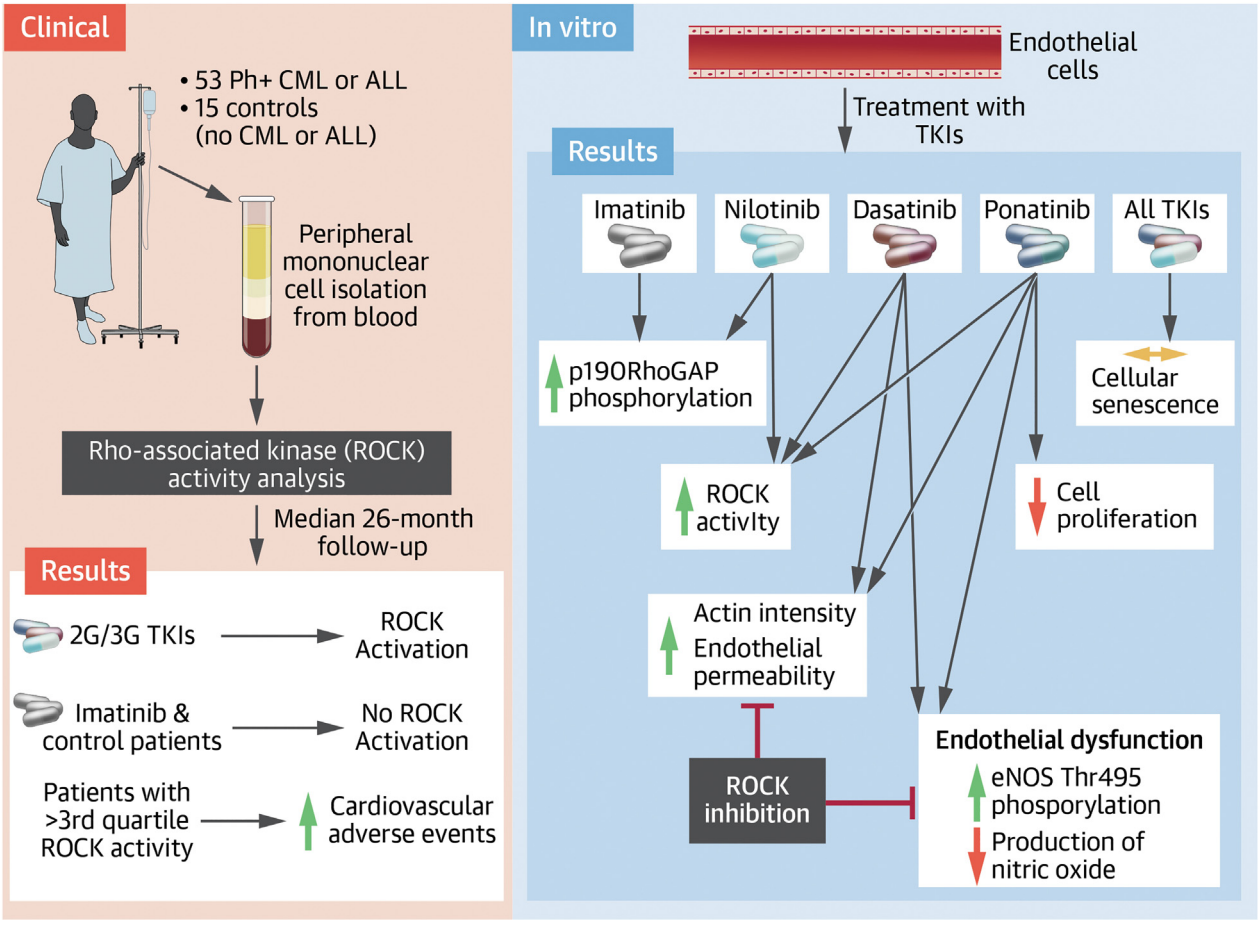


(A) Immunoblotting and **(B)** quantification of phosphorylation of Tyr^{1105} on p190RhoGAP to total p190RhoGAP after 250 nmol/L treatment of imatinib, nilotinib, dasatinib, or ponatinib at 15 minutes, 2 hours, and 24 hours in human aortic endothelial cells. n = 6 per treatment. Error bars represent SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

FIGURE 4 Continued

Immunoblots and quantification of Tyr^{495} endothelial nitric oxide synthase (eNOS) phosphorylation to total eNOS in human aortic endothelial cells treated with 0 to 1,000 nmol/L **(A)** imatinib (ima), **(B)** nilotinib (nilo), **(C)** dasatinib (dasa), and **(D)** ponatinib (pona) with/without 20 μmol/L ROCK inhibitor Y-27632 (Y) for 24 hours. **(E)** Visualization of nitric oxide concentration in endothelial cells with the fluorescent probe 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM). **(F)** Quantification of DAF-FM fluorescence. **(G)** Quantification of nitrite via the Griess reaction per 50,000 endothelial cells. For **E** and **F**, all treatments were performed with 250 nmol/L of each respective TKI (ima, nilo, dasa, and pona) with and without 20 μmol/L Y-27632 for 24 hours while control (con) indicates treatment with dimethyl sulfoxide for 24 hours. n = 3 to 6 per treatment. Error bars represented as SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations as in Figure 1.

CENTRAL ILLUSTRATION **Activation of Rho-Associated Coiled-Coil Containing Kinase by Second-Generation/ Third-Generation Tyrosine Kinase Inhibitors Can Increase Cardiovascular Adverse Events**



Yu B, et al. J Am Coll Cardiol CardioOnc. 2022;4(3):371-383.

Rho-associated coiled-coil containing kinase (ROCK) may play a role in the cardiotoxicity of second-/third-generation (2G/3G) tyrosine kinase inhibitors (TKIs). **(Left)** Our clinical results indicate that patients with Philadelphia chromosome-positive (Ph+) leukemia on 2G/3G TKIs have elevated ROCK activity compared with control patients without Ph+ leukemia and patients receiving imatinib. Patients above the third quartile of ROCK activity have a higher incidence of cardiovascular adverse events. **(Right)** Our in vitro results suggest that each TKI can also exert specific effects on endothelial cells. Imatinib and nilotinib lead to p190RhoGAP phosphorylation. Nilotinib, dasatinib, and ponatinib lead to ROCK activation. Dasatinib and ponatinib lead to increased actin intensity, endothelial permeability, and endothelial dysfunction. Pharmacologic ROCK inhibition can reverse TKI-induced changes in endothelial function. Increase **(green up arrow)**, decrease **(red down arrow)**, and no change **(yellow horizontal arrow)**.

DISCUSSION

Since their introduction to clinical practice about 20 years ago, BCR-ABL1 TKIs have yielded remarkable benefits for patients with Ph+ leukemia. However, the 2G and 3G TKIs were subsequently found to be associated with significant cardiovascular toxicity.[2,4,6,20] Meta-analyses indicate that patients receiving 2G and 3G TKIs do not lead to increased survival compared with patients receiving imatinib.[1] This may be partially attributed to their cardiovascular toxicity leading to increased mortality and morbidity despite their putative beneficial effects on Ph+ leukemia.[1]

ROLE OF ROCK IN TKI-INDUCED CARDIOTOXICITY. ROCK activity assayed from PBMCs of patients has been associated with cardiovascular diseases such as CAD, heart failure, and vasospastic angina[10,11]; it predicts future cardiovascular events[21]; and the inhibition of ROCK improves endothelial function in

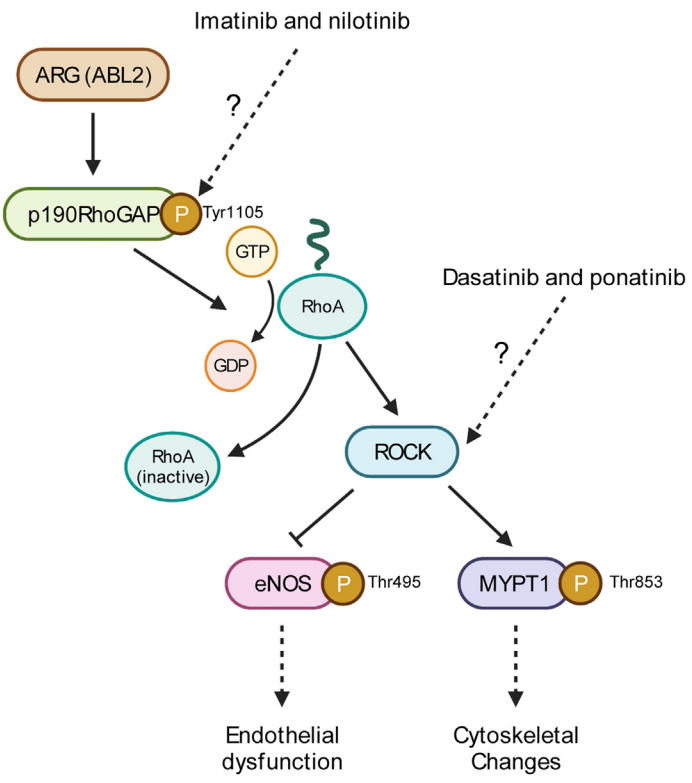
patients with atherosclerosis.[22] In this study, we provide clinical evidence and in vitro mechanisms showing that the cardiovascular toxicity associated with BCR-ABL1 TKIs may occur through the acute activation of ROCK (**Central Illustration**). First, we show that Ph+ leukemia patients actively receiving 2G and 3G TKIs have elevated ROCK activity in PBMCs compared with control patients without Ph+ leukemia and Ph+ leukemia patients receiving imatinib. Increased ROCK activity is associated with CVAEs, even after adjustment for age and BMI, 2 confounders of ROCK activity.[13,14] These results are consistent with the cardiotoxic profiles of each TKI.[1,4] We also show that 2G and 3G TKIs lead to increased ROCK activity in vitro. Both dasatinib and ponatinib caused increased actomyosin formation and endothelial permeability in a ROCK-dependent manner. Ponatinib and dasatinib also led to dose-dependent phospho-inhibition of Thr^{495} on eNOS, which corresponded to decreased nitric oxide production. These effects on eNOS were reversed by pharmacologic ROCK inhibition. Finally, we provide exploratory evidence for the differential regulation of ROCK and endothelial function by TKIs through differential phosphorylation patterns of p190RhoGAP.

Patients with newly diagnosed Ph+ CML before starting TKI therapy had similarly elevated ROCK activity as patients on 2G and 3G TKIs (**Figure 1A**). A possible explanation for the elevated ROCK activity in newly diagnosed CML patients is that the blood samples from newly diagnosed patients contain predominantly Ph+ cells (ie, more BCR-ABL1 transcripts). BCR-ABL1 contains a RhoGEF domain that activates RhoA, an upstream activator of ROCK.[23] Therefore, more BCR-ABL1 transcripts would be expected to be associated with an increase in ROCK activity. Indeed, the increase in ROCK activity in patients with newly diagnosed Ph+ CML before TKI therapy is consistent with the increased prevalence of cardiovascular disease in CML patients who are not on TKIs.[24]

Interestingly, imatinib appears to decrease ROCK activity in Ph+ leukemia patients (**Figure 1A**). Although imatinib has been shown to offer slight dose-dependent cardioprotection in a clinical study,[4] it is unlikely that this is due solely to its effects on ROCK activity. Other possibilities include its ability to cause a decline in BCR-ABL1 transcripts, the inhibition of ROCK activity by phosphorylating p190RhoGAP (**Figures 5A and 5B**), and the differential binding affinities of imatinib to different kinases in comparison with 2G and 3G TKIs.[7]

ROLE OF p190RhoGAP IN TKI-INDUCED ENDOTHELIAL DYSFUNCTION. Although dasatinib and ponatinib do not lead to increases in phosphor-Tyr^{1105} p190RhoGAP, imatinib and nilotinib do increase phosphor-Tyr^{1105} p190RhoGAP. Why this occurs is uncertain because the Tyr^{1105} site is directly phosphorylated by several kinases associated with off-target TKI inhibition, including ABL2 (**Figure 6**).[9] A study looking at the crystal structure and structural dynamics of imatinib bound to another important kinase of Tyr^{1105}, c-Src, suggested that the imatinib-c-Src complex contains structural changes indicative of c-Src activation.[25] Moreover, nilotinib is based on the imatinib scaffold,[15] which may provide clues for why Tyr^{1105} is hyperphosphorylated after imatinib and nilotinib treatment. In contrast, dasatinib and ponatinib do not show Tyr^{1105} hyperphosphorylation. Because phosphorylation of Tyr^{1105} is necessary for the

FIGURE 6 BCR-ABL1 TKIs and the Rho/ROCK Signaling Pathway



ARG can directly phosphorylate p190RhoGAP at tyrosine 1105, which leads to cycling of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) on RhoA, a small GTPase, inactivating RhoA.[9] Our study indicates that imatinib and nilotinib lead to hyperphosphorylation of p190RhoGAP, although the exact mechanisms underlying this are not well characterized. GTP-bound RhoA leads to ROCK activation, which affects diverse signaling pathways, including directly phosphorylating eNOS at threonine 495[18] and MYPT1 at threonine 853.[12] Our study indicates that dasatinib and ponatinib lead to ROCK activation. However, the mechanisms underlying this are unclear and likely to include effects that are independent of p190RhoGAP phosphorylation. Abbreviations as in **Figures 1 and 4**.

downstream functions of p190RhoGAP, including ROCK inhibition,[9] our findings may indicate that the lack of p190RhoGAP phosphorylation in ponatinib- and dasatinib-treated cells may drive ROCK activity and the adverse phenotype we observed in endothelial cells (**Figures 3 and 4**).

OTHER POTENTIAL PATHWAYS MEDIATING TKI-INDUCED CARDIOVASCULAR TOXICITY. Other off-target potential mechanisms involved in the cardiovascular toxicity associated with BCR-ABL1 TKIs include modulation of vascular endothelial growth factor, c-Src, and thrombogenic signaling pathways.[20] However, these prior studies do not show that these pathways are indeed modulated in the clinical setting. Furthermore, these proposed mechanisms also do not fully explain why imatinib, a potent inhibitor of vascular endothelial growth factor receptor,[8] does not also result in increased cardiovascular events.

STUDY LIMITATIONS. Our clinical results are limited by the small sample size, particularly among patients receiving ponatinib treatment. The number of CVAEs in our sample was small, preventing meaningful interpretation of ROCK activity measurements in relation to outcomes. Additionally, a subset of our patients (28%) was actively receiving statin therapy. Statins have been shown to decrease ROCK activity, but our sample size was too small to detect such an effect. Larger studies are needed to assess whether TKI-enhanced ROCK activity can be used as a practical biomarker for the prediction of CVAEs in patients with Ph+ leukemia. For our in vitro studies, imatinib and nilotinib were used at lower concentrations than their steady-state maximal plasma concentrations and, therefore, may understate their effects on endothelial cells in vivo.

CONCLUSIONS

We provide evidence for a novel mechanism underlying the cardiovascular toxicity associated with 2G and 3G BCR-ABL1 TKIs through the activation of ROCK. This may provide rationale for clinical studies exploring the use of ROCK inhibitors for prophylaxis and/or treatment of CVAEs experienced in patients receiving 2G and 3G BCR-ABL1 TKIs.

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ADDRESS FOR CORRESPONDENCE: Dr James K. Liao, Section of Cardiology, Department of Medicine, University of Chicago, 5841 South Maryland Avenue, MC 6080, Chicago, Illinois 60637, USA. E-mail: jliao@medicine.bsd.uchicago.edu.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Dasatinib, nilotinib, and ponatinib are associated with an increased risk of cardiovascular toxicity. Identifying prognostic indicators and/or potential mechanisms mediating the cardiotoxicity among newer-generation TKIs may lead to therapies that decrease the incidence of CVAEs in these patients. We identified ROCK as a potential mediator of CVAEs that is activated in patients receiving second- and third-generation TKIs. Mechanistically, the inhibition of ROCK in vitro ameliorates TKI-induced endothelial dysfunction.

TRANSLATIONAL OUTLOOK: If validated, ROCK activity may be used as a prognostic indicator of future CVAEs in patients receiving BCR-ABL1 TKIs. However, it remains to be determined whether ROCK inhibition can reduce CVAEs in patients receiving BCR-ABL TKIs.

REFERENCES

1. Douxfils J, Haguet H, Mullier F, Chatelain C, Graux C, Dogné J-M. Association between BCR-ABL tyrosine kinase inhibitors for chronic myeloid leukemia and cardiovascular events, major molecular response, and overall survival: a systematic review and meta-analysis. *JAMA Oncol*. 2016;2:625–632.

2. Giles FJ, Mauro MJ, Hong F, et al. Rates of peripheral arterial occlusive disease in patients with chronic myeloid leukemia in the chronic phase treated with imatinib, nilotinib, or non-tyrosine kinase therapy: a retrospective cohort analysis. *Leukemia*. 2013;27:1310–1315.

3. Cortes JE, Kim D-W, Pinilla-Ibarz J, et al. Ponatinib efficacy and safety in Philadelphia chromosome–positive leukemia: final 5-year results of the phase 2 PACE trial. *Blood*. 2018;132:393–404.

4. Jain P, Kantarjian H, Boddu PC, et al. Analysis of cardiovascular and arteriothrombotic adverse events in chronic-phase CML patients after frontline TKIs. *Blood Adv*. 2019;3:851–861.

5. Lipton JH, Chuah C, Guerci-Bresler A, et al. Ponatinib versus imatinib for newly diagnosed

chronic myeloid leukaemia: an international, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2016;17:612–621.

6. Kim TD, Rea D, Schwarz M, et al. Peripheral artery occlusive disease in chronic phase chronic myeloid leukemia patients treated with nilotinib or imatinib. *Leukemia*. 2013;27:1316–1321.

7. Uitdehaag JC, de Roos JA, van Doornmalen AM, et al. Comparison of the cancer gene targeting and biochemical selectivities of all targeted kinase inhibitors approved for clinical use. *PLoS One*. 2014;9:e92146.

8. Davis MI, Hunt JP, Herrgard S, et al. Comprehensive analysis of kinase inhibitor selectivity. *Nat Biotechnol*. 2011;29:1046–1051.

9. Sfakianos MK, Eisman A, Gourley SL, et al. Inhibition of Rho via Arg and p190RhoGAP in the postnatal mouse hippocampus regulates dendritic spine maturation, synapse and dendrite stability, and behavior. *J Neurosci*. 2007;27:10982–10992.

10. Yu B, Sladojevic N, Blair JE, Liao JK. Targeting Rho-associated coiled-coil forming protein kinase (ROCK) in cardiovascular fibrosis and stiffening. *Expert Opin Ther Targets*. 2020;24:47–62.

11. Sladojevic N, Yu B, Liao JK. ROCK as a therapeutic target for ischemic stroke. *Expert Rev Neurother*. 2017;17:1167–1177.

12. Kimura K, Ito M, Amano M, et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*. 1996;273:245–248.

13. Noma K, Goto C, Nishioka K, et al. Roles of rho-associated kinase and oxidative stress in the pathogenesis of aortic stiffness. *J Am Coll Cardiol*. 2007;49:698–705.

14. Liu P-Y, Chen J-H, Lin L-J, Liao JK. Increased Rho kinase activity in a Taiwanese population with metabolic syndrome. *J Am Coll Cardiol*. 2007;49:1619–1624.

15. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell*. 2005;7:129–141.

16. Laufs U, Endres M, Stagliano N, et al. Neuroprotection mediated by changes in the endothelial actin cytoskeleton. *J Clin Invest*. 2000;106:15–24.

17. Hiroi Y, Noma K, Kim HH, et al. Neuroprotection mediated by upregulation of endothelial nitric oxide synthase in Rho-associated, coiled-coil-containing kinase 2 deficient mice. *Circ J*. 2018;82:1195–1204.

18. Sugimoto M, Nakayama M, Goto TM, Amano M, Komori K, Kaibuchi K. Rho-kinase phosphorylates eNOS at threonine 495 in endothelial cells. *Biochem Biophys Res Commun*. 2007;361:462–467.

19. Ming XF, Viswambharan H, Barandier C, et al. Rho GTPase/Rho kinase negatively regulates endothelial nitric oxide synthase phosphorylation through the inhibition of protein kinase B/Akt in human endothelial cells. *Mol Cell Biol*. 2002;22:8467–8477.

20. Moslehi JJ, Deininger M. Tyrosine kinase inhibitor-associated cardiovascular toxicity in chronic myeloid leukemia. *J Clin Oncol*. 2015;33:4210–4218.

21. Dong M, Liao JK, Fang F, et al. Increased Rho kinase activity in congestive heart failure. *Eur J Heart Fail*. 2012;14:965–973.

22. Nohria A, Grunert ME, Rikitake Y, et al. Rho kinase inhibition improves endothelial function in human subjects with coronary artery disease. *Circ Res*. 2006;99:1426–1432.

23. Sahay S, Pannucci NL, Mahon GM, et al. The RhoGEF domain of p210 Bcr-Abl activates RhoA and is required for transformation. *Oncogene*. 2008;27:2064–2071.

24. Lang K, McGarry LJ, Huang H, Dorer D, Kaufman E, Knopf K. Mortality and vascular events among elderly patients with chronic myeloid leukemia: a retrospective analysis of linked SEER-Medicare data. *Clin Lymphoma Myeloma Leuk*. 2016;16:275–285.e1.

25. Tsutsui Y, Deredge D, Wintrode PL, Hays FA. Imatinib binding to human c-Src is coupled to inter-domain allostery and suggests a novel kinase inhibition strategy. *Sci Rep*. 2016;6, 30832-30832.

KEY WORDS **chronic myeloid leukemia, endothelial dysfunction, Philadelphia chromosome, Rho-associated protein kinase, tyrosine kinase inhibitor**

APPENDIX **For an expanded Methods section and supplemental tables and figures, please see the online version of this paper.**