Regorafenib Induces Rapid and Reversible Changes in Plasma Nitric Oxide and Endothelin-1

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BACKGROUND

Hypertension is a toxicity of antiangiogenic therapies and a possible biomarker that identifies patients with superior cancer outcomes. Understanding its mechanism will aid in treatment and could lead to the development of other biomarkers for predicting toxicity and anticancer efficacy. Recent evidence implicates nitric oxide (NO) suppression and endothelin-1 (ET-1) stimulation as potential mechanisms leading to antiangiogenic therapy-induced hypertension. The aim of this study was to evaluate the effects of regorafenib, a novel broad-spectrum kinase inhibitor with activity against multiple targets, including vascular endothelial growth factor receptor 2 inhibition, on NO and ET-1 levels.

METHODS

Regorafenib was administered to 32 subjects with gastrointestinal stromal tumor on a 3-week-on, 1-week-off basis. Plasma levels of NO and ET-1 were measured at baseline, 2, 4, and 6 weeks of therapy. Data analysis was by Wilcoxon rank-sum and paired *t*-tests.

RESULTS

Twenty subjects (63%) developed regorafenib-induced hypertension. Two weeks after starting regorafenib therapy, plasma ET-1 levels increased (25% increase, $P < 0.05$) and NO was suppressed (20% decrease, $P < 0.05$). These normalized after 1-week washout but ET-1 rose again by 30% ($P < 0.05$) and NO fell by 50% ($P < 0.05$) after restarting regorafenib.

CONCLUSIONS

These findings indicate that regorafenib induces a coordinated and reversible suppression of NO and stimulation of ET-1. Whether NO and ET-1 might predict therapeutic efficacy in these patients requires further study.

Keywords: *antiangiogenic therapy; blood pressure; endothelin-1; hypertension; nitric oxide*

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Antiangiogenic therapies target the vascular endothelial signaling pathway (VEGF) among other proangiogenic pathways,[1] and they are effective for many solid tumors.[2–5] Hypertension is a common, occasionally dose-limiting toxicity of antiangiogenic therapies.[6–10] Importantly, emerging clinical evidence suggests that the development of antiangiogenic therapy-induced hypertension may be related to superior outcomes in select populations.[9,11–13] However, whether this association can be exploited for clinical benefit remains unclear and how best to identify patients with treatment-induced hypertension is an open question. Indeed, the utility of blood pressure as a predictive biomarker is limited by variation in office-based blood pressure measurement and the early institution of antihypertensive medications. Surrogate biomarkers of hypertension might overcome these limitations.

Preclinical and clinical evidence implicates inhibition of VEGF-mediated regulation of vascular tone in the etiology of antiangiogenic therapy-induced hypertension. Specifically, blockade of the VEGF-signaling pathway reduces vasodilatory nitric oxide (NO), and increases vasoconstrictive endothelin-1 (ET-1).[14–20] VEGF stimulates endothelial NO synthase via the phosphatidylinositol 3-kinase-Akt pathway.[21] However, how antiangiogenic therapies modulate ET-1 release is less clear. NO suppresses ET-1 release by inhibiting preproendothelin synthesis[22] and ET-receptor antagonist attenuates the effects of NOS inhibition,[23] suggesting an interaction between these two pathways. Whether ET-1 stimulation by antiangiogenic therapies might be a consequence of NO inhibition remains to be elucidated. Regardless of the specific mechanism, circulating levels of these vasoactive factors might represent surrogate biomarkers for antiangiogenic therapy-induced hypertension that could be used to predict tumor response.

Antiangiogenic therapies currently available include anti-VEGF monoclonal antibodies, multitargeted tyrosine kinase inhibitors with activity against the VEGF receptor (VEGFR) and VEGF Trap.[24] Of note, tyrosine kinase inhibitors target

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not only the VEGF receptor, but also other kinase receptors including platelet-derived growth factor receptor and epithelial growth factor receptor.[24] The use of these broad-spectrum agents enhances their therapeutic usefulness by expanding the actions to oncogenic targets as well. However, whether individual antiangiogenic therapies with different kinase activity spectrums also have different effects on the NO and ET-1 pathways is uncertain. Given the expanding use and wide variety of tyrosine kinase inhibitors with antiangiogenic activity, there is a need to evaluate the effects of individual antiangiogenic therapies on these pathways.

Regorafenib (BAY-73-4506) is a novel broad-spectrum kinase inhibitor with activity against multiple targets including VEGFR1, 2, 3, platelet-derived growth factor receptor, KIT, TIE2, RET, FGFR1, RAF, and p38 MAPK.[25] In this study, we prospectively investigated whether regorafenib induces changes in circulating levels of NO and ET-1 in patients with metastatic and/or unresectable gastrointestinal stromal tumor (GIST). Secondary aims were to explore whether NO suppression and ET-1 stimulation were associated with high blood pressure and better tumor response (i.e., clinical benefit).

METHODS

Study population. Plasma samples were collected from subjects enrolled in a single arm, open label, multicenter phase II study evaluating regorafenib in subjects with metastatic and/or unresectable GIST (NCT0106878769).[26] Of the 34 subjects enrolled in this phase II trial, 32 subjects had plasma collected and were included in this analysis.

The inclusion criterion for the study required a histologically confirmed diagnosis of metastatic and/or unresectable GIST with prior therapeutic failure (defined as progression of disease or intolerance) to imatinib and disease progression on sunitinib. Patients may have received any number of prior therapies, including kinase inhibitors, with the exception of prior sorafenib. Key exclusion criteria were use of unapproved tyrosine kinase inhibitors or investigational agents within 2 weeks before study entry, uncontrolled hypertension at baseline (defined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg) despite optimal medical management, persistent grade 3 proteinuria (defined as >3.5 g of proteins in 24 h), clinically significant arrhythmias or cardiac disease, or renal failure requiring dialysis.[26]

All patients received oral regorafenib on a daily basis, with a starting daily dose of 160 mg, following a 3-week-on drug, 1-week-off drug regime per cycle. Regorafenib dose was adjusted for toxicity.

Data collection. Plasma samples collected at baseline, cycle 1 day 15 (2 weeks), cycle 2 day 1 (at 4 weeks, after a 1-week washout period) and cycle 2 day 15 (6 weeks) were evaluated. Plasma samples were collected in EDTA-containing tubes, centrifuged for 5 min at 1,500*g* within 30 min of collection and stored at −80 °C until analysis.

Baseline clinical information was collected by chart review, including age, sex, body mass index, previous history of hypertension, antihypertensive medications, diabetes diagnosis, use of statins and estimated glomerular filtration rate (eGFR) by the modification of diet in renal disease formula.[27] Blood pressures measurements, heart rate, eGFR, addition of new antihypertensive medications and/or dose changes at all time points of plasma collection were also obtained by chart review.

All patients provided written informed consent to collect plasma samples for research use. The study was approved by the Dana Farber Cancer Institute Institutional Review Board.

Measurement of biomarkers. The NO pathway was evaluated in plasma by measuring NO metabolites (nitrates and nitrites). ET-1 levels were directly measured in plasma. All markers were evaluated using commercially available kits. NO metabolites were measured by using a colorimetric assay (Parameter; R&D Systems, Minneapolis, MN) and ET-1 by chemiluminescent ELISA (QuantiGlo; R&D Systems). Inter- and intra-assay coefficients of variations for all assays were <15%.

Blood pressure measurement and hypertension definition. Single oscillometric blood pressure measurements taken at each visit day were recorded. Regorafenib-induced hypertension was defined as recurrent or persistent systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg, requiring medical intervention, or having increases in blood pressure <20 mm Hg associated with symptoms, if previous blood pressure was within normal limits. This definition includes all subjects developing grade 2 or higher hypertension as per the Common Terminology Criteria for Adverse Events Version 4.0.[28]

Assessment of tumor response. Tumor response was assessed by clinical benefit defined by the composite of complete response, partial response, and stable disease lasting ≥16 weeks per RECIST 1.1[29] as a measure of disease control. Briefly, response is defined relative to the sum diameters of target lesions at baseline. Complete response refers to the disappearance of all target lesions as well as a reduction to <10 mm in short axis of any pathological lymph nodes. On the other hand, partial response refers to a decrease of at least 30% in the sum of diameters of target lesions relative to baseline. Finally, stable disease refers to the clinical situation where the increase or shrinkage in target lesions is not sufficient to qualify for progressive disease or partial response, respectively.[29]

Statistical analysis. Descriptive statistics were employed to define the study group. Continuous variables were evaluated using means and standard deviations for normally distributed data and medians and interquartile ranges for non-normal data. Categorical variables were summarized using percentages. We evaluated whether regorafenib induces NO suppression and ET-1 stimulation by comparing biomarkers levels at different time points relative to baseline by using Wilcoxon rank-sum tests. We evaluated whether NO suppression and ET-1 activation were associated with mean blood pressure (MAP) rise by using Spearman's correlation

tests. Specifically, we compared changes in NO and ET-1 from baseline to 2 weeks (ΔNO_{0-2wks} and ΔET_{0-2wks}) to mean arterial pressure (MAP) from baseline to 2 weeks (ΔMAP_{0-2wks}) as well as changes in NO and ET-1 from baseline to 6 weeks (ΔNO_{0-6wks} and ΔET_{0-6wks}) to MAP from baseline to 6 weeks (ΔMAP_{0-6wks}). We evaluated whether NO suppression and ET-1 activation were associated with clinical benefit by comparing absolute changes in NO and ET-1 from baseline to 6 weeks (ΔNO_{0-6wks} and ΔET_{0-6wks}) among subjects who achieved clinical benefit and those who did not achieve clinical benefit by using Wilcoxon rank-sum test. Statistical significance was set at <0.05. All analyses were performed using GraphPad Prism5 and SAS v9.2.

RESULTS

Subject characteristics

Thirty-two subjects were evaluated in this study. The median age of the participants was 56 years, 60% were male and 88% were white. All patients had an eGFR >60 ml/min/1.73 m^2 at study entry (median 88 ml/min/1.73 m^2). Sixty five percent of the subjects had a diagnosis of hypertension at baseline (Table 1).

Table 1 | Clinical characteristics at baseline ($n = 32$)

Variable	Baseline value
Age, year[a]	56 (48–61)
Male[b]	19 (60)
Race[b]	
White	28 (88)
Black	3 (9)
Hispanic	1 (3)
Asian	1 (3)
BMI, kg/m^2[c]	27.7 (6.6)
eGFR, ml/min/1.73 m^2[a]	88 (78–110)
Diabetes[b]	3 (9)
Statins[b]	10 (30)
Prior HTN[b]	20 (65)

BMI, body mass index; eGFR, estimated glomerular filtration rate; HTN, hypertension. [a]Median (interquartile range). [b]*n* (%).[c]Mean (s.d.).

Biomarkers

Circulating NO levels decreased by 20% 2 weeks after regorafenib was started ($P < 0.05$) but returned to baseline 1-week off therapy. Two weeks later, after resuming regorafenib, systemic NO fell by 50% compared with baseline (Figure 1a) ($P < 0.05$). Systemic levels of ET-1 rose by 25% at 2 weeks while on regorafenib ($P < 0.05$) and by 30% as compared with baseline at 6 weeks ($P < 0.05$). ET-1 also returned to baseline after the washout period (Figure 1b). Plasma samples were collected in 31, 30, 31, and 28 of 32 subjects at baseline, 2, 4, and 6 weeks, respectively.

Relationship between blood pressure and biomarkers

Sixty-three percent of subjects developed regorafenib-induced hypertension at any point during the phase II study. MAP increased by 4 mm Hg two weeks after regorafenib therapy was started ($P < 0.05$). During the washout period, MAP returned to baseline levels. At 6 weeks, the MAP was increased by 6 mm Hg ($P < 0.05$) (Figure 2). Within the first 2 weeks, 8 patients (25%) required increasing doses of antihypertensive medications or addition of new antihypertensive medication, which were started before the first blood pressure measurement was documented in our study. By 6 weeks, 4 (13%) additional subjects required the addition or increase of antihypertensive medications. No changes in heart rate or eGFR were observed while patients were on or off regorafenib (Table 2). No correlations were observed between increasing MAP and the plasma levels of NO or ET-1.

Relationship between tumor response and biomarkers

Since hypertension has been reported to be an efficacy biomarker in some patients treated with antiangiogenic therapy, we assessed whether there were any correlations between changes in biomarker levels and clinical response. Data on clinical benefit was available for 27 subjects. Twenty-three subjects (85%) achieved clinical benefit and only 4 subjects (15%) did not achieve clinical benefit. There was no statistically significant association for either biomarker, although there was a trend towards a greater decrease in plasma NO and increase in ET-1 at 6 weeks in those who did achieve clinical benefit (ΔNO_{0-6wks}: −15 µmol/l vs. −8 µmol/l, $P = 0.24$, ΔET_{0-6wks}: 1 pg/ml vs. −0.04 pg/ml, $P = 0.27$). Of note, this analysis was

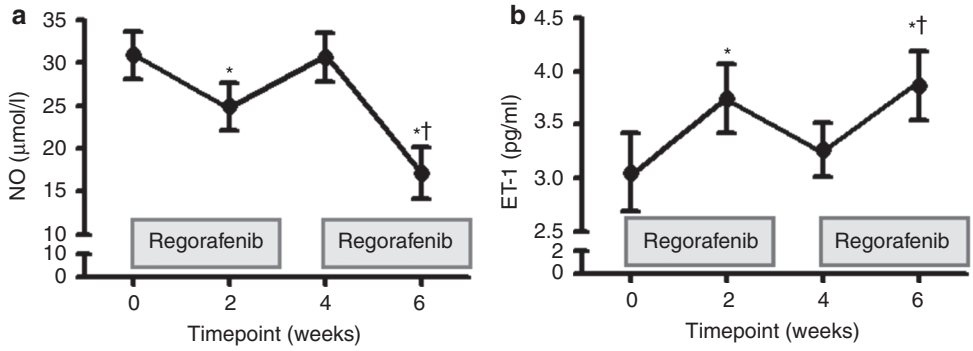

Figure 1 | Variations in circulating levels of (**a**) nitric oxide (NO) and (**b**) endothelin-1 (ET-1) in patients with gastrointestinal stromal tumor on regorafenib at 0, 2, 4, and 6 weeks after starting therapy. Wilcoxon rank-sum test. *$P < 0.05$; †$P < 0.05$ to 4 weeks.

underpowered. The power to detect differences with this number of patients was 28%; if the ratio between respondents and nonrespondents remains the same, we would need 101 patients to have 80% power to detect a significant relationship between biomarker level and clinical response.

DISCUSSION

This study shows that administration of regorafenib, a novel broad-spectrum kinase inhibitor, induces a coordinated and rapidly reversible suppression of NO and stimulation of ET-1 in patients with metastatic and/or unresectable GIST. These pathways have been suggested as the major mediators of antiangiogenic therapy-induced hypertension, primarily via VEGF pathway blockade.[14] For patients treated with regorafenib, these pathways represent rational targets for antihypertensive therapy. Given that drug-related hypertension has been associated with better clinical outcomes,[10–13] circulating levels of these factors are also potential candidates for biomarkers of therapeutic efficacy.

Whether all antiangiogenic therapies cause increased ET-1 is an open question. Sunitinib is another broad-spectrum tyrosine kinase inhibitor that induces both hypertension and increased in ET-1,[16] and ET-1 receptor blockade abrogates sunitinib-induced blood pressure rise in preclinical models.[17,18,30] By contrast, in subjects treated with sorafenib, no changes in plasma ET-1 levels were reported,[31] although baseline values were measured during the first two weeks of therapy, and our data here suggests that ET-1 levels rise quickly. Whether the specific tyrosine kinase inhibitory profile determines the effect of a given antiangiogenic agent on ET-1 and hypertension or whether these are universal properties of this class remains to be seen. Since ET-1 is under consideration as a biomarker of efficacy, this is an important question.[32]

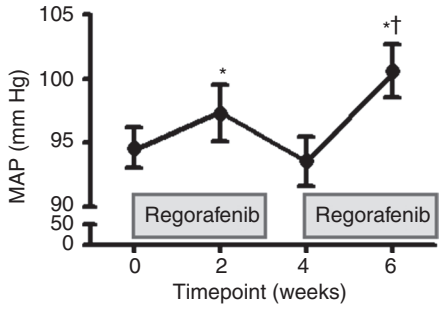

Figure 2 | Mean arterial pressure (MAP) in patients with gastrointestinal stromal tumor treated with regorafenib at 0, 2, 4, and 6 weeks after starting therapy. Paired *t*-test. *$P < 0.05$; †$P < 0.05$ to 4 weeks.

In contrast to our results, NO bioavailability was not suppressed in a study of sunitinib-induced hypertension in swine.[18] However, in a rodent model, anti-VEGF receptor 2 antibody treatment led to both suppressed NO synthase expression in kidney and caused hypertension.[15,19] Also, in human subjects treated with vandetanib, an antiangiogenic agent with different tyrosine kinase spectrum, a small decrease in circulating NO levels was reported. In our study, we observed NO suppression by as much as 50% by week 6, which is the largest decrease in circulating NO yet reported in association with antiangiogenic therapy. Together, these findings suggest that biomarker changes may vary according to the individual antiangiogenic drug administered.

Similar to other antiangiogenic therapies, regorafenib caused hypertension with 63% of the subjects developing hypertension at some point during our study. However, the rise in blood pressure observed at 2 and 6 weeks of regorafenib therapy was mild and, as in other studies,[16,19] no correlation was observed between blood pressure changes and NO suppression or ET-1 increases. This may reflect the early treatment of hypertension based on home monitoring in our study since most patients who developed hypertension were treated before the office visits at 2 and 6 weeks.

There are no reliable biomarkers to predict response in patients treated with antiangiogenic therapies and circulating biomarkers of antiangiogenic therapy-induced hypertension are attractive biomarkers for several reasons. Office-based blood pressure measurement is subject to variation due to "white coat hypertension," pain and other factors, and while ambulatory blood pressure monitoring overcomes this limitation, it is not practical.[6] Measurement of a biomarker, or several of them, might allow for more accurate readout of appropriate *in vivo* VEGF blockade. Furthermore, patients that develop antiangiogenic therapy-induced hypertension are typically treated aggressively with antihypertensive medications to reduce their blood pressure,[33] thus reducing the utility of blood pressure as a predictive marker. Circulating biomarkers may not be subject to this limitation. Finally, it is unclear which is more predictive of clinical outcomes: hypertension itself (defined as blood pressure >140/90 mm Hg), an individual patient's absolute blood pressure rise (even if they do not develop hypertension), or a change at the tumor level for which hypertension is a surrogate marker.[13] The measurement of biomarker changes induced by antiangiogenic therapy might offer an alternative to evaluate predictive efficacy.

Table 2 | Clinical characteristics of patients at baseline and 2, 4, and 6 weeks after starting regorafenib

Variable	Baseline	2 weeks	4 weeks	6 weeks	*P* value[a] (each week to baseline)
Pulse, bpm, median (IQR)[a]	81 (72–86)	74 (67–80)	79 (69–95)	68 (60–82)	ns
eGFR, ml/min/1.73 m^2, median (IQR)[a]	88 (78–110)	91 (80–114)	90 (83–122)	98 (83–119)	ns

bpm, beats per min; eGFR, estimated glomerular filtration rate; IQR, interquartile range.
[a]Wilcoxon rank-sum test.

In this study, we explored whether NO suppression and ET-1 stimulation were associated with clinical benefit. We did not observe a statistically significant association for either biomarker, although there was a trend towards a greater decrease in plasma NO and increase in ET-1 at 6 weeks in those who achieved clinical benefit. Notably, our ability to find an association between biomarker levels and clinical benefit was limited by the large proportion of patients achieving clinical benefit compared to those who did not, a conclusion supported by our power analysis. Future prospective studies that are larger will be required to evaluate the usefulness of these biomarkers in predicting tumor response.

This study has other limitations. First these patients were all tolerant to and previously failed to sunitinib therapy, making our population of patients a selected population, which might potentially limit the generalization of our conclusions to other patients receiving antiangiogenic therapy. We also lacked dietary information and dietary nitrate content could affect could affect plasma levels of NO metabolites. The reversibility of NO suppression off regorafenib makes this possibility less likely; however, it cannot be excluded.

In conclusion, regorafenib induces rapid and reversible changes in both NO and ET-1 in patients with GIST. These findings support continued investigation of these biomarkers in larger studies, in order to assess their predictive efficacy for cancer outcomes. Whether these changes are observed in other antiangiogenic therapies with broad-spectrum tyrosine kinase activity also requires further evaluation.

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