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Effects of Vascular Endothelial Growth Factor Signaling Inhibition on Human Erythropoiesis

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Vascular endothelial growth factor A • Biological markers • Erythrocyte count • Erythropoietin • Axitinib • Bevacizumab • Sorafenib

Abstract

Inhibition of vascular endothelial growth factor (VEGF) signaling increases red blood cell (RBC) counts, and erythropoiesis markers have been proposed to guide antiangiogenic therapy in humans. We analyzed RBC measurements in patients enrolled in three studies: a phase II trial of axitinib in thyroid cancer; a study of sorafenib in advanced solid tumors; and a randomized trial of fluorouracil, hydroxyurea, and radiation with and without bevacizumab for head and neck cancer. In the sorafenib trial, plasma erythropoietin concentrations were measured at baseline, day 8, and day 35. Over the first 84 days of treatment, RBC counts increased for each day on sorafenib (2.7 M/μL [95% confidence interval (CI), 1.5–3.9]) and axitinib (4.3 M/μL [95% CI, 2.2–6.5]). RBCs declined over the first 68 days of cytotoxic chemoradiotherapy alone (−12.8 M/μL per day [95% CI, −15.7 to −9.8]) but less so with added bevacizumab (−7.2 M/μL per day [95% CI, −9.5 to −4.9]). Erythropoietin levels increased, on average, by 9.5 mIU/mL between day 8 and day 35 of sorafenib exposure. No significant relationships between elevations in RBCs and changes in volume status or blood pressure or between elevations in erythropoietin and smoking status were found. VEGF signaling inhibition is associated with increased RBC and erythropoietin production in humans. The effects of these changes are subtle at physiologic doses and are unlikely to be clinically useful biomarkers for guiding the administration of or predicting treatment responses to VEGF pathway inhibitors. ***The Oncologist*** 2013;18:965–970

Implications for Practice: Studies of preclinical models often uncover unexpected physiologic effects of new drugs. For the VEGF signaling inhibitors, one study suggested that they stimulated hepatic synthesis of erythropoietin and erythrocytosis. The investigators hypothesized that erythropoietin or red blood cell counts could be a pharmacodynamic biomarker with which to personalize VEGF inhibitor therapy in cancer patients. In this study, we detected subtle, consistent effects of axitinib, bevacizumab, and sorafenib on red blood cell counts. We further investigated the changes in circulating erythropoietin after initiation of sorafenib therapy. Although an average effect was detectable, the measured changes were small and variable. We concluded that although the preclinical models detected this physiologic effect, it is unlikely that these measures will be effectively developed to guide VEGF inhibition therapy in human cancer patients.

Introduction

Vascular endothelial growth factor (VEGF) signaling is crucial to numerous adult physiologic functions but more so in conditions of pathologic angiogenesis such as cancer [1, 2]. Disruption of VEGF signaling has demonstrated therapeutic benefit [3–13]; however, given the severity of the conditions for which these agents were developed, their use and dosing were determined empirically. Although much is known about the biology of angiogenesis and angiogenesis inhibition by disruption of VEGF signaling, no noninvasive biomarker has been sufficiently developed to guide the optimal and safe administration of VEGF signaling pathway (VSP) inhibitors [14–16].

In cynomolgus monkeys that received the VSP inhibitor aflibercept, Tam et al. observed erythrocytosis [17]. To explain this observation, the investigators administered various modalities of VEGF inhibition to rodents and demonstrated (a) that VEGF inhibition stimulates production of erythropoietin by hepatocytes, (b) that this occurs through a mechanism independent of hypoxia-inducible factor 1α and is associated with suppression of renal erythropoietin mRNA expression, (c) that these effects are dose dependent and are readily detected through measurement of circulating red blood cell (RBC) counts or plasma erythropoietin, and (d) that the increases are cumula-

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tive and detectable after 4 weeks of chronic VSP inhibition with aflibercept. The authors concluded "that levels of erythropoietin and erythrocytosis could represent noninvasive surrogate markers for stringent blockade of VEGF in vivo," but they acknowledged that "whether the high degree of VEGF blockade associated with erythrocytosis . . . would be either safe or desirable remains to be seen." Notably, aflibercept was recently approved by the U.S. Food and Drug Administration (FDA) for second-line therapy of metastatic colorectal cancer in combination with 5-fluorouracil, leucovorin, and irinotecan at a dose of 4 mg/kg every 2 weeks (equivalent to one-ninth of the dose administered to cynomolgus monkeys) [11, 18, 19].

For erythropoietin levels or RBC counts to serve as clinically relevant biomarkers, this effect of VSP inhibitors should be detected in human subjects within the typical range of therapeutic doses. Several studies in cancer patients have observed increased hemoglobin levels associated with the use of VEGF inhibitors including bevacizumab, sunitinib, sorafenib, and axitinib [20–24]. In addition, studies have shown reduced risk of anemia with bevacizumab in patients receiving chemotherapy [25]. These studies verify in humans the observation that VSP inhibitor therapy increases RBC counts and erythropoietin levels; however, developing these measurements as biomarkers for prediction of treatment outcomes with these agents or improving the precision of dosing these drugs for individual patients requires a stepwise evaluation process [26]. During the past decade, the FDA provided guidance on biomarker development [27] that has been increasingly incorporated into National Cancer Institute [28] and industry-sponsored investigations [29]. A key element of the approach is to distinguish biomarkers that are measured in an analytical test system with well-established performance characteristics for which there is a scientific framework that elucidates the clinical significance of the results as "probable valid biomarkers." This initial step is important for promoting a biomarker from simply exploratory to a candidate worth further investigation and investment.

We analyzed complete blood counts from patients at our institution who received axitinib, sorafenib, or bevacizumab within early clinical trials. In addition, we measured erythropoietin in prospectively collected, stored, frozen plasma specimens to determine the time course and magnitude of changes during sorafenib treatment. Our findings confirm that VSP inhibitor therapy is associated with increases in RBCs and erythropoietin, but these changes are subtle and variable, making further development of these biomarkers unlikely to be useful for individualized treatment.

Methods

Clinical Trials

Patients were enrolled in one of three clinical trials. In "A Pharmacokinetic, Pharmacodynamic, and Pharmacogenetic Study of Sorafenib and Blood Pressure Elevation in Patients with Advanced Malignancies," patients received sorafenib 400 mg twice daily (57 patients) [30]. In "A Study of the Anti-Angiogenesis Agent Axitinib in Patients with Metastatic Thyroid Cancer," patients received axitinib 5 mg twice daily (16 patients) [31]. In "A Study of Chemoradiotherapy for Intermediate Stage/Selected Stage IV Cancers of the Head and Neck," patients received biweekly chemoradiation: twice daily radiation and continuous infusion 5-fluorouracil 600 mg/m^2 per day for 5 days and 500 mg hydroxyurea twice daily for 6 days with (14 patients) or without (8 patients) bevacizumab 10 mg/kg on day 1 of each treatment week [32]. In the sorafenib and axitinib trials, no patient required transfusion and growth factors were not permitted. To isolate the pharmacodynamic effects of bevacizumab from disease and cytotoxic treatment effects, patients who required administration of packed RBCs or erythropoiesis-stimulating agents ($n = 3$) were excluded from the analysis. This research was approved by the University of Chicago institutional review board, and patients provided written informed consent for relevant specimen collections and retrospective data analysis.

RBC and Erythropoietin Measurements

To determine whether disruption of VEGF signaling in humans is associated with increased erythropoiesis, we analyzed RBC measurements in patients at a single institution. All RBC count data were processed at the University of Chicago Hospitals with the Coulter LH755 Hematology Workcell (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com). RBC count was obtained by direct measurement of erythrocytes with multiplication by calibration factor. Reportable ranges using the Coulter LH755 are 0.00 to 8.00 $\times$ 10^6/μL. Mean difference, a measure of accuracy, is $\pm$0.05.

Erythropoietin plasma concentrations were measured only for patients in the sorafenib study at baseline and on days 8 and 35. Erythropoietin levels were measured in these stored plasma samples using the Immulite 2000 assay (Arup Laboratories, Salt Lake City, UT, http://www.aruplab.com/), according to the manufacturer's specifications. Thirty-two subjects were included in the analysis.

Ambulatory Blood Pressure and Creatinine Clearance Measurements

Ambulatory blood pressure measurement was collected based on previously published methods [30] and was performed at baseline, once between days 6–12 of sorafenib treatment, and once during weeks 5–7 (typically on day 35). For 19 patients, 24-hour urine collections and same-day plasma creatinine measurements were also available. All plasma and urine creatinine measurements were determined at the University of Chicago Hospitals Analytical Laboratory with the Roche Hitachi P800 Analyzer (Roche, Indianapolis, IN, http://www.roche.com), according to the manufacturer's specifications. The reportable ranges of plasma and urine creatinine measurements are 0.2–25 mg/dL and 4.0–650 mg/dL, respectively.

Collection of Smoking History

Patient smoking history was collected from retrospective chart review of physician's initial social history assessment at presentation to the institution and, if the patient was treated at University of Chicago prior to enrollment in the clinical trial, again on the study pretreatment visit note.

Statistical Analysis

Linear mixed models with random intercept were used to determine the effect of VEGF signaling inhibitors on RBC measurements. Study day was included as a fixed effect, and subject was included as a random effect. Changes over time in weight and creatinine clearance were also analyzed using linear mixed models. Repeated measures analysis of variance

with the Greenhouse-Geisser adjustment was used to examine whether erythropoietin levels changed significantly over time. Because of the skewness of the distribution, erythropoietin levels were natural log transformed prior to analysis. Pearson's correlation coefficient was used for determination of the association between changes (week 5–7 vs. baseline) in blood pressure and RBC concentration. Changes in erythropoietin level among those with a history of smoking versus those who never smoked were analyzed with a two-sample *t* test using log-transformed erythropoietin values. Statistical significance was defined as $p < .05$. All statistical analyses were performed with Stata 10 (StataCorp, College Station, TX, http://www.stata.com).

Results

Increased RBC Levels With Exposure to VEGF Inhibitors

RBC measurements were collected from patients in the studies previously described. Over the first 84 days of treatment, RBC counts increased for each day on sorafenib (2.7 M/μL; 95% confidence interval [CI], 1.5–3.9; $p < .001$) and axitinib (4.3 M/μL; 95% CI, 2.2–6.5; $p < .001$) (Fig. 1A, 1B). RBC counts declined over the first 68 days of chemoradiation only (−12.8 M/μL per day; 95% CI, 15.7 to −9.8) but less so (−7.2 M/μL per day; 95% CI, −9.5 to −4.9) with added bevacizumab ($p = .003$ for the study day by treatment interaction) (Fig. 1C). Women had lower RBC counts than men ($p = .002$). For patients enrolled in the sorafenib study, the test of gender by time interaction on change in RBC count was not statistically significant. Because sample size was small, this interaction was not calculated for patients enrolled in the axitinib or bevacizumab studies.

Increased Erythropoietin With Exposure to Sorafenib

To determine whether VEGF inhibition in humans had effects similar to those in monkeys, baseline, day 8, and day 35 erythropoietin measurements were collected from patients enrolled in the sorafenib trial. Thirty-two patients with complete data were included in this analysis. There was a significant difference in erythropoietin levels across the three visits ($p = .027$). The highest levels, on average being reached at day 35, were 9.5 mIU/mL greater than on day 8 of sorafenib exposure (Fig. 2A). The individual trajectories, however, were highly variable (Fig. 2B). Data on smoking history, antihypertensive use, and mean corpuscular volume (MCV) are included in Table 1 (pulse oximetry and reticulocyte count data were not collected in the clinical trials and were not available for analysis). There was no significant change in MCV for patients receiving sorafenib or axitinib. Notably, 34 of 57 patients receiving sorafenib had data on RBC counts as well as erythropoietin levels at baseline and at day 35. For these patients, there was not a significant positive correlation between change in erythropoietin and change in RBC count. Pharmacokinetic analyses were performed on 32 subjects who had steady-state plasma total sorafenib minimum concentration measurements available. There was no correlation between sorafenib concentration and change in erythropoietin ($r^2 = .05$, $p = .24$).

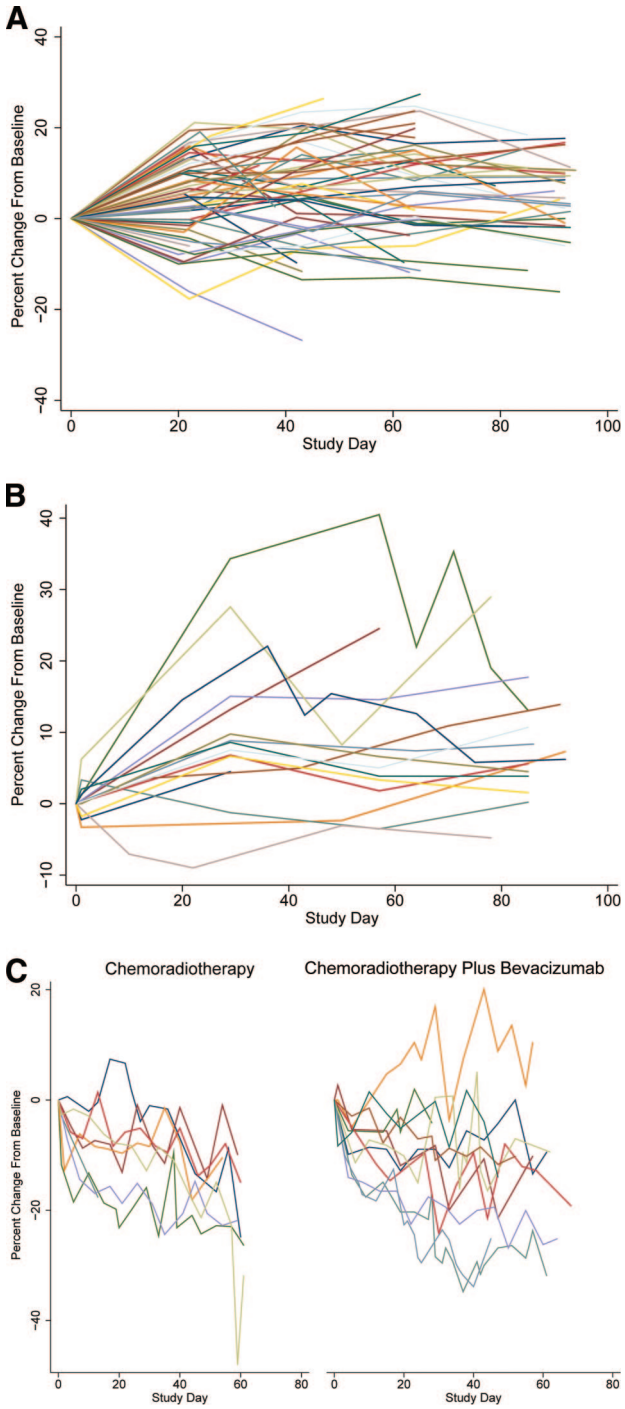

Figure 1. Evidence that red blood cell (RBC) levels increase with exposure to vascular endothelial growth factor inhibition. **(A):** Percent change in RBC counts (in million per microliter) in patients receiving sorafenib. **(B):** Percent change in RBC counts in patients receiving axitinib. **(C):** Bevacizumab inhibits declines in RBC counts due to chemotherapy. In Figure 1A–1C, each colored line represents an individual patient's observed change over time.

RBC Elevation Does Not Correlate Significantly With Blood Pressure Elevation

In initial reports, investigators hypothesized that RBC elevation was secondary to loss of plasma volume or to blood pressure elevation rather than a direct effect of VEGF signaling inhibition [33]. Consequently, we examined changes in

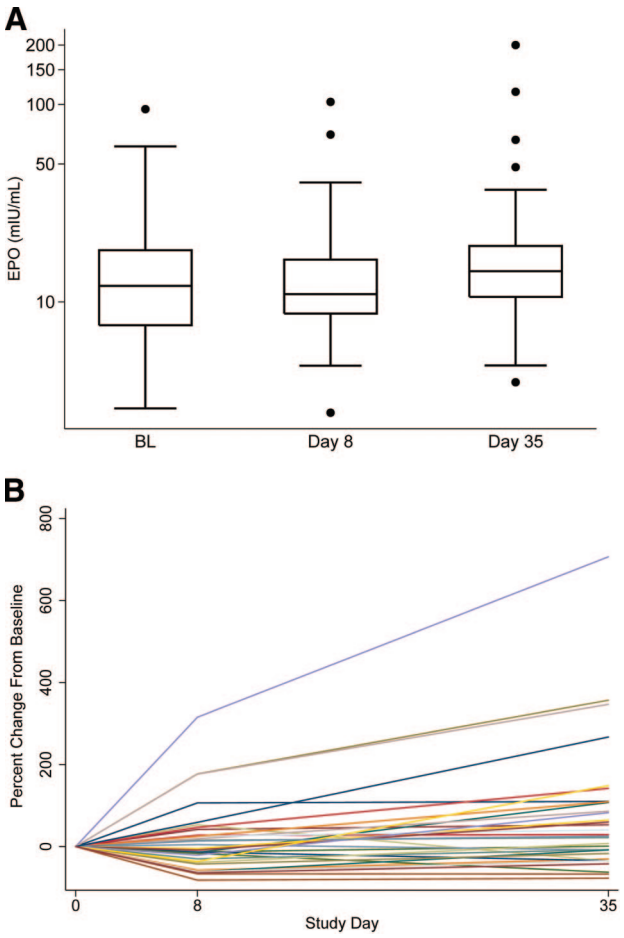

Figure 2. Erythropoietin increases subtly with sorafenib exposure. **(A):** After steady-state concentrations of sorafenib are reached (on approximately day 8), plasma EPO concentrations increase by day 35. **(B):** Percent change of each individual subject's EPO measurements (in milli-international units per milliliter) are depicted. There is significant interindividual variability among these trajectories.

Abbreviation: Erythropoietin, EPO.

Table 1. Characteristics of 32 patients receiving sorafenib for whom erythropoietin values were analyzed

Characteristics	*n* (%)
Sex	
Male	20 (63)
Female	12 (37)
Smoking Status	
Never	17 (53)
Former	13 (41)
Current	2 (6)
No. of antihypertensive agents used	
At baseline	
0	22 (69)
1	10 (31)
At day 35	
0	19 (59)
1	10 (31)
2	3 (9)
Laboratory measures	Mean value (SD)
Erythropoietin, mIU/mL	
Baseline	17.4 (18.0)
Day 8	16.7 (20.1)
Day 35	26.2 (38.4)
Change (day 35 vs. day 8)	9.5 (19.5)
RBCs, M/μL	
Baseline	4.2 (0.6)
Day 35	4.5 (0.8)
Change (day 35 vs. baseline)	0.3 (0.5)
Corpuscular volume, fL	
Baseline	87.6 (8.4)
Day 35	87.6 (8.3)
Change(day 35 vs. baseline)	0.0 (2.4)

Abbreviation: RBC, red blood cell.

weight, creatinine clearance, and precise ambulatory diastolic blood pressure (DBP) in sorafenib-treated patients. Weight decreased 0.4 kg for each week on treatment (95% CI, 0.3–0.5; $p < .001$; $n = 36$). In addition, there was no statistically significant change in urine creatinine clearance over time (−0.08 mL per minute per day; 95% CI, −0.20 to 0.03; $p = .16$; $n = 19$). To determine whether the RBC elevation was associated with DBP elevation, ambulatory data were collected from 43 patients. There was a weak correlation between change in DBP and change in RBC count. The Pearson's correlation coefficient was 0.10 (95% CI, −0.21 to 0.39; $p = .52$) (Fig. 3).

Smoking Status Is Not Significantly Correlated With Changes in Erythropoietin Levels

Active and prior cigarette smoking can lead to a relative pro-erythropoietic state. Of the 34 subjects who had erythropoietin measurements available, 15 patients reported a prior history of smoking and 19 patients reported never smoking (Table 1). Changes in erythropoietin levels from baseline to week 5 were compared between the never-smokers and the patients with a history of smoking. There was a mean increase of 5.6 mIU/mL (standard deviation [SD]: 29.6) in the group of patients who had a prior history of smoking versus 12.9 mIU/mL (SD: 42.7) in those who had never smoked. There was no significant association between smoking status and rise in erythropoietin levels with sorafenib treatment ($p = .39$).

Discussion

This retrospective analysis across studies of three different VSP inhibitors demonstrated consistent but small effects of these agents on erythropoiesis. Monotherapy with sorafenib and axitinib led to measurable increases in RBC, whereas the presence of bevacizumab in a small randomized study of chemoradiotherapy appeared to protect against the severity of anemia typically caused by the myelosuppressive therapy. Some might argue that grouping these agents assumes they affect RBC counts by the same mechanism. VSP inhibition effects on RBCs are reproducible across species and consistent across methods of pathway inhibition (e.g., antibodies to the VEGF ligand and small molecule inhibitors of the VEGF receptor), suggesting a mechanism-based phenomenon. These

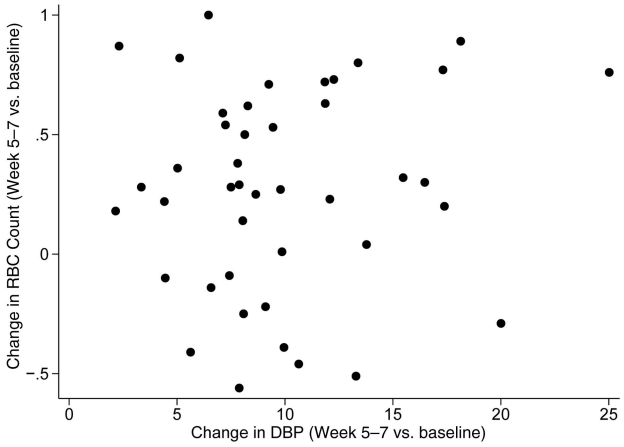

Figure 3. Relationship between change in diastolic blood pressure and change in red blood cell count. Pearson's correlation between change in DBP and change in RBC is 0.10 (95% confidence interval, −0.21 to 0.39; $p = .52$).

Abbreviations: DBP, diastolic blood pressure; RBC, red blood cell.

findings previously suggested that measures of erythropoiesis might be strong candidate biomarkers of VSP inhibition, but the RBC changes that we have measured are subtle and variable. In fact, the majority of patients receiving these agents will be unlikely to show clear evidence of erythropoiesis. Consequently, these markers are unlikely to be developed successfully as clinically relevant biomarkers of the extent of VSP inhibition in human subjects.

Our results are consistent with the findings of Tam et al. in cynomolgus monkeys [17]. At the time of their publication, this effect had not been reported in humans, but subsequently, evidence of erythrocytosis as a pharmacodynamic effect of VSP inhibitors in cancer patients was published [20, 33]. Similar to the preclinical studies, collectively, our studies and others have verified that this is a detectable effect of VSP inhibition in humans [21, 22, 25]. Although Tam et al. clearly demonstrated hepatic erythropoietin synthesis as a putative mechanism, the clinical investigators suggested alternative mechanisms such as volume contraction reflected by rapid changes in blood pressure with exposure and removal of sunitinib [33]. Our study of continuous administration of sorafenib included more precise and sensitive measurement of ambulatory blood pressure changes and 24-hour urine collections. We did not find evidence of significant volume contraction over the course of sorafenib exposure and found no strong correlation between the increase in RBC and the increase in blood pressure. We did detect evidence of increases in circulating erythropoietin after steady-state concentrations of sorafenib exposure were reached (between days 8 and 35), consistent with the hypothesis of Tam et al. that VSP inhibition led to increased erythropoietin production, which in turn led to erythrocytosis.

Although this study was a collection of small studies, the analytical methods applied to the available data suggested that the qualitative effect of VSP inhibition on increased erythropoiesis was identified, but the magnitude of effect was less apparent than in the mouse and primate models. We hypothesize that this may be due to differences in the relative dosage of VSP inhibitors. The recommended dose for humans in the FDA-approved label for aflibercept, for example, is 4 mg/kg every 2 weeks, and this dose achieves approximately 1.7-fold the exposures achieved with a 3-mg/kg dose in monkeys [19]. To achieve the elevations in hematocrit observed in the monkeys, however, Tam et al. administered aflibercept 15 mg/kg twice weekly, or 60 mg/kg every 14 days. This dosage roughly approximates a human-equivalent dose of 36 mg/kg over 14 days, or approximately 9-fold the labeled dose. Although there is some variability among different VSP inhibitors in the intensity of VEGF inhibition at the standard doses, none are likely to be equivalent to 9-fold the dose of another. At the tolerable doses of the different VSP inhibitors included in this study, the subtle, reproducible, but variable effects on human erythropoiesis likely reflect fractional inhibition of the signaling pathways that lead to the greater erythropoietin production and RBC elevations observed in the animal models.

Conclusion

We have demonstrated that the proposed candidate markers, RBC count and erythropoietin level, will not achieve status as valid or "probable valid" biomarkers. In this case, our findings suggest that humans are receiving drug dose equivalents at the lower end of the dose-response relationship studied in detail in animal models. In future development of novel anticancer agents, a systematic strategy for developing mechanism-dependent toxicities as biomarkers might routinely include assessment of the reproducibility and precision of measurements as an initial consideration of the costs and ultimate utility of such markers for individualizing therapy. In the case of VSP inhibitors, we conclude that changes in RBC count and erythropoietin level, although biologically related to VSP inhibition, are not valid biomarkers for VSP inhibitors.

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Disclosures

Michael L. Maitland: AbbVie, Amgen, Bayer, BMS, EMD-Serono, Genentech (RF); **Mark J. Ratain:** Genentech (C/A); Bristol-Myers Squibb (RF); **Tanguy Y. Seiwert:** Boehringer Ingelheim (H); Boehringer Ingelheim, Genentech (RF); **Everett E. Vokes:** Genentech, Pfizer (RF). The other authors indicated no financial relationships.

(C/A) Consulting/advisory relationship; (RF) Research funding; (E) Employment; (H) Honoraria received; (OI) Ownership interests; (IP) Intellectual property rights/inventor/patent holder; (SAB) Scientific advisory board

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