Effects of sorafenib dose on acquired reversible resistance and toxicity in hepatocellular carcinoma.

Running title – Sorafenib levels and resistance in HCC

Elizabeth A. Kuczynski¹, Christina R. Lee², Shan Man², Eric Chen³, Robert S. Kerbel¹²

¹. Department of Medical Biophysics, University of Toronto, Toronto, Canada

². Biological Sciences Platform, Sunnybrook Research Institute, Toronto, Canada

³. Department of Medical Oncology, Princess Margaret Hospital, Toronto, Canada

Corresponding Author: R.S. Kerbel, Biological Sciences Platform, Sunnybrook Research Institute, Department of Medical Biophysics, University of Toronto, S-217, 2075 Bayview Avenue, Toronto, ON, Canada, M4N 3M5. Phone: 416-480-5711; Fax: 416-480-5884.

Financial Support: Canadian Liver Foundation graduate studentship (EAK); Canadian Institutes of Health Research Grant #5814 and International Association for Cancer Research (Worldwide Cancer Research) (RSK)

The authors disclose no potential conflicts of interest.
ABSTRACT

Acquired evasive resistance is a major limitation of hepatocellular carcinoma (HCC) treatment with the tyrosine kinase inhibitor (TKI) sorafenib. Recent findings suggest that resistance to sorafenib may have a reversible phenotype. Additionally, loss of responsiveness has been proposed to be due to a gradual decrease in sorafenib plasma levels in patients. Here, the possible mechanisms underlying reversible sorafenib resistance were investigated using a Hep3B-hCG orthotopic human xenograft model of locally advanced HCC. Tissue and plasma sorafenib and metabolite levels, downstream anti-tumor targets and toxicity were assessed during standard and dose-escalated sorafenib treatment. Drug levels were found to decline significantly over time in mice treated with 30 mg/kg sorafenib coinciding with the onset of resistance but a greater magnitude of change was observed in tissues compared to plasma. Skin rash also correlated with drug levels and tended to decrease in severity over time. Drug level changes appeared to be partially tumor-dependent involving induction of tumoral CYP3A4 metabolism, with host pre-treatment alone unable to generate resistance. Escalation from 30 to 60 mg/kg sorafenib improved anti-tumor efficacy but worsened survival due to excessive body weight loss. Microvessel density was inhibited by sorafenib treatment but remained stable over time and dose increase. In conclusion tumor CYP3A4 induction by sorafenib is a novel mechanism to account for variability in systemic drug levels, however declining systemic sorafenib levels may only be a minor resistance mechanism. Escalating the dose may be an effective treatment strategy, provided toxicity can be controlled.
INTRODUCTION

The oral anti-angiogenic tyrosine kinase inhibitor (TKI) sorafenib remains the only approved systemic treatment for advanced hepatocellular carcinoma (HCC). Sorafenib is an inhibitor of Raf serine/threonine kinases and receptor tyrosine kinases associated with vascular endothelial growth factor receptors (VEGFR) 2 and 3, platelet derived growth factor receptor (PDGFR) β, Flt-3 and c-Kit (1) and other signaling pathways including STAT3 (2). In two randomized phase III trials in advanced HCC patients, sorafenib treatment improved the time to progression and extended overall survival by 2.8 (3) and 2.3 months (4) compared to placebo. These benefits are unfortunately modest, with upfront (innate/intrinsic) and acquired (evasive/secondary) drug resistance being major contributing factors. Toxicity is also an issue leading to a high rate of dose reductions and treatment interruptions in patients(5, 6).

Proposed mechanisms for resistance, based primarily on preclinical studies, are diverse and numerous. Typically the host tumor microenvironment has been shown to be involved in an adaptive or evasive response to anti-angiogenic treatment leading, for instance, to re-induction of tumor vascularization and adaptation or escape from tumor hypoxia (7). Cancer cells intrinsically may also drive resistance, particularly in HCC (8).

There has also been the suggestion that ‘pharmacokinetic resistance’ could develop to TKIs (9, 10). Standard doses of oral sorafenib leads to high (~50%) inter-individual variability in drug exposure (11-13) suggesting that some patients may be under-dosed. The absence of certain toxicities that are associated with improved clinical outcomes (e.g. hand-foot skin reaction (HSFR) (14, 15)) may also indicate under-exposure and dosing (16, 17). Inadequate target inhibition and tumor-regrowth could result, appearing as intrinsic resistance. Additionally, sorafenib plasma levels have been shown to decline over time. In a study of 15 HCC patients,
sorafenib exposure was found to decrease significantly from one month of treatment (area under the curve, AUC 60.3 mg·h/L) to the time of disease progression (33.2 mg·h/L; p=0.007) (10). This trend was later reported in other malignancies (12, 18, 19). Such drug level changes could lead to reduced toxicities and acquired resistance and managed simply by longterm dosage adjustments. The cause of declining sorafenib exposure is unknown.

Additionally, resistance to TKIs occasionally presents as a reversible rather than permanent phenotype (20). Patients with renal cell carcinoma (RCC) who had progressed on/acquired resistance to sorafenib (21) or sunitinib (22) have been reported to respond to rechallenge with the same agents. It has been argued that clinical trials evaluating the strategy of switching to another anti-angiogenic TKI after ‘resistance’ has developed should contain an arm evaluating continuation of treatment with the same TKI since this strategy is often efficacious (23). Reversible sorafenib resistance has been modeled preclinically in HCC using mice bearing intrahepatic human xenografts of Hep3B-hCG cells (24). These mice demonstrate initial marked sensitivity to sorafenib which is followed by tumor rebound after one month, based on levels of the secreted urinary protein tumor biomarker βhCG (β human chorionic gonadotrophic hormone) and tumor weight changes (24). Once ostensibly resistant tumor cells were adapted to culture and re-implanted into new hosts, the resultant tumors were completely re-sensitized to retreatment (24). Reversible resistance may be a common but under-appreciated phenomenon but how it relates to other resistance mechanisms is unclear.

Using the Hep3B-hCG model of HCC, mechanisms underlying reversible resistance to sorafenib were investigated. Similar to patients, sorafenib plasma levels were found to gradually decline – an effect that was found to be partially tumor-dependent. Dose escalation inhibited tumor growth, but was unable to prevent further tumor progression and was too toxic without
incorporation of therapy breaks. This study supports the involvement of declining sorafenib plasma levels as a minor or contributing resistance mechanism.

MATERIALS AND METHODS

Orthotopic mouse model

Athymic nude mice (nu/nu; Harlan) were used for experiments assessing sorafenib concentrations. CB17 severe combined immunodeficient (SCID) mice (Charles River) were used for sorafenib ‘pre-conditioning’ and in-house bred yellow fluorescent protein (YFP) expressing CB17 SCID mice were used for transfer of resistant phenotype experiments (breeding pairs a gift from Dr. Janusz Rak). Mice were male aged 6-8 weeks. Animal procedures were in accordance with institutional animal care and maintenance guidelines.

The Hep3B-hCG model of HCC was described previously (25). Briefly, HCCs were established by injecting 1-2x10^6 Hep3B-hCG cells/10 µL volume into the left liver lobe of anesthetized mice (25). Individual mouse urine βhCG levels (henceforth, “hCG”) normalized to creatinine served as a noninvasive surrogate biomarker for tumor burden (25). The Hep3B-hCG cell line was authenticated by STR DNA analysis (Genetica DNA Laboratories) and was found to be mycoplasma-free (Lonza).

Sorafenib dosing and toxicity monitoring

Sorafenib tosylate was obtained from Bayer with the assistance of Dr. Dennis Healy and was prepared according to manufacturer’s recommendations. Unless otherwise indicated, oral gavage treatment of 30 mg/kg sorafenib or vehicle control began at hCG>0 (tumor diameter ~1-2
Tumor response was defined as hCG stabilization (decline from prior assessment and/or hCG<200 mIU/mg) and a progression defined as hCG>200 mIU/mg. In cases where mice progressed early, treatment was continued to confirm tumor response. Dose was switched to 60 mg/kg on treatment day 29 approximating the average time of tumor progression.

Therapy was temporarily stopped at 10% average body weight loss in SCID mice and re-initiated following recovery to ≤5% (3-5 day therapy breaks) mimicking the way toxicity is frequently managed clinically (5). For sorafenib pre-conditioning, tumor-free SCID mice were treated with sorafenib or vehicle as per tumor-bearing mice for 45 or 65 days, were given a 2-day washout period then were orthotopically implanted with parental Hep3B-hCG cells and randomized. After 5 days of surgery recovery, treatment was resumed according to the schedule of the group experiencing the most toxicity. Continuous daily dosing was possible for athymic nu/nu mice which tolerate TKIs better than SCID mice (24). Skin toxicity was monitored biweekly by briefly anesthetizing and photographing nu/nu mice. Rash was graded as ‘rash’, ‘no rash’, or ‘resolved rash’. Resolved rash appeared as clear skin or rashes that diminished significantly in area (>~95%) and redness from prior assessment.

Plasma and tissue sampling

Flash-frozen tumor and heparin-plasma samples (by cardiac puncture) were initially obtained 24h after dosing (c_{trough}). In subsequent experiments, mice were fasted for 3h, dosed, and heparin-plasma was obtained from the retro-orbital sinus 3h later to achieve maximal drug/metabolite concentrations (t_{max} 1-3 h in mice (26, 27)). Plasma samples were taken from different tumor-free and HCC mice 2 weeks pre-treatment, days 1, 4, 7 and 11. Serial biweekly samples were obtained from a different set of mice for weeks 2+. Weighed flash-frozen liver and tumor and formalin-fixed tumor samples were obtained at sacrifice (3h after dosing) on day 14.
(sensitive) and at endpoint (control, resistance and dose escalation phases). Plasma or homogenized tissue samples (100 µL volume) were analyzed by HPLC tandem mass spectrometry (HPLC-MS/MS) to determine sorafenib and estimate N-oxide metabolite levels as previously described (28, 29).

**Protein analysis**

Protein expression in liver and tumor lysates was analyzed by Western blot (40µg protein/well) using the following antibodies: phospho-STAT3 (Tyr705; 4113), STAT3 (4904), mouse/human CYP3A4 (13384), phospho-ERK (Thr202/Tyr204; 4376), ERK (4696; all Cell Signaling Technology), and βactin (A5441; Sigma-Aldrich).

**Immunohistochemistry**

Formalin fixed, paraffin-embedded tumor sections were immunostained using the following reagents: For microvessel density (MVD; CD34+ and CD31+ vessels), 1:150 CD34 (LS-C47878; LifeSpan Biosciences), 1:100 CD31 (sc-1506; Santa Cruz Biotechnology), Cy3- and Alexa488-conjugated secondary antibodies (Jackson Immunoresearch) and DAPI (Invitrogen); for cell proliferation (human Ki67), 1:1000 Ki67 antibody (VP-K451; Vector Laboratories), LSAB+ and DAB+ kits (Dako) and hematoxylin (Surgipath). ~20 images/section were obtained (n=4-9/group) at 100X (Ki67) or 200X (MVD). CD34+ or CD31+ microvessel counts and Ki67 stain were normalized to nuclear stain using ImageJ (v.1.46r) software.

**Statistical analysis**

Experimental group differences were evaluated by Student’s T-test or one-way ANOVA with Bonferroni’s correction for multiple comparisons. The effects of tumor presence and time on drug concentrations were assessed by two-way ANOVA. Correlations between hCG and drug
concentrations were determined by linear regression. Survival was evaluated by log-rank test using GraphPad Prism (v.4.00). Data is presented as the mean and standard error of the mean. Significance level was set at p=0.05.

RESULTS

Plasma sorafenib declines over time

Variants from in vivo sorafenib (30 mg/kg) resistant Hep3B-hCG tumors were first assessed in vitro for resistant properties; however no indication of resistance was observed (Supplementary Fig. S1A-E). Since the resistant phenotype was also lost by transferring resistant tumor fragments or chronically sorafenib-exposed variants into tumor-naïve hosts, host treatment appeared important for resistance (Supplementary Fig. S1F-G and unpublished results). Host-wide pharmacokinetic changes are associated with disease progression in patients (10, 12, 18, 19), therefore this was investigated as a mediator of the reversible sorafenib-resistant phenotype in mice.

Drug levels in tumor and plasma samples were analyzed from athymic nu/nu mice bearing Hep3B-hCG xenografts treated daily with 30 mg/kg sorafenib. Samples were obtained from sensitive (treatment day 13, n=6), pre-progression (day 33, n=6), and acquired resistant mice (days 47-88, n=8; Fig. 1A). Trough (c_{trough}, 24h) measurements were taken to estimate steady state levels (shown to correlate with TKI treatment outcome (30, 31)), and to overcome the obstacle of obtaining multiple timed plasma samples. As per HCC patients (10), a significant reduction in plasma sorafenib concentration was observed in mice that had acquired resistance relative to responsive mice (70.6% decline, p<0.05; ANOVA p=0.0204; Fig. 1B). Tumor
sorafenib levels tended to decline but this was not statistically significant (ANOVA p=0.309; Fig. 1C). Similar trends were also observed in four mice that progressed without a prolonged initial response to sorafenib (plasma p=0.0451; tumor p=0.142; Supplementary Fig. S2A-B). Overall, declining systemic drug levels correlated with resistance in mice with HCC.

**The presence of tumor intensifies declining sorafenib levels**

Both host and tumor factors could conceivably contribute to the observed decline in sorafenib. In the host, 5% of sorafenib is oxidated by hepatic p450 enzyme CYP3A4 (32) and 15% glucuronidated by UGT1A9 (33) contributing to fecal and urinary elimination, respectively. The oxidated N-oxide metabolite is pharmacologically active but more hydrophobic than the parent compound and represents the dominant circulating metabolite in humans (9-16% of total sorafenib) (33) and pharmacological induction of CYP3A4 has been shown to decrease systemic sorafenib concentrations (32, 34). In contrast, drug efflux transporters P-glycoprotein and ABCG2 are thought to play minor roles in sorafenib pharmacokinetics (35, 36). In the tumor, drugs may accumulate in cancer cells (such as in acidic lysosomes for sunitinib) (9) or cancer cells may themselves highly express drug metabolizing enzymes (37) thereby potentially influencing exposure levels. Thus, concentrations of sorafenib and its major metabolite were determined in the presence and absence of tumor to explore the mechanism of sorafenib decline.

Tumor-free and Hep3B-hCG tumor-bearing (HCC) mice were treated daily with sorafenib (30 mg/kg) and after 29 days half of mice were switched to 60 mg/kg, and plasma was sampled 3h after dosing to maximize drug concentrations. Peak plasma sorafenib was achieved days 4 to 7 in mice treated with 30 mg/kg sorafenib (Fig. 2A) which then declined in both HCC ($R^2=0.723$, $p=0.0075$) and tumor-free mice ($R^2=0.813$, $p=0.0055$), however total sorafenib levels tended to be higher in tumor-free mouse plasma (Fig. 2B). Both time ($p<0.0001$) and tumor
presence (p=0.0196) were significantly associated with plasma levels by two-way ANOVA. Tumor presence also significantly impacted %N-oxide levels (p=0.0063 vs. time p=0.1107) as %N-oxide peaked by day 11 in tumor-bearing mice only, but thereafter remained marginally higher than in tumor-free mice. The decline in sorafenib concentrations from sensitive (day 14) to resistance (endpoint) phases were more striking in HCC mouse tissues: a 52.1% (p=0.0192) and 31.7% drop (p=0.00184) was observed in tumors and in livers, respectively, while a non-significant 19.4% decline was observed in tumor-free livers (p=0.232; Fig. 2C). Tissue levels of %sorafenib N-oxide did not significantly change (p>0.05; Supplementary Figure S3A).

The host effect on resistance is minimal

To determine the extent to which host-induced changes contributed to resistance, tumor-free SCID mice were pre-conditioned with sorafenib for 45 or 65 days prior to tumor implantation, corresponding to early and late onset of resistance (Supplementary Fig. S3B). SCID mice were given brief toxicity-associated therapy breaks allowing extended treatment times (24). Sorafenib pre-conditioning worsened weight loss but did not accelerate the onset of resistance relative to vehicle pre-conditioning (Fig. 2D, Supplementary Fig. S3C). Thus, the presence of HCC tumor worsened the gradual decline in sorafenib but the host effect alone appeared minimal as host treatment was insufficient to generate resistance.

Incidence of mouse skin toxicity parallels decline of sorafenib levels

Skin toxicities, including rash and HSFR, are the most commonly reported adverse events in sorafenib-treated patients (5). Skin toxicity has been correlated with drug exposures (17, 38) and improved clinical outcomes (14, 39) but has paradoxically been reported to decrease in severity over time (12, 40, 41). 74% of HCC (n=43) and 75% of tumor-free mice (n=20)
developed a non-irritated red skin on the ventral skin surface, ranging from small spots to nearly the entire surface (Fig. 2E). Rash was a treatment effect since stopping therapy caused complete rash resolution (and weight gain) in 7/7 mice within 1-2 weeks (results not shown). While weight loss was slow and progressive in sorafenib (30 mg/kg) treated mice, rash initially developed weeks 2 to 6 but tended to resolve beginning week 4 (Fig. 2F). 42% of HCC mice treated >6 weeks (n=12) and 44% tumor-free mice (n=9) showed rash improvement. Although delayed in time, this result may reflect declining tissue drug levels.

**Tumor-mediated sorafenib metabolism correlates with declining drug levels**

The effect of the tumor on drug levels was further explored. Negative correlations were observed between sorafenib concentrations and tumor burden/hCG. This relationship was strongest in the tumor (R²=0.248, p=0.0419) and approached significance in the liver (R²=0.1507, p=0.0820) and the plasma (R²=0.0498, p=0.074; Fig. 3A). Contrastingly, plasma %N-oxide correlated positively with hCG (R²=0.280, p<0.0001). Thus, local tumor drug levels and plasma %N-oxide associated well with the degree of tumor burden, but associations with plasma drug concentrations and tumor responses were weak.

Tumor and liver lysates were analyzed to determine expression levels of CYP3A4 enzyme. CYP3A4 protein levels were found to increase during sorafenib treatment in tumors (individual and pooled lysates) compared to vehicle-treated controls (Fig. 3B-C) and was further induced in dose-escalated tumors. In contrast, CYP3A4 remained stable in HCC and tumor-free livers (Fig. 3C). While dose escalation increased plasma sorafenib concentrations (Fig. 3D), it also induced %N-oxide metabolite which appeared to relate to CYP3A4 levels (Fig. 3E). Thus, auto-induction of sorafenib metabolism may explain, at least in part, the tumor’s contribution to systemic sorafenib level decline.
Sorafenib dose escalation inhibits tumor growth at the expense of increased toxicity

When the dose of sorafenib was doubled to 60 mg/kg on day 29 (beginning at the time of c_{trough} decline Fig. 1B and average hCG progression) plasma levels increased by only 23.6% from pre-escalation levels. This escalation strategy appeared effective in correcting drug level decline since plasma concentrations were restored to early timepoints (Fig. 3D) and concentrations significantly increased in the livers (p=0.00895) and tumors (p=0.0387) of HCC mice (Fig. 4A; though this did not correspond with significant %N-oxide increases except in tumor-free livers; Supplementary Figure S4A).

Dose escalation was evaluated for its ability to treat or prevent resistant disease. By day 29, progressive disease was achieved in 17/43 mice, a difference that appeared weakly (non-significantly) to lower tissue sorafenib levels (Supplementary Fig. S4B-C). In all mice dose escalation inhibited hCG (Fig. 4B) in addition to tumor plus liver mass (p<0.01; Supplementary Fig. S4D). This strategy was not however effective in reversing resistance since disease continued to progress at a rate similar to non-escalated mice. Dose escalation also resulted in excessive weight loss (Fig. 4C) which was the primary reason for termination (20% weight loss endpoint), leading to significantly decreased median survival (log rank p=0.002; Fig. 4D). Tumor presence had no significant impact on weight loss or survival (p>0.05; Supplementary Fig. S4E-G), therefore drug toxicity was a critical factor in survival. Dose escalation worsened skin rash by week 6, then 58% of HCC mice (n=12) and 50% of tumor-free mice (n=8) experienced rash improvement (Fig. 4E).

Inhibition of angiogenesis is not associated with drug level changes
The possibility that circulating drug concentrations were suboptimal for target inhibition and anti-tumor effect was investigated. No inhibition in Raf (ERK), VEGFR2, or PDGFRβ signaling by sorafenib treatment was detected in tumor lysates (Supplementary Fig. S5A-B). Only P-STAT3 was inhibited by initial sorafenib treatment which subsequently increased during resistance in livers from both HCC and tumor-free mice (Fig. 5A, Supplementary Fig. S5C), potentially related to changes in local sorafenib levels. Similar findings were observed in tumors although initial P-STAT3 inhibition was not observed. Escalation to 60 mg/kg sorafenib had little effect on P-STAT3 despite increases in tissue drug concentrations (Fig. 5A).

Of those tested, the predominant anti-tumor mechanism for sorafenib treatment appeared to be anti-angiogenesis. Sorafenib-treated tumors were less hemorrhagic than controls, but dose-escalated tumors were smaller and appeared more white/necrotic (Fig. 5B). Sorafenib treatment significantly inhibited microvessel density (p<0.001 vs. controls, ANOVA p<0.0001) but during resistance there was no evidence of resumption of angiogenesis. Likewise, hypoxia-responsive carbonic anhydrase IX (CAIX) protein remained elevated throughout treatment (Supplementary Fig. S5D). In contrast, tumor cell proliferation was not significantly affected by treatment (ANOVA p=0.082; Fig. 5C), except for minor inhibition during dose escalation (T-test p=0.0135 vs. resistance). Altogether, systemic drug levels appeared sufficient for inhibiting angiogenesis therefore factors other than an increase in microvessel density must be responsible for causing HCC tumor progression.

**DISCUSSION**
In recent clinical studies sorafenib exposure was reported to decline by up to 50% at the time of disease progression in small groups of patients with HCC (10), melanoma (19) and other solid tumors (12, 18). A similar observation has been made in GIST patients treated with the TKI imatinib (42), but as with sorafenib, the underlying causes are unknown. Here, clinical observations were confirmed in mice bearing HCC xenografts and a possible mechanism was identified. However, the involvement of this phenomenon in reversible resistance appears complex.

Mechanism of reduced drug exposure over time

In patients pharmacological induction of CYP3A4 by anti-epileptic drugs or rifampin significantly decreased sorafenib exposure (32, 34) suggesting a key role for this metabolic pathway. CYP3A4 inhibition by ketoconazole or midazolam had little effect on exposure except for reducing metabolite levels (43, 44), which is expected given that only 5% of an oral dose is metabolized by CYP3A4 (32). Auto-induction of drug metabolism in the tumor has largely been an under-appreciated contributor to resistance (37). *In vivo* tumor induction of CYP3A4 by sorafenib was found to be a possible contributor to declining drug levels for the first time. This result is consistent with findings that TKIs gefitinib and sunitinib could induce expression of p450 enzyme CYP1A1 in cancer cell lines (45, 46), which has not yet been shown for sorafenib. Despite the key role for hepatic metabolism, no liver induction of CYP3A4 was observed during treatment, in agreement with prior observations (26, 27).

Tumoral CYP3A4 induction does not explain the weaker sorafenib decline in tumor-free mice. It is possible that additional factors, such as drug-binding plasma proteins levels, decreased intestinal absorption, and involvement of other metabolic pathways (e.g. UGT1A9) contribute to sorafenib level changes in the host, and in patients overall. Due to the high tumor:body mass...
ratio of mice in xenograft studies, the importance of a tumor-dependent mechanism may have been exaggerated here, further highlighting the need for follow-up studies.

The sorafenib dose used here (30 mg/kg) is considered low following conversion to a human equivalent dose (47). Plasma concentrations (~20,000mg/ml) were in the range of mouse studies (26) but higher than in patients (<10,000 ng/mL with 400 mg b.i.d, (11)). In humans the N-oxidation pathway is more pronounced than in mice (27), which is validated by the N-oxide levels observed here (1-3% vs. 9-16% in humans (33)). While higher %N-oxide might be expected from CYP3A4 induction, it cannot be ruled out that generated metabolite was cleared too rapidly for direct quantification. Alternatively, the observed metabolite levels may indicate a minor impact of the tumor on drug levels. Indeed P-STAT3 and rash patterns (potential pharmacodynamic markers) were similar regardless of tumor presence, but the relationship between pharmacokinetics and pharmacodynamics can be complex.

**Dose escalation as a therapeutic strategy**

In selected populations of RCC and HCC patients, slowly ramping up the dose of sorafenib (41) or increasing the dose at progression (48, 49) have demonstrated tolerability and anti-tumor activity. Dose escalating may also serve to re-establish adequate exposure levels (10, 12, 18, 19). Here, dose was doubled concurrently for all mice regardless of weight loss status. This did not prove to be effective: anti-tumor activity increased but tolerability was poor. Lower-than-predicted plasma drug levels also occurred as is common in dose-escalated patients, which may indicate saturated drug absorption (11, 13) or poor drug solubility at higher doses (27).

Based on the data presented here, therapeutic plasma drug monitoring could underrepresent drug levels changes within the tissues, which could mean missed opportunities to
optimize the dosage. Dose escalating according to toxicity is an alternative. For the first time shown here, skin rash in mice recapitulated observations of rash and HSFR in TKI-treated patients. Rash developed at a high rate for up to 6 weeks correlating with early treatment response (14, 15) and tended to improve in ~50% of cases mimicking the reported decreasing severity of skin toxicity (12, 40, 41). Rash improvement may therefore be directly related to declining drug levels as suggested by correlations with AUC in patients (12, 19). The uncoupling between patterns of weight loss and rash appears consistent with clinical findings and may relate to higher unabsorbed drug concentrations in the gut (19). To manage excessive weight loss, dose interruptions should be incorporated, which may also help re-sensitize the tumor (20). Optimized strategies that combine dose increases with brief therapy breaks may hold at least some anti-tumor activity while prolonging survival. Extensions in PFS and OS have been achieved in a retrospective study using a similar strategy with sunitinib in RCC (50).

**Declining sorafenib levels as a resistance mechanism**

At first glance systemic drug level changes appear to correlate with resistance and hence provide a possible mechanism for the reversible resistant phenotype that cannot be propagated to new hosts (24). Tissue sorafenib levels related to tumor progression and P-STAT signaling. Interaction between the host and tumor cells was also found to be critical for resistance. However, the lack of microvessel density change during treatment suggests that drug levels remained sufficient for long term angiogenesis inhibition, assuming that microvessel density is directly correlated to the anti-tumor effect. Thus, declining drug levels could be a minor or a contributing factor to resistance. Likewise, dose escalation slowed tumor growth but did not directly prevent tumor progression or effectively treat resistant disease. This might explain why the benefits of sorafenib dose escalation are often transient (18, 41).
In conclusion, sorafenib levels declined over time in mice but its impact on resistance is unclear. Escalating the dose may be an effective strategy however more tolerable regimens are needed, particularly for HCC patients with impaired liver function. A relationship was also observed between skin rash in ‘nude’ mice and drug levels. Given the frequency of rash as a side effect of many biologic anti-cancer agents, these results could be extended to study the impact of rash as a potential biomarker of drug efficacy. While drug resistance remains a complex issue, individualizing treatment regimens with toxicity-guided approaches has potential to enhance the activity of currently available TKIs for cancer treatment.

ACKNOWLEDGEMENTS

The authors thank Bayer through Dr. Dennis Healy for providing sorafenib, Dr. Terence Tang for developing the Hep3B-hCG model and Dr. Georg Bjarnason for helpful discussion.
REFERENCES


FIGURE LEGENDS

Figure 1. Decline in plasma sorafenib levels is associated with resistance in HCC. A) Athymic nu/nu mice bearing intrahepatic xenografts of Hep3B-hCG cells eventually acquire resistance to daily 30 mg/kg sorafenib treatment as demonstrated by urinary hCG levels normalized to creatinine. Plasma and tumors were obtained 24h after dosing for drug level assessment from drug sensitive, pre-progression and acquired resistant mice (days 13, 33 and 47-88 respectively; n=6-8). B) Sorafenib plasma concentration was found to decline significantly from sensitive to resistant time points (ANOVA p=0.0204, *p<0.05), indicating a potential pharmacokinetic resistance mechanism. C) Corresponding tumor concentrations of sorafenib also tended to decline but not significantly (ANOVA p=0.309).

Figure 2. Tumor impact on drug concentrations and resistance, and variation in rash development. A) Total sorafenib plasma concentrations sampled 3h after dosing tended to decline in tumor-free (R^2=0.813 days 7-70, p=0.0055) and Hep3B-hCG-bearing (HCC) mouse plasma (R^2=0.723 days 4-70, p=0.0075) but drug levels were generally higher in tumor-free mice. The tumor (p=0.0196) and time (p<0.0001) significantly impacted drug levels (two-way ANOVA). B) The %N-oxide metabolite/total sorafenib was marginally higher in HCC mouse plasma (two-way ANOVA p=0.0063 tumor, p=0.1107 time) and peaked in HCC mice day 11 (T-test p=0.0372). C) Sorafenib concentrations declined significantly from sensitive to resistant phases in the liver (p=0.00184; n=10-11) and tumor (p=0.0192; n=8-9) of HCC mice but not in tumor-free mouse livers (p=0.232; n=6). D) SCID mice pre-conditioned for 45 days with 30 mg/kg sorafenib and subsequently implanted orthotopically with parental tumors developed resistance at the same rate as vehicle preconditioned mice (n=4-5). E) Skin rash toxicity
frequently developed in tumor-free and HCC athymic *nu/nu* mice (arrow) when mice were sorafenib-responsive. **F**) Rash eventually improved weeks 4+ in mice, potentially relating to drug level decline.

**Figure 3. Sorafenib levels are associated with tumoral drug metabolism.** A) Tumor burden (hCG) in 30 mg/kg sorafenib-treated mice (all time points for plasma; sensitive and resistance time points for tissues) correlated significantly with tumor total sorafenib concentrations ($R^2=0.248$, $p=0.0419$) and plasma %N-oxide ($R^2=0.280$, $p<0.0001$) suggesting a link with drug metabolism. Correlations for total sorafenib approached significance in the liver ($R^2=0.151$, $p=0.0820$) and plasma ($R^2=0.0498$, $p=0.074$). **B**) CYP3A4 protein in individual tumors was induced by 30 mg/kg sorafenib treatment and re-induced by escalation to 60 mg/kg. **C**) Analysis of pooled lysates confirmed induction of CYP3A4 in tumor but not in livers (controls $n=4$, HCC $n=9-10$, tumor-free $n=5-6$). **D**) Dose escalation increased sorafenib plasma concentrations and **E** the %N-oxide metabolite in HCC mice, consistent with CYP3A4 expression ($n=10-12$; *$p<0.05$, **$p<0.01$ vs. 30 mg/kg). Arrow= switch to 60 mg/kg.

**Figure 4. Impact of sorafenib dose escalation.** A) Dose-escalating sorafenib to 60 mg/kg significantly increased drug levels in the livers ($p=0.0089$; $n=11$, 9) and tumors ($p=0.0387$; $n=8-9$) of HCC mice, but not in the livers of tumor-free mice ($p=0.161$; $n=5-6$) *$p<0.05$. **B** Escalating the dose at disease progression slowed tumor growth ($n=5$ control, 21-22 sorafenib). **C** Sorafenib-treated mice lost weight (~10% loss day 44) which was accelerated by dose escalation and led to animal termination. **D** Mice maintenance on 30 mg/kg sorafenib had
superior survival (median 60 days) compared to switching to 60 mg/kg (48 days, log-rank p=0.002; n=21-22). **E)** Skin rash (arrowhead) worsened by week 6 after dose escalation then tended to improve. Arrow=switch to 60 mg/kg.

**Figure 5. Effects of sorafenib on cell signaling and anti-tumor activity.** **A)** Sorafenib treatment inhibited phosphorylation of downstream target STAT3 (P-STAT3) in livers (pooled lysates). P-STAT3 increased in tumors and livers of resistance phase HCC and tumor-free mice, but remained high in dose-escalated tissues, indicating some correlation with local drug levels. **B)** Fixed tumor cross-sections show hemorrhagic control tumors and increasingly pale treated tumors. Dose escalated tumors were small and appeared necrotic/white. **C)** Tumor cell proliferation (human Ki67 immunostaining) did not significantly change during treatment (ANOVA p=0.082) but tended to decrease during dose escalation (T-test p=0.0135 vs. resistant). Tumor microvessel density (CD31 (green) and CD34 (red) vessel counts normalized to DAPI (blue)) was significantly inhibited throughout 30 and 60 mg/kg sorafenib treatment (ANOVA p<0.0001; ***p<0.001), showing little association with drug levels. Representative images are shown at right. Bar=500 µm.
Figure 1

A. Tumor burden (hCG) in mice for Drug Level Assessment

B. Plasma Sorafenib

C. Tumor Sorafenib
Figure 2

A. **Plasma Sorafenib**

B. **Plasma %Sorafenib N-Oxide**

C. **Tissue Sorafenib**

D. **hCG: 45 Day Sorafenib Pre-Conditioned Mice**

E. **Skin Rash**

F. **Rash: HCC**

---

**D**hCG: 45 Day Sorafenib Pre-Conditioned Mice

**Figure 2**

- A. **Plasma Sorafenib**
  - Graph showing total sorafenib (ng/ml) over day of treatment for Tumor-free and HCC groups.

- B. **Plasma %Sorafenib N-Oxide**
  - Graph showing % sorafenib N-oxide in urine over day of treatment for Tumor-free and HCC mice.

- C. **Tissue Sorafenib**
  - Graph showing total sorafenib (ng/g) for Liver (tumor-free), Liver (HCC), and Tumor (HCC).

- D. **hCG: 45 Day Sorafenib Pre-Conditioned Mice**
  - Graph showing urinary hCG/creatinine (mIU/mg) over day of treatment for different treatment intervals.

- E. **Skin Rash**
  - Images showing skin rash in HCC, week 4 and Tumor-free, week 4.

- F. **Rash: HCC**
  - Bar chart showing the number of mice with different skin rash types over weeks 0 to 10.

**Author Manuscript Published OnlineFirst on April 23, 2015; DOI: 10.1158/0008-5472.CAN-14-3687**
Figure 3

A

Tumor

Liver

Plasma

Plasma (%N-oxide)

B

Tumor CYP3A4

C

CYP3A4 (Pooled Lysates)

D

Plasma sorafenib

E

Plasma sorafenib N-oxide

Day of treatment

Day of treatment
Figure 4

A. Tissue sorafenib
- Liver (tumor-free)
- Liver (HCC)
- Tumor (HCC)

B. hCG: Dose Escalation Day 29
- Vehicle control
- 30 mg/kg sorafenib
- 30 to 60 mg/kg sorafenib

C. Weight Loss: Dose Escalation
- 30 mg/kg
- 30 to 60 mg/kg

D. Survival: Dose Escalation
- 30 mg/kg
- 30 to 60 mg/kg

E. Skin Rash
- HCC mouse (dose-escalated)
- Week 2
- Week 4
- Week 6
- Week 8

Legend:
- Died without a rash
- Died with a rash
- No Rash
- Rash resolved
- Rash

% weight loss vs. day of treatment

% survival vs. day of treatment

# of mice vs. week
Figure 5

A. Tissue STAT3 Signaling

<table>
<thead>
<tr>
<th></th>
<th>Tumor</th>
<th>Liver (HCC)</th>
<th>Liver (TF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escalated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Histological images showing tissue samples from different conditions.

C. Microvessel Density (MVD) and Tumor Cell Proliferation

<table>
<thead>
<tr>
<th></th>
<th>MVD</th>
<th>Ki67</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitive (30 mg/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant (60 mg/kg)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Escalated (60 mg/kg)</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

Ki67/hematoxylin

CD31/CD34/DAPI

Downloaded from cancerres.aacrjournals.org on April 5, 2017. © 2015 American Association for Cancer Research.
Effects of sorafenib dose on acquired reversible resistance and toxicity in hepatocellular carcinoma.

Elizabeth A Kuczynski, Christina R. Lee, Shan Man, et al.

Cancer Res  Published OnlineFirst April 23, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-3687

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2015/04/25/0008-5472.CAN-14-3687.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.