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Supplemental Data

Report

Accelerated Metastasis after Short-Term Treatment with a Potent Inhibitor of Tumor Angiogenesis

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Supplemental Experimental Procedures

Drug Dosing

For sunitinib, doses of 120 mg/kg/day (given for consecutive 7 days) or 60 mg/kg/day (given continuously) were prepared and administered once a day by gavage as described previously (Ebos et al., 2007), and are referred to throughout the manuscript as 'short-term' or 'sustained' sunitinib therapy, respectively. One week short-term treatment with SU10944 at doses of 225 mg/kg/day was adapted and modified from Patel et al. (Patel et al., 2003) while a sorafenib dose of 150 mg/kg/day was based on acceptable toxicity profiles and molecular marker changes observed in the plasma after 7 days of treatment in non-tumor bearing mice, similar to those described previously with sunitinib (data not shown).

Bioluminescent Imaging

Bioluminescent imaging was performed with a highly sensitive, cooled CCD camera mounted in a light-tight specimen box (IVIS™; Xenogen) as previously described (Jenkins et al., 2005; Ebos et al., 2008). Briefly, mice were injected intraperitoneally with substrate D-luciferin at 150 mg/kg in Dulbecco's Phosphate Buffered Saline (Invitrogen) and anesthetized (4% isoflurane in oxygen for induction, 2% for maintenance) after a 10 minute interval. One to five mice were then placed onto the warmed stage inside the light-tight camera box with continuous exposure to 1–2% isoflurane and imaged for 1 minute. Light emitted from the bioluminescent cells was detected by the IVIS™ camera system with images quantified for tumor burden using a log-scale color range set at $5x10^4$ to $1x10^7$ and measurement of total photon counts per second (photons/sec) using Living Image® software (Xenogen). The only exception to the aforementioned log-scale color range settings occur in Figure 1C (top panel) where a range of $7x10^3$ to $5x10^5$ was used to detect low amounts of cells collected in the lungs 24 hours after i.v. tumor cell injection. For bioluminescent quantification of individual organs described in Figure 3B, a limit of 40-fold greater than control was used for graphing purposes only.

Measurement of Primary and Metastatic Tumor Growth

For primary tumors orthotopically grown in the mammary fat pad (LM2-4^{LUC+}) and in the dermis (B16 and MeWo), tumor size was assessed regularly with Vernier calipers using the formula $width^2 \times length \times 0.5$. For monitoring spontaneous and experimental metastasis growth, and in confirmation with studies previously described by others (Jenkins et al., 2005; Paroo et al., 2004;

Jenkins et al., 2003), bi-weekly monitoring of LM2-4^{LUC+} tumor bearing mice showed bioluminescence paralleled overall tumor burden which, in turn, strongly correlated with overall survival (data not shown). Highly divergent bioluminescence values between mice, likely related to the inherent variability of metastatic disease such as exponential tumor growth rates and differences in metastatic distribution, limited statistical significance to be reached in most cases of bioluminescent quantification. Therefore, bioluminescence was used as a monitor of tumor burden and growth kinetics, while measurement of overall survival was relied on for statistical analysis and interpretation.

Tissue Processing and Immunostaining

Mouse organs were surgically removed and immediately placed in Tissue-Tek optimum cutting temperature compound (Miles Inc.), frozen in dry ice, and then kept at –70°C. Simultaneously, separate tissues were fixed in 10% zinc-buffered formalin (Z-FIX, Anatech Ltd.) for 24 hours, then stored in 70% ethanol. For frozen sections, to distinguish between mouse and human tumor tissue, five micron-thick cryosections were stained with rabbit monoclonal anti-human vimentin antibody (α-vimentin-A; 1:70 dilution; ab16700, Abcam) followed by a FITC-conjugated anti-rabbit antibody (1:200 dilution, Jackson Laboratories). Sections were counterstained with DAPI (1 μg/ml dilution, Molecular Probes). Staining with mouse IgG2a (DakoCytomation) was used as a negative control. Five micron paraffin-embedded tissues were deparaffinized and subjected to detection and counterstaining with hematoxylin using LSAB+ kit, HRP following manufacturer's instructions (LSAB+ System-HRP, DakoCytomation; Code K0679) and a monoclonal mouse anti-vimentin antibody (α-vimentin-B; 1:50 dilution; DakoCytomation; Clone V9).

Tissue Image Acquisition and Quantification

Sections were visualized under a Zeiss Axioplan 2 microscope using a bright-field filter or the FITC (470 nm) and DAPI (350 nm) fluorescence filters. Images were captured with a Zeiss Axiocam camera connected to the microscope using AxioVision 3.0 software. Tissue sections were imaged at 25,100 or 400X (2.5, 10 or 40X objective/10X eyepiece). For table in Figure 3B, micrometastases in organs were defined as tumors < 1 mm diameter. Tissue sections were scored either positive or negative based on the presence or absence of detectable micrometastases. Excluding the lung, organ macrometastases (tumor > 1 mm diameter) were not detected in mice in Figure 3B, with one MeWo liver metastasis being the only exception (shown in Figure 3C, lower panel).

Supplemental References

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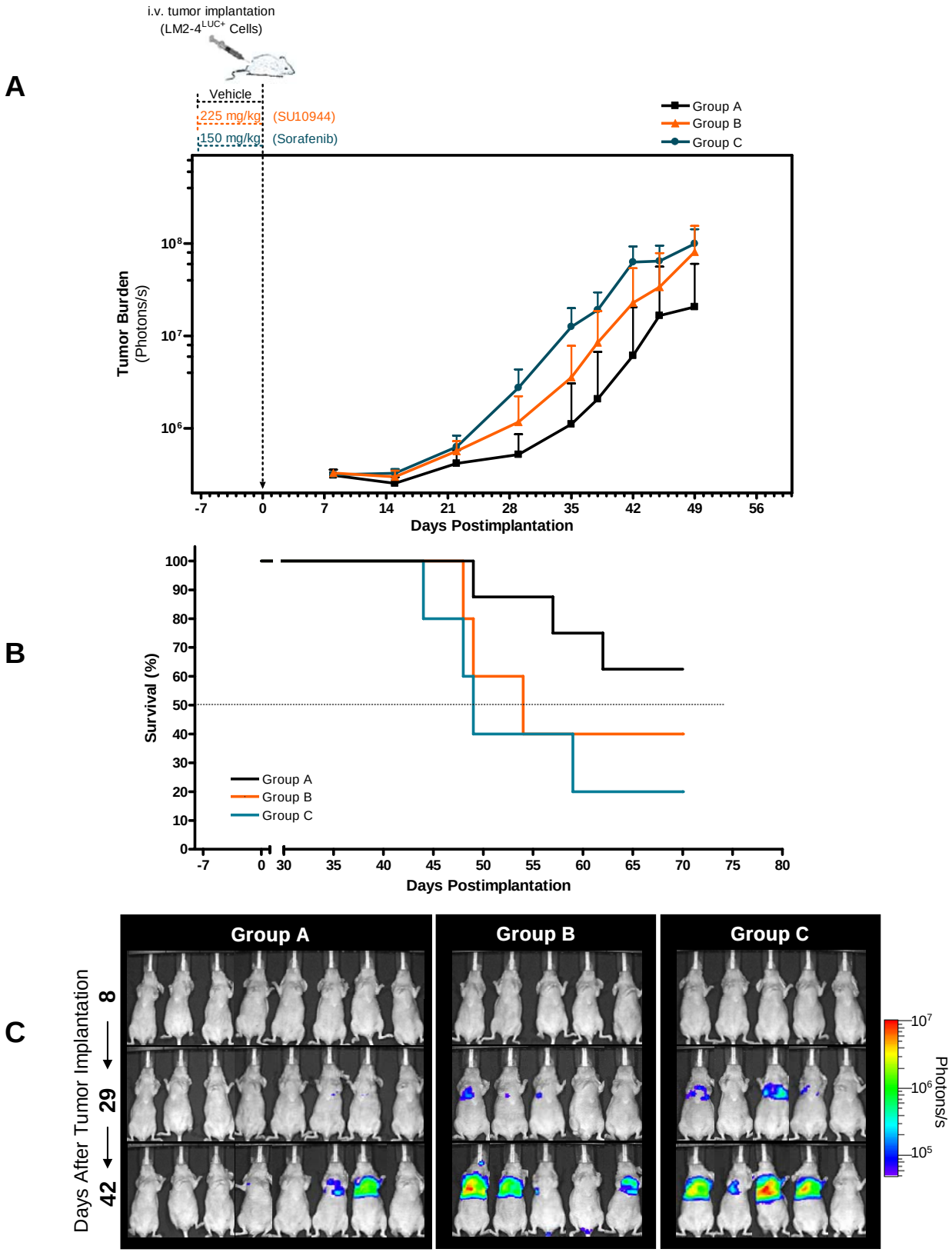
A
i.v. tumor implantation
(LM2-4LUC+ Cells)
Vehicle
225 mg/kg (SU10944)
150 mg/kg (Sorafenib)
Group A
Group B
Group C
Tumor Burden
(Photons/s)
Days Postimplantation
B
Survival (%)
Group A
Group B
Group C
Days Postimplantation
C
Group A
Group B
Group C
Days After Tumor Implantation
8
29
42
Photons/s

Figure S1.

Figure S1. Short-Term Treatments with Sorafenib and SU10944 before Intravenous Tumor Inoculation Accelerate Experimental Metastasis and Decrease Survival in *nu/nu* Mice

(A) $1x10^6$ human metastatic variant breast cells expressing luciferase ($231/LM2\text{-}4^{LUC+}$) were injected into the tail vein of *nu/nu* mice that had received vehicle (Group A), SU10944 treatment (225 mg/kg/day), or short-term sorafenib treatment (150 mg/kg/day daily for 7 days) before tumor inoculation (Groups B and C, respectively). Quantification of bioluminescence showed accelerated tumor growth in Groups B and C compared with controls in Group A. Group A (n = 8), Group B (n = 5), Group C (n = 5), with Group A treatments comprising both Vehicle 1 and 2 formulation (n = 4, respectively). Data are shown as mean ± SD.

(B) Kaplan-Meier survival curve of corresponding mice from Figure S1A show decreased median survival of mice in Group B (log rank test; p = 0.2850) and Group C (log rank test; p = 0.0627), when compared with vehicle-treated controls in Group A.

(C) Representative images for each group taken 8, 29 and 42 days post-tumor implantation with increased metastasis visible in SU10944- and sorafenib-treated mice.

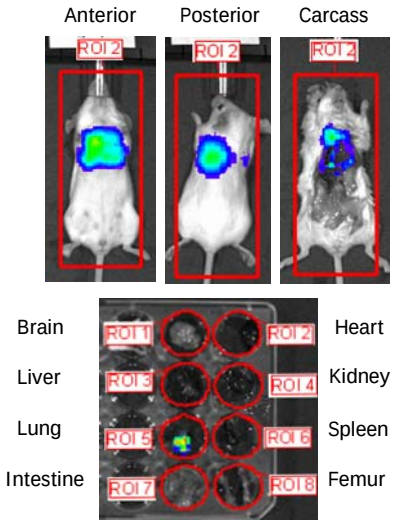

Figure S2. Bioluminescent Measurement of Multiorgan Metastasis in Mice after Short-Term Sunitinib Treatment

For quantification of organ bioluminescence as described in Figure 3B, bioluminescent images were taken of anterior and posterior regions, with individual organs and remaining carcass imaged immediately following sacrifice (representative image shown).

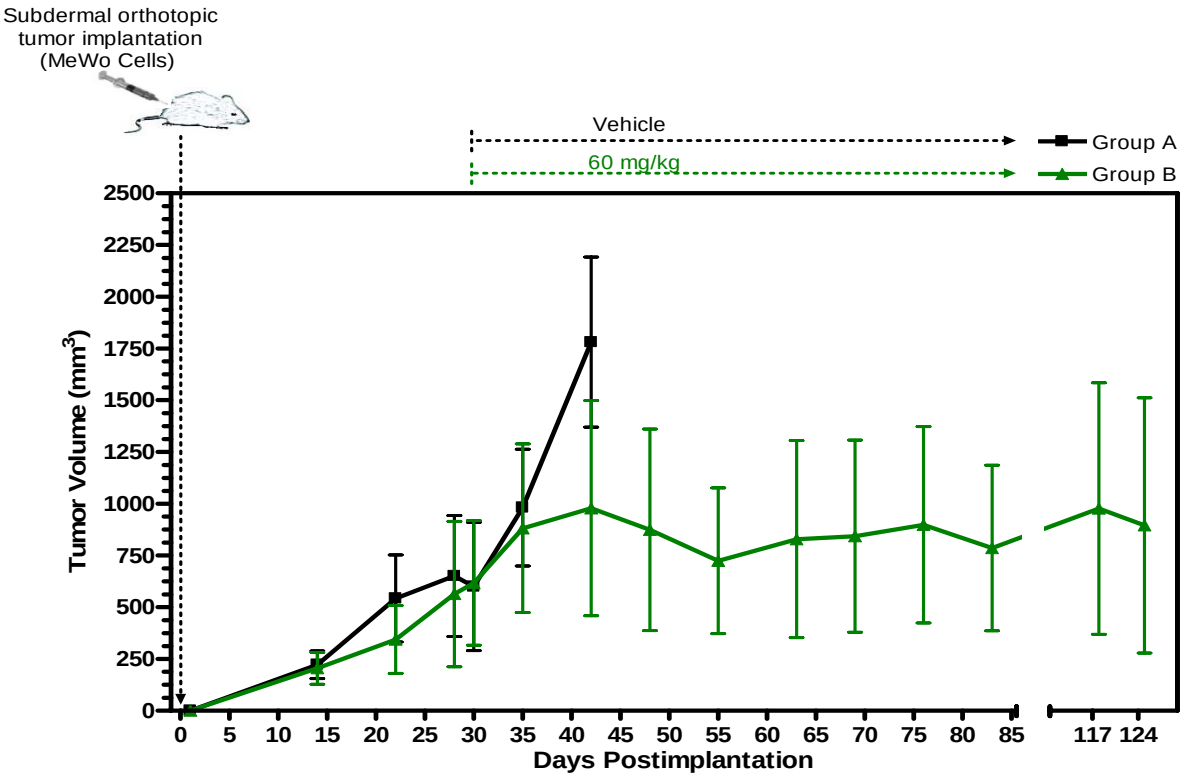

Figure S3. Tumor Growth Delay after Sustained Sunitinib Treatment in Orthotopically Grown Human MeWo Melanoma Xenografts

Human melanoma MeWo cells ($2x10^6$) implanted orthotopically into the dermis of nu/nu mice (Day 0) received either daily vehicle (Group A) or daily sunitinib (Group B - 60 mg/kg/day) continuously when tumors reached an average of 600 mm^3. Group B showed delayed primary tumor growth compared to Group A. Group A (n = 5), Group B (n = 5).

Data are shown as mean ± SD.