Convection-enhanced delivery of sorafenib and suppression of tumor progression in a murine model of brain melanoma through the inhibition of signal transducer and activator of transcription 3

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OBJECTIVE Despite recent advances, metastatic melanoma remains a terminal disease, in which life-threatening brain metastasis occurs in approximately half of patients. Sorafenib is a multikinase inhibitor that induces apoptosis of melanoma cells in vitro. However, systemic administration has been ineffective because adequate tissue concentrations cannot be achieved. This study investigated if convection-enhanced delivery (CED) of sorafenib would enhance tumor control and survival via inhibition of the signal transducer and activator of transcription 3 (Stat3) pathway in a murine model of metastatic brain melanoma.

METHODS Melanoma cells treated with sorafenib in vitro were examined for signaling and survival changes. The effect of sorafenib given by CED was assessed by bioluminescent imaging and animal survival.

RESULTS The results showed that sorafenib induced cell death in the 4 established melanoma cell lines and in 1 primary cultured melanoma cell line. Sorafenib inhibited Stat3 phosphorylation in HTB65, WYC1, and B16 cells. Accordingly, sorafenib treatment also decreased expression of Mcl-1 mRNA in melanoma cell lines. Because sorafenib targets multiple pathways, the present study demonstrated the contribution of the Stat3 pathway by showing that mouse embryonic fibroblast (MEF) Stat3+/+ cells were significantly more sensitive to sorafenib than MEF Stat3−/− cells. In the murine model of melanoma brain metastasis used in this study, CED of sorafenib increased survival by 150% in the treatment group compared with animals receiving the vehicle control (p < 0.01). CED of sorafenib also significantly abrogated tumor growth.

CONCLUSIONS The data from this study indicate that local delivery of sorafenib effectively controls brain melanoma. These findings validate further investigation of the use of CED to distribute molecularly targeted agents.

http://thejns.org/doi/abs/10.3171/2015.3.JNS132040

KEY WORDS sorafenib; convection-enhanced delivery; Stat3; melanoma; brain; mice; oncology

D espite the low incidence of melanoma compared with other cancers, melanoma is the third most common type of cancer to metastasize to the brain. Patients with malignant melanoma have a 37% chance of being diagnosed with brain metastasis and a 75% chance of harboring this disease in the brain at autopsy.29 When brain metastasis occurs, treatment is extremely difficult due to high resistance to radiation and chemotherapy; median survival with maximal treatment is 5–9 months. Ipilimumab, an anticytotoxic T-lymphocyte-associated protein 4 antibody,9 and vemurafenib, an inhibitor of mutant B-Raf,20 are 2 exciting targeted therapies that have been recently developed. However, due to tumor escape mechanisms, these drugs only extend survival by 2–6 months and, for unclear reasons, ipilimumab and vemurafenib have at most a modest effect on melanoma brain metastasis.7

New insights into the biology of this cancer and state-of-the-art drug delivery methods may provide a frame-
work for novel therapeutic approaches that combine molecular and physical targeting. Of particular interest is signal transducer and activator of transcription 3 (Stat3), an essential signaling molecule in carcinogenesis found to be highly overexpressed in melanomas. Stat3's transcriptional targets include genes mediating apoptosis (survivin, Bcl-xl, Mcl-1, cellular FLICE-like inhibitory protein); proliferation (c-fos, c-myc, cyclin D1); invasion (matrix metalloproteinase-2); and angiogenesis (vascular endothelial growth factor). Functionally, Stat3 activation has been associated with chemoresistance and radioresistance.

Sorafenib was developed as a c-Raf kinase inhibitor and, by extension, was expected to be potent because the Ras-Raf pathway has a prominent role in the biology of melanomas. Interestingly, our group and Delgado et al. recently demonstrated that sorafenib inhibits Stat3 signaling in medulloblastomas and esophageal carcinoma, respectively. Thus, given its inhibitory targets, sorafenib would seem to be a promising drug for the treatment of melanomas. Indeed, in vitro testing demonstrated good activity. However, in clinical trials, maximal doses of orally administered sorafenib showed limited activity against metastatic melanoma as a single agent.

The failure of systemically administered sorafenib in clinical trials highlights a challenge of this form of drug delivery. Systemic delivery of therapeutic agents generally results in increased toxicity to normal tissues and organs. Additionally, drug distribution to protected organs, such as the brain, is limited. Hence, there is good rationale for using local delivery methods to target bulky metastasis, which often causes neurological compromise. Accordingly, convection-enhanced delivery (CED), a highly controlled form of microinjection developed for regional drug delivery to the CNS, has received increasing interest due to the potential advantages that it provides when compared with other delivery methods. One is that CED is able to bypass the blood-brain barrier, allowing for targeted delivery into a designated area with limited neurotoxicity. Another potential advantage resides in its capability to distribute therapeutic agents homogeneously over large volumes of normal or pathological tissue, as demonstrated by preclinical and clinical studies. As an aside, nanoparticles as large as viruses have been conjugated for gene therapy of Parkinson's disease. In this study, we investigated CED of sorafenib in a murine model of metastatic brain melanoma. We demonstrated that sorafenib in vitro causes cytotoxicity in melanoma cells, at least partially through inhibiting Stat3 phosphorylation. The results of this study also revealed that CED of sorafenib into melanomas within the brain effectively abrogated tumor growth.

Methods

Cell Culture

The B16 murine melanoma cell line and 3 human melanoma cell lines (A2058, HTB65, and HTB72) were obtained from the American Type Culture Collection. WYC1, a primary melanoma culture, was a generous gift from Dr. James Wang. All cells were maintained in media with 10% fetal bovine serum and the antibiotics. For B16, WYC1, and A2058 cell lines, Dulbecco's Modified Eagle's Medium (DMEM) was used. For HTB65 and HTB72, Eagle's Minimum Essential Medium (EMEM) was used.

Cell Viability Assay

Cytotoxicity was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega). Each well of 96-well plates was seeded with 1000 cells in culture medium with 10% fetal bovine serum. After overnight culture, the cells were treated with different concentrations of sorafenib (Onyx and Bayer Pharmaceuticals) and melphalan. Controls were treated with vehicle control (dimethyl sulfoxide [DMSO]). After 96 hours of treatment, the assay was performed according to the supplier's protocol and measured using a luminescent plate reader. Experiments were performed in quadruplicate and repeated independently at least twice.

Immunoblotting Analysis

All antibodies were obtained from Cell Signaling. Total protein (20 µg) was denatured and separated on 4%–12% gradient Tris-Glycine gels (Invitrogen). Protein samples were transferred to polyvinyl difluoride membranes (Invitrogen). The membranes were blocked for 1 hour at room temperature in blocking buffer (5% skim milk; Invitrogen). After gel electrophoresis, the proteins were transferred to polyvinyl difluoride membranes (Invitrogen). The membranes were incubated with primary antibodies in 1× phosphate-buffered saline (PBS) with 0.1% Tween 20 with 5% bovine serum albumin. The membranes were then incubated with horseradish peroxidase–labeled antimouse or antirabbit secondary antibodies for 30 minutes at room temperature. Immunoreactivity was detected with SuperSignal West Pico substrate (Pierce).

Firefly Luciferase–Expressing B16 Melanoma Cell Line

For the animal study, pGL4.51[2uc23cmvneo] vector (Promega) encoding firefly luciferase was stably transfected into B16 melanoma cells to facilitate noninvasive bioluminescent imaging. Individual stable clones were selected in the presence of 1 mg/ml G418 (Sigma).

Sorafenib

Sorafenib was dissolved in DMSO and Tween 20 at a concentration of 50 mmol/L and stored at −20°C before use for in vitro and in vivo experiments. For oral administration, sorafenib was freshly prepared in DMSO and Tween 20 at a concentration of 10 mg/ml.

Synergy Calculations

We used a method described by Hata et al. and Zhang et al. to determine drug synergy. Predicted survival was compared with actual survival values at each dose. Predicted survival (c) was calculated from the equation $c = (a \times b)/100$, where a and b represent survival values for a single agent. The 2 drugs were considered synergistic if the actual survival values were less than 70% of the predicted survival values.
B16 Melanoma Xenograft Model

Six to 8 mice were used for experiments. All animals were housed and handled in accordance with the City of Hope Research Animal Care Committee guidelines. Six- to 8-week-old female C57BL/6 (Charles River Laboratories) mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. Mice were shaved on the head above the nape of the neck. Animals were placed in a Kopf stereotactic frame. A 5-mm skin incision was made along the sagittal suture and a bur hole was drilled into the skull. Firefly luciferase–expressing B16 (B16-ffluc) melanoma cells (1.5 × 10⁶ cells/mouse) suspended in 10 μl PBS were stereotactically implanted into the right striatum, 0.5 mm anterior to and 2.5 mm to the right of the bregma and 3 mm below the dural surface. The incision was sealed with Nexaband glue after tumor implantation.

Convective-Delivery Rate of Sorafenib

C57BL/6 mice with B16-ffluc melanoma had sorafenib injected into their striatum at the same incision site using CED. The mice were anesthetized by exposure to 4% isoflurane for 2 minutes, and then ketamine and xylazine were administered by intraperitoneal injection to maintain anesthesia. The CED system was set up by connecting an injection syringe, which was filled with sorafenib or its carrier, to an external pump with an injecting rate range of 0.03–10 μl/min. A 21-gauge needle was used in CED for drug infusion throughout the target region. Animals were placed in a Kopf stereotactic frame and secured using ear bars. A sagittal incision was made and the 21-gauge needle was stereotactically guided to 0.5 mm anterior to and 2.5 mm to the right of the bregma, 3 mm below the brain surface. Delivery was initiated by activating the pump. The delivery rate was 0.2 μl/min. After that, the needle was withdrawn, and the incision was glued. For postoperative pain, buprenorphine was subcutaneously administered to provide analgesia.

Animal Experiments and Imaging

Dose-response curves of CED of varying sorafenib concentrations (10, 100, 300, 500, and 1000 μM) were generated in non–tumor-bearing C57BL/6 mice to determine the maximal tolerated dose in normal brain. Based on clinical observation and histological analysis (supplemental data), CED of 100 μM sorafenib was used for further experiments.

In tumor-bearing animals, CED was performed once or twice after tumor inoculation. In the former group, 10 μl of 100 μM sorafenib or vehicle was convected at a rate of 1 μl/min into the brain tumor 7 days after tumor inoculation, using previously described methods. The same stereotactic coordinates used for tumor implantation were used for CED of the sorafenib. For bioluminescent imaging, anesthetized mice were given 150 mg/kg D-luciferin intraperitoneally. Five minutes after injection, images were acquired using the Xenogen in vivo imaging system. Bioluminescent signal was measured as photons per second per square centimeter in defined regions of interest using Living Image software (Xenogen). Imaging was performed on Day 6 after tumor implantation immediately prior to treatment, and on Days 1, 2, and 3 after CED of the sorafenib. Survival was measured from the time of tumor inoculation. To prevent undue suffering, euthanasia was performed if mice developed significant distress.

In the group in which animals were treated twice, 10 μl of 100 μM sorafenib was convected on Days 4 and 8 after B16-ffluc injection. Xenogen imaging was performed 2 days after each treatment. In the group in which animals were treated with oral administration of sorafenib, the mice received 50 mg/kg/day for 7 days by gavage, starting on Day 4 after tumor inoculation.

Statistical Analysis

One-way ANOVA was used to establish whether significant differences existed between groups. Statistical analyses were performed using SPSS version 14.0 (IBM). A p < 0.05 was considered statistically significant.

Results

Sorafenib Cytotoxicity In Vitro

We first assessed the potency of sorafenib in vitro to confirm its described cytotoxicity. Dose-response curves were generated using 4 established melanoma cell lines (A2058, HTB65, HTB72, and B16) and 1 primary cultured melanoma cell line (WYC1). Toxicity occurred in the micromolar range, with median inhibitory concentrations (IC50) ranging from 4.5 μM to 12 μM (Fig. 1A, supplementary Fig. 1). At doses approaching 20 μM, sorafenib was able to cause at least a 90% reduction in cell viability. These results, showing a relatively high IC50, confirmed our suspicion that systemic delivery would not be able to achieve sufficient concentrations to kill melanoma cells. To treat melanoma, the potency of sorafenib would have to be increased by modifying its pharmacodynamics or pharmacokinetics.

Synergism Between Sorafenib and Melphalan

Melphalan is a chemotherapeutic agent that has been used in the past to treat melanoma, with modest results. Clinical studies also indicate that systemically administered sorafenib is ineffective. Both drugs have had minimal success in patients. We rationalized that coadministration of sorafenib and melphalan may be synergistic because sorafenib-mediated inhibition of the Ras-Raf kinase pathway has been shown to enhance the chemosensitivity of cancer cells to other chemotherapeutic agents. While the sorafenib dose was held constant (approximately the IC50 dose), the melphalan dose was increased. Thus, if there was no synergism, then the predicted toxicity at each dose of melphalan would be the additive effect of the sorafenib and the melphalan. With a few minor exceptions, the amount of synergism seen in the HTB65 and WYC1 melanoma cell lines was negligible (Fig. 1B). We also found no evidence of synergy between sorafenib and temozolomide, another drug commonly used to treat melanoma, in A2058, HTB65, HTB72, and WYC1 cells (data not shown). Thus, further experiments used sorafenib alone.
Sorafenib Inhibits Stat3 Phosphorylation in Melanoma Cell Lines

Because Stat3 is highly overexpressed in melanomas and has recently been shown to be a sorafenib target in other cancers, we investigated whether Stat3 inhibition accounts for at least part of sorafenib’s toxicity against melanomas. HTB65, WYC1, and B16 cells were harvested at 5 and 24 hours after sorafenib treatment and then assessed for phosphorylation status of Stat3 by Western blot (Fig. 2A–C). In HTB65 cells, phosphorylation of Stat3 (pStat3) was only inhibited by sorafenib after 24 hours (Fig. 2A). In WYC1 and B16 cells, pStat3 was significantly inhibited at both 5 and 24 hours after treatment (Fig. 2B and C).

Interleukin 6 (IL-6) is a strong activator of the Jak/Stat3 pathway, which often occurs through an autocrine loop in cancer cells. A measure of sorafenib’s anti-Stat3 activity is whether it can inhibit Stat3 in the presence of IL-6 stimulation. HTB65 cells were serum starved for 6 hours before treatment with 2 μM sorafenib. After 5 or 24 hours of sorafenib exposure, the melanoma cells were subjected to 1 or 4 hours of IL-6 stimulation prior to analysis. Serum starvation abrogated baseline pStat3 expression (Fig. 2D and E). IL-6 stimulation resulted in pStat3 expression with or without sorafenib under all conditions. These results indicate that although sorafenib targets Stat3, its suppression of Stat3 is readily overcome by IL-6.

Sorafenib Preferentially Targets Stat3 +/+ Cell Lines

Small-molecule inhibitors often target multiple pathways, making it difficult to determine whether suppression of any particular pathway contributes to a drug’s cytotoxicity. To assess the importance of Stat3 targeting for sorafenib, we examined the cytotoxicity caused by the drug in mouse embryonic fibroblast (MEF) Stat3 +/+ and MEF Stat3 −/− cells. MEF Stat3 +/+ was significantly more sensitive to both 6 μM (Fig. 3A) and 12 μM (Fig. 3B) sorafenib (p < 0.05). If Stat3 was not an important sorafenib target, then one would have expected MEF Stat3 −/− cells to have been equally, if not more, sensitive.

FIG. 1. Sorafenib potency and synergism. The cytotoxicity of sorafenib on melanoma cell lines. Dose-response curves were generated by measuring cell viability 96 hours (HTB65 and WYC1) or 48 hours (B16) after sorafenib treatment (A). Dose-response curves of melphalan alone, melphalan plus sorafenib (10 μM; the IC50 dose), and the predicted additive cytotoxicity of melphalan plus sorafenib (B). Significant synergism occurred when survival of melphalan plus sorafenib cells was less than 70% of the predicted value, an event that seldom occurred.

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Z. Zou et al.

**Mcl-1 and Bcl-xl Expression After Sorafenib Treatment**

The antiapoptotic genes *Mcl-1* and *Bcl-xl* are well-known downstream Stat3 targets. Western blot to determine expression of these 2 genes was performed in HTB65, WYC1, and B16 cells 5 and 24 hours after sorafenib treatment (Fig. 4). In HTB65 and B16 cells, *Mcl-1* was downregulated after 24 hours. In WYC1 cells, downregulation of *Mcl-1* was observed after 5 hours. Contrary to expectations, *Bcl-xl* was not downregulated by sorafenib treatment in either cell line.

**Convection-Enhanced Delivery of Sorafenib In Vivo**

Although CED is a very accurate form of microinfusion, a small amount of leakage of the infusate into healthy surrounding brain tissue is unpreventable. Therefore, it was critical to examine normal brain tolerance for directly injected sorafenib for when leakage occurs. Non–tumor-bearing C57BL/6 mice that underwent CED of 10 μM or 100 μM sorafenib into normal brain were observed for up to 1 month for toxicity. The mice tolerated both doses very well, displaying no weight loss, neurological deficits, or behavioral abnormalities (supplementary Table 1). H & E staining of the brain tissue also revealed no evidence of neurotoxicity (Fig. 2).

Because CED has not been used in melanomas previously, we then optimized parameters for CED into our intracranial melanoma model, using Evans Blue as a surrogate marker. Distribution of 10 μl of Evans Blue into normal brain and melanomas achieved a volume of distribution that encompassed the entire tumor (supplementary Fig. 3).

Next, we examined the effect of CED of sorafenib into B16-fluc+ melanomas implanted intracranially in C57BL/6 mice. Bioluminescent imaging revealed that CED of sorafenib 6 days after implantation decreased the tumor volume compared with CED of the vehicle control (p < 0.05) (Fig. 5A). On Day 3 after treatment, average tumor bioluminescence in animals given sorafenib via
Convective delivery of sorafenib for brain melanoma

CED was (100 ± 81) × 10⁴ photons/second, whereas average tumor bioluminescence in vehicle controls was (500 ± 300) × 10⁴ photons/second (Fig. 5C). Correspondingly, the treatment group survived 150% longer than the control animals (p < 0.01, Fig. 5B).

Because a single treatment showed significant antitumor activity, we investigated if sequential treatments in the same animal would further improve results. CED of sorafenib or vehicle control was then performed on Days 4 and 8 after tumor implantation. To highlight the advantages of CED, we added another control group, in which mice were treated with high doses of orally administered sorafenib. Survival of mice given 2 doses of sorafenib via CED was not significantly different than of those given 1 dose (Fig. 6). Survival of mice given orally administered sorafenib was not significantly different than of animals that received CED of the vehicle control.

Discussion

Treatment of melanoma brain metastasis is hampered by the cancer’s intrinsic resistance to therapy and the protection from systemic agents afforded by the blood-brain barrier. The use of CED alleviates these issues. The technique bypasses the blood-brain barrier and simultaneously allows precise delivery of high concentrations of drugs such as sorafenib, which have limited efficacy at lower doses.

Despite its middling potency, sorafenib is an attractive antimelanoma agent because of its molecular targets. These include the Raf kinases, which are highly active in the majority of melanomas. In our study, we demonstrated that sorafenib also inhibits Stat3, an active pathway in many cancers, but one that has particularly high levels of expression in melanomas. Perhaps indicative of sorafenib’s mechanism of cytotoxicity, Stat3 activation was inhibited at 6 μM (Fig. 2), which is within the range of the IC50s (4.5–12 μM) determined in multiple melanoma cell lines (Fig. 1).

Stat3 signaling can increase chemoresistance by preventing apoptosis through positive upregulation of Mcl-1 and Bcl-xl in diverse human tumor cell types. Previous studies have also indicated that sorafenib can downregulate the expression of Stat3 and Mcl-1 in several types of tumor cells. The effect of sorafenib on these antiapoptotic genes in melanomas has not been previously described. Our results showed that sorafenib treatment reduced Mcl-1, but not Bcl-xl, expression in melanoma cell lines (Fig. 4). This is consistent with previous studies, which indicate that the relationship between Stat3, Mcl-1, and Bcl-xl in melanomas is complex and often contextual. Our data also suggest that inhibition of the Stat3/Mcl-1 pathway plays a role in sorafenib’s cytotoxicity. Further supporting this hypothesis, we found that MEF Stat3+/+ cells were significantly more sensitive to sorafenib than MEF Stat3−/− cells (Fig. 3). If Stat3 was not an important target of sorafenib, one would expect that the MEF Stat3 knockouts would have been equally, if not more, susceptible to sorafenib’s effects, particularly because Stat3 is a prosurvival factor.

Because sorafenib is able to suppress multiple pathways important to melanoma survival, we hypothesized that there may be synergy with other chemotherapeutic agents. Indeed, sorafenib is synergistic with agents such as vorinostat, sunitinib, and gemcitabine. However, we found no such synergy with either melphalan or temozolomide. Because both of these drugs are alkylating agents, it is possible that the 2 pathways through which these drugs and sorafenib exert cytotoxicity do not cross and could even interfere with one another. For example, some evidence suggests that there may be an antagonistic effect because alkylating agents upregulate Mcl-1 expression in myeloid leukemia cells.

Because IC50s were in the 4.5–12 μM range, this concentration of drugs would be extraordinarily difficult...
FIG. 5. Regression of established melanoma xenografts after CED of sorafenib. Mice bearing intracranial B16-fflucl tumors were treated by CED of sorafenib or vehicle 6 days after tumor implantation. Bioluminescent imaging of intracranial tumor burden at Days 0, 1, and 2 after treatment show a dramatic effect of CED of sorafenib (A). Kaplan-Meier analysis showed improved survival for the mice treated by CED of sorafenib (15 ± 2.58 days) versus vehicle control (10 ± 1.51 days) (**p < 0.01; n = 6–8) (B). Average bioluminescent signal from tumors in animals treated with sorafenib was significantly decreased compared with animals treated with vehicle (*p < 0.05; n = 6–8) (C).

FIG. 6. Effect of serial CED of sorafenib in a murine model of melanoma brain metastasis. CED of sorafenib (100 μM) or vehicle control was performed on Days 4 and 8 after B16-fflucl+ implantation. This was compared with a group of animals in which sorafenib was administered orally (50 mg/kg/day) for 7 days. Bioluminescent imaging done on Days 6 and 10 after tumor implantation showed responses to convected and systemically given sorafenib and control (A). Kaplan-Meier analysis showed improved survival for mice that received CED of sorafenib compared with CED of vehicle or oral administration (**p < 0.01; n = 6–8) (B). Graph indicating that CED of sorafenib significantly decreased tumor growth (**p < 0.01; n = 6–8) (C).
to achieve in any particular tissue, much less the brain. Highlighting this fact is the failure of high doses of orally administered sorafenib to have an effect on tumor size or survival in mice with intracranial melanoma implants (Fig. 6). This contrasts sharply with the animals that were treated by CED of sorafenib. These animals exhibited 150% greater survival, with corresponding responses seen in noninvasive imaging (Figs. 5 and 6). To improve on this result, we investigated if serial treatments would further prolong survival. Surprisingly, this approach did not cause the desired effect (Fig. 6). We speculated that the cells that were sensitive to sorafenib were already killed by the first treatment, leaving behind cells resistant to the second treatment. Indeed, in clinical practice, rapid tolerance occurs to small-molecule inhibitors used for melanoma therapy.

Overall, our results demonstrate that CED of sorafenib into melanomas within the brain can decrease tumor size and increase survival in a murine model. However, predicting the utility of this technique in the clinical setting is difficult. Convective distribution of a drug in a rodent brain tumor is more readily achievable than in the human counterpart. One of the main underlying causes of the discrepancy is the difference in size between rodent and human brain tumors. Rodent brain tumors are so small that even when convective flow does not achieve ideal drug distribution, diffusion of the drug can still cause a major effect. Human brain tumors are usually up to an order of magnitude larger and thus demand more of the convective, rather than the diffusive, component of CED for drug distribution. Furthermore, the larger target volume introduces more tissue heterogeneity, which adversely affects the predictability of convective distribution. Therefore, effects of CED could be overestimated in a murine model. It is also difficult to estimate side effects of CED of drugs into the brain by using a murine model. Despite these limitations, numerous human studies using CED have confirmed that the fundamental properties of CED established in the murine model remain the same. CED of small-molecule inhibitors appears to be a promising approach warranting further investigation and refinement.

Conclusions

Our study suggests that sorafenib’s inhibition of Stat3 in melanoma cells is one mechanism through which it causes cellular death. Convective delivery of sorafenib is feasible and augments its effectiveness in an intracranial model of melanoma metastasis. In light of the results of recent clinical trials, it would be particularly interesting to investigate CED of more potent antimelanoma small-molecule inhibitors, such as ipilimumab or vemurafenib.

Acknowledgment

This project was supported by the National Institutes of Health K12, Clinical Oncology Career Development Grant (CA001727).

References

Disclosure
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: Chen, Zou, Yang, Jove, Li. Acquisition of data: Zou, Yin, Hsu, Li, Brandon. Analysis and interpretation of data: Chen, Zou, Li. Reviewed submitted manuscript on behalf of all authors: Chen. Statistical analysis: Chen, Jandial, Li. Reviewed submitted version of manuscript: Chen, Lin. Approved the final version of the manuscript on behalf of all authors: Chen. Statistical analysis: Chen, Zou. Administrative/technical/material support: Chen. Study supervision: Chen, Li.

Supplemental Information
Online-Only Content
Supplemental material is available with the online version of the article.

Supplementary Table 1 and Supplementary Figures 1–3.

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