

Tris (Dibenzylideneacetone) Dipalladium, a *N*-Myristoyltransferase-1 Inhibitor, Is Effective against Melanoma Growth *In vitro* and *In vivo*

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Abstract Purpose: Melanoma is a solid tumor that is notoriously resistant to chemotherapy, and its incidence is rapidly increasing. Recently, several signaling pathways have been shown to contribute to melanoma tumorigenesis, including constitutive activation of mitogen-activated protein kinase, Akt, and Stat-3. The activation of multiple pathways may account in part for the difficulty in treatment of melanoma. In a recent screen of compounds, we found that an organopalladium compound, Tris (dibenzylideneacetone) dipalladium (Tris DBA), showed significant antiproliferative activity against melanoma cells. Studies were carried out to determine the mechanism of action of Tris DBA.

Experimental Design: Tris DBA was tested on efficacy on proliferation of human and murine melanoma cells. To find the mechanism of action of Tris DBA, we did Western blot and gene array analyses. The ability of Tris DBA to block tumor growth *in vivo* was assessed.

Results: Tris DBA has activity against B16 murine and A375 human melanoma *in vivo*. Tris DBA inhibits several signaling pathways including activation of mitogen-activated protein kinase, Akt, Stat-3, and S6 kinase activation, suggesting an upstream target. Tris DBA was found to be a potent inhibitor of *N*-myristoyltransferase-1, which is required for optimal activity of membrane-based signaling molecules. Tris DBA showed potent antitumor activity *in vivo* against melanoma.

Conclusion: Tris DBA is thus a novel inhibitor of *N*-myristoyltransferase-1 with significant antitumor activity and is well tolerated *in vivo*. Further preclinical evaluation of Tris DBA and related complexes is warranted.

Melanoma is one of the most common solid tumors and is notoriously difficult to treat. Recently, constitutive activation of several signaling pathways has been shown in melanoma. Many melanomas carry mutations in B-raf, which cause constitutive activation of mitogen-activated protein kinase (MAPK; refs. 1, 2). Even melanomas that do not carry activated B-raf show activation of MAPK, and constitutive expression of activated MAPK kinase is sufficient to transform melanocytes to melanoma (3–5). Other pathways that are known to be activated in advanced melanoma include phosphoinositol

3-kinase (PI3K)/Akt and nuclear factor- κ B (6–10). All of these pathways confer survival and proliferative advantages to melanoma, such as induction of angiogenic factors, including vascular endothelial growth factor (VEGF), interleukin-8, survivin, IAP, and mcl-1 (11–13).

Platinum compounds have been the mainstay of many solid tumor regimens, especially testicular cancer. However, platinum compounds, including cisplatin and carboplatin, have also shown activity in melanoma and have been incorporated into melanoma treatment regimens (14). Other inhibitors, such as sorafenib, a B-raf inhibitor, have had modest effects on melanoma with B-raf mutation despite robust inhibition of B-raf (15). This may be due to the ability of aggressive tumors to switch signaling pathways (16). We have observed this phenomenon in Burkitt's lymphoma, in which MAPK is activated when nuclear factor- κ B is down-regulated (17). Similarly, inhibition of nuclear factor- κ B with Velcade has had modest effects in melanoma (18, 19).

In our screens for angiogenesis inhibitors, we have identified a small-molecule palladium complex, which has structural similarities to curcumin and chalcones, compounds with known chemopreventive activity (20, 21). Although chemopreventive agents are effective against preneoplastic lesions in mice and man, they are less effective against established tumors (22, 23). Analysis of Tris (dibenzylideneacetone) dipalladium (Tris DBA)-treated melanoma cells by gene array revealed reduction of *N*-myristoyltransferase-1 (NMT-1), which was confirmed by quantitative reverse-transcription PCR (RT-PCR).

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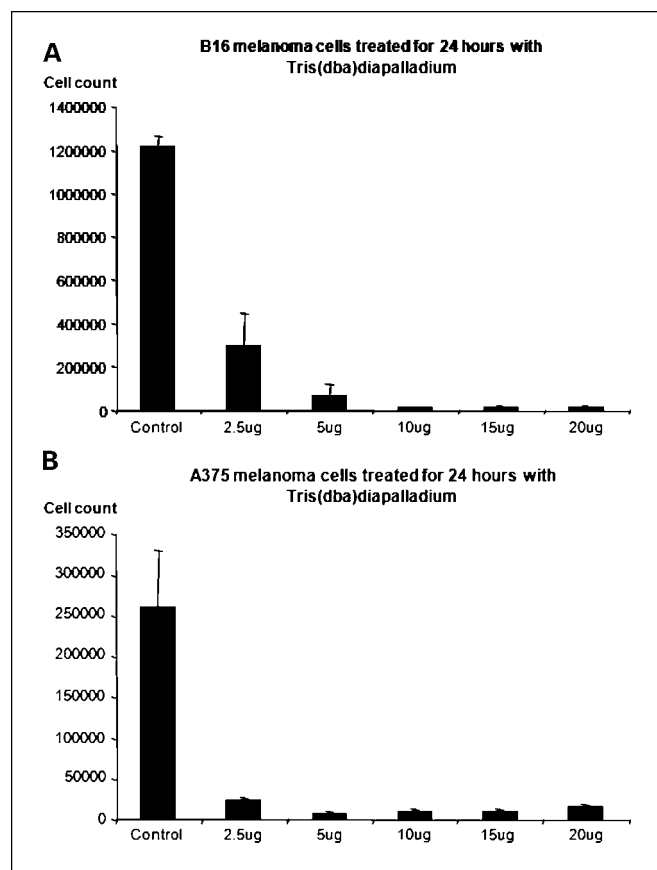


Fig. 1. Tris DBA inhibits murine melanoma cell proliferation (A) and human melanoma cell proliferation (B) *in vitro*. Tris DBA decreases B16 and A375 cell number in a dose-dependent fashion. Control is with the vehicle, DMSO. Columns, average of triplicate experiments; bars, SE.

Myristoylation done by NMT-1 is required for most membrane-based signaling molecules. C-src is a candidate molecule that requires myristoylation for optimal activity. Tris DNA reduced expression of c-src, which is a substrate of NMT-1. Consistent with inhibition of c-src/NMT-1, Tris DBA inhibited downstream signaling pathways, including MAPK kinase, PI3K, and Stat-3. Tris DBA has activity *in vivo* against A375 and B16 melanoma *in vivo*. Further preclinical evaluation of Tris DBA is warranted.

Materials and Methods

Cells. B16 melanoma cells were cultured in DMEM (1,000 mg glucose/L; Sigma-Aldrich) supplemented with 10% fetal bovine serum, L-glutamine (14 mL/L), and antibiotic/antimycotic (14 mL/L; Sigma-Aldrich). A375 cells were cultured in DMEM (4,500 mg glucose/L; Sigma-Aldrich) supplemented with 10% fetal bovine serum, L-glutamine (14 mL/L) and antibiotic/antimycotic (14 mL/L; Sigma-Aldrich).

Cell proliferation assays. To evaluate the potential of Tris DBA as an antitumor agent, a proliferation assay was done using B16 cells (Fig. 1A) and A375 cells (Fig. 1B). The assay was done according to the method described previously by the Arbiser laboratory (24, 25). Ten thousand cells were plated per well in a 24-well plate. After incubation for 24 h at 37°C and 5% CO₂, the cells were treated at 2.5, 5, 10, 15,

and 20 µg/mL from a stock solution of 10 mg/mL Tris DBA dissolved in DMSO, also used as the control. Experiments were done in triplicate. The cells were allowed to incubate for an additional 24 h and then counted using a Coulter counter.

Western blot analysis. For signal transduction analysis, B16 cells, untreated and treated with 10 µg/mL Tris DBA at timed intervals, were lysed in NP-40 lysis buffer (1% NP-40, 150 mmol/L NaCl, 10% glycerol, 20 mmol/L HEPES, 1 mmol/L phenylmethylsulfonyl fluoride, 2.5 mmol/L EDTA, 100 µmol/L Na₃VO₄, and 1% aprotinin). The lysate was spun in microfuge, and the pellet was discarded. Protein concentration of the supernatant was determined by the Eppendorf BioPhotometer. Samples were treated with Laemmli sample buffer and heated to 90°C for 5 min before SDS-PAGE (National Diagnostics) and was transferred to nitrocellulose membranes. The membranes were then blocked with 5% nonfat dry milk in 10 mmol/L Tris/0.1% Tween 20/100 mmol/L NaCl and were subsequently incubated with p42/44 MAPK antibody, phospho-p44/42 MAPK (Thr²⁰²/Tyr²⁰⁴) antibody, phospho-Akt (Ser⁴⁷³), and phospho-p70 S6 kinase (Thr⁴²¹/Ser⁴²⁴) antibody (Cell Signaling Laboratories). Monoclonal anti-β-tubulin antibody (Sigma) was used as a loading control and detected using horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Quantitative RT-PCR for and VEGF in B16 and A375 cells and NMT-1 in A375 cells treated with vehicle control and 10 µg/mL Tris DBA. B16 and A375 cells were seeded equally into two T-25 flasks each and

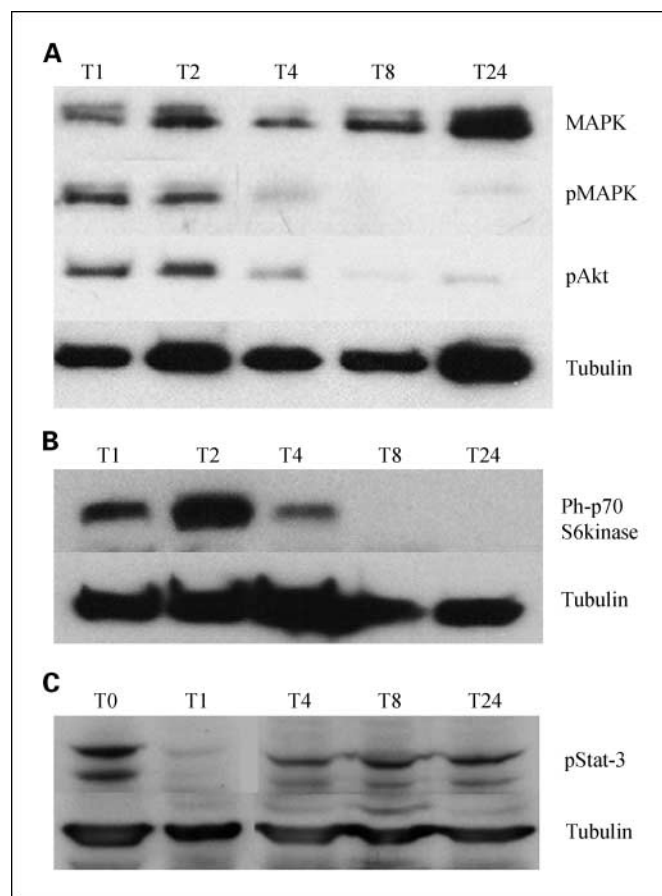


Fig. 2. Western blot analysis using phosphorylated forms of MAPK, Akt (A), p70 S6 kinase (B), and Stat-3 (C). Western blot analysis of B16 and A375 cells treated with 10 µg/mL Tris DBA at T₀, T₁, T₂, T₄, T₈, and T₂₄. Cells were lysed and analyzed by using antibodies specific for the unphosphorylated form of MAPK and phosphorylated forms of MAPK, Akt, p70 S6 kinase, and Stat-3. Tubulin was used as the loading control by using monoclonal anti-β-tubulin antibody.

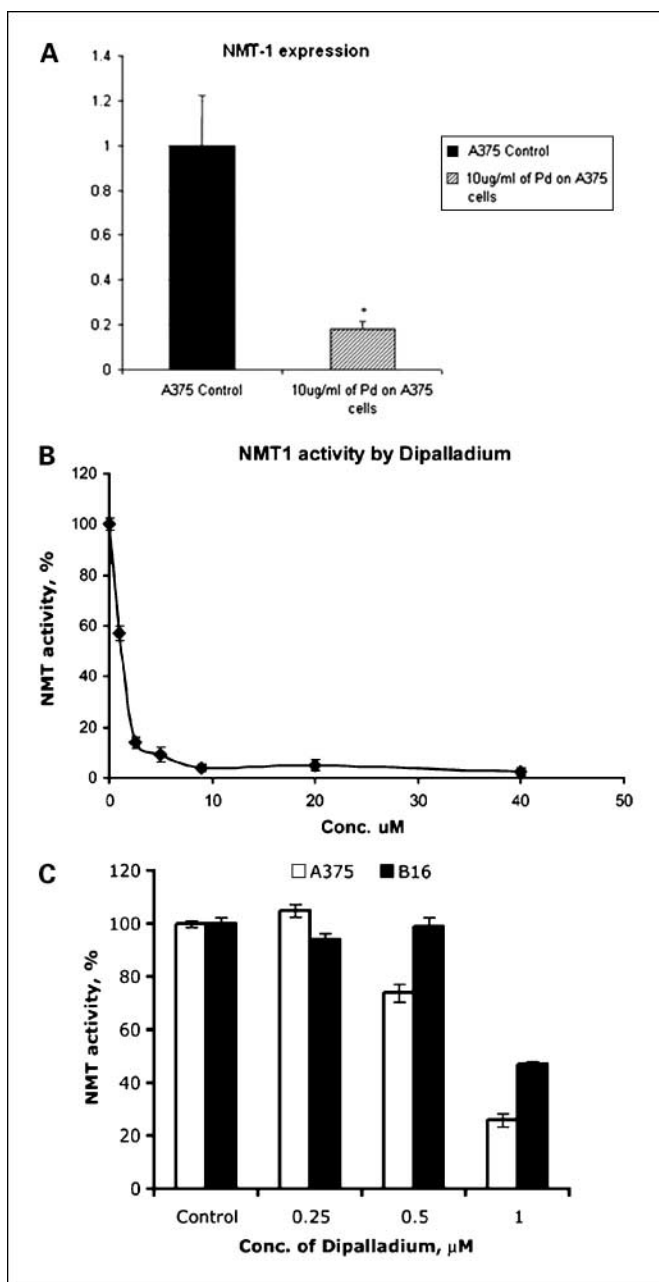


Fig. 3. Inhibition of human NMT expression at the level of RNA and protein by Tris DBA. *A*, treatment of A375 cells with 10 µg/mL Tris DBA decreases levels of NMT-1 mRNA (corrected for 18S RNA). Columns, average of triplicate experiments; bars, SE. $P = 0.0002$. *B*, inhibition of human NMT-1 by Tris DBA. The purified recombinant human NMT-1 (0.2 µg/assay) was incubated with various concentrations of Tris DBA using cyclic AMP – dependent protein kinase – derived peptide as a substrate described earlier. Representative data from three independent experiments, with SD from three determinations. *C*, effect of Tris DBA on NMT-1 and NMT-2 in melanoma cells (A375 and B16). Cells were treated with 0.25, 0.5, and 1.0 µM concentrations of Tris DBA. After 48 h, cells were lysed and analyzed by using NMT-1 and NMT-2 antibodies. β -Actin was used as the loading control by using monoclonal anti- β -actin antibody. Representative data from three independent experiments.

24 h later were treated with 0 and 10 µg/mL Tris DBA (Aldrich) in DMSO for 24 h. RNA was extracted and purified using Qiagen RNeasy mini kit and measured using spectrophotometer (Perkin-Elmer UV/visible). RNA (1 µg) was used for DNase amplification (Invitrogen) followed by first-strand synthesis for RT-PCR (SuperScript). Optical Reaction Plate (96 wells; 7500 Fast Real-Time PCR) was used for the

RT-PCR. The template (2.5 µL), which had been diluted 1:10 in cross-linked water, was used in each well and the experiment was done in triplicate. VEGF-a (Applied Biosystems Taqman Gene Expression Assay, Mm00437304_ml), NMT-1 (Applied Biosystems Taqman Gene Expression Assay, Hs00221506_m1) and 18S (Applied Biosystems Taqman Gene Expression Assay, Hs99999901_s1) primers were used along with cross-linked molecular-grade water (Cellgro) and master mix [Applied Biosystems Taqman Fast Universal PCR Master Mix (2 \times)]. Reaction was set up at the 7500 Applied Biosystems Reader for Absolute Quantification for 96-well plate. Ct values were analyzed by $\Delta\Delta$ Ct method, and the SE was calculated (see Fig. 4).

NMT-1 assay. [3 H]Myristic acid (39.3 Ci/mmol) was obtained from NEN Life Science Products. *Pseudomonas* acyl-CoA synthetase and coenzyme A were obtained from Sigma-Aldrich Canada. The peptide based on the NH₂-terminal sequence of the type II catalytic subunit of cyclic AMP – dependent protein kinase (GNAAAARKRR) was obtained from Alberta Peptide Institute, University of Alberta. The expression and purification of recombinant human NMT-1 were undertaken as described previously (26). The NMT activity was measured as described previously (27, 28). For the standard enzyme assays, the reaction mixture contained 0.4 µmol/L [3 H]myristoyl-CoA, 50 mmol/L Tris-HCl (pH 7.8), 0.5 mmol/L EGTA, 0.1% Triton X-100, 500 µmol/L synthetic peptide, and purified human NMT-1 in a total volume of 25 µL. The reaction was initiated by the addition of radiolabeled [3 H]myristoyl-CoA and incubated at 30°C for 10 to 30 min. The reaction was terminated by spotting aliquots of incubation mixture onto P81 phosphocellulose paper discs and drying them under a stream of warm air. The P81 phosphocellulose paper discs were washed in three changes of 40 mmol/L Tris-HCl (pH 7.3) for 90 min. The radioactivity was quantified in 7.5 mL Beckman Ready Safe Liquid Scintillation mixture using a Beckman Liquid Scintillation Counter. One unit of NMT activity was expressed as 1 pmol myristoyl-peptide formed/min/mg protein. The human NMT-1 inhibitory assay was carried out using Tris DBA according to the method described earlier (Fig. 5A; ref. 28). A control experiment was done in the absence of Tris DBA and the human NMT-1 activity was considered as 100%.

In vivo tumor growth. To determine if a compound that inhibits melanoma growth *in vitro* would also inhibit tumor formation *in vivo*, we injected 1 million B16 melanoma cells and 1 million A375 cells s.c. into six nude mice, respectively. Beginning 2 days later, the mice received i.p. injections three times per week of either Tris DBA or control. Tris DBA (40 mg/kg/d) was suspended in 0.3 mL peanut oil, and control was 0.3 mL peanut oil alone. Neither local nor systemic toxicity was observed in any of the nude mice as a result of treatment. A

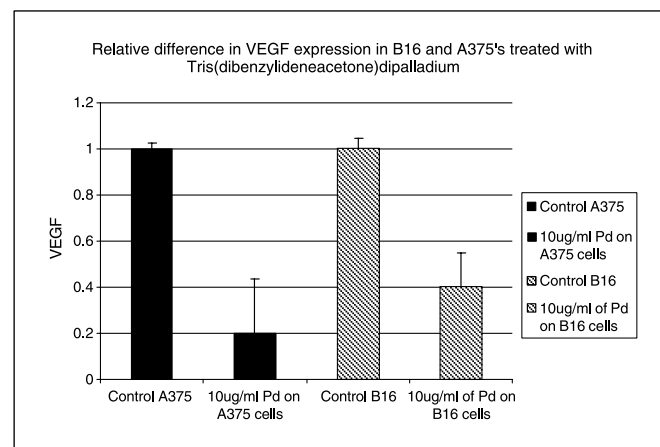


Fig. 4. Treatment of B16 and A375 cells with 10 µg/mL Tris DBA decreases levels of VEGF mRNA (corrected for 18S RNA). Columns, average of triplicate experiments; bars, SE. Differences are significant ($P < 0.05$).

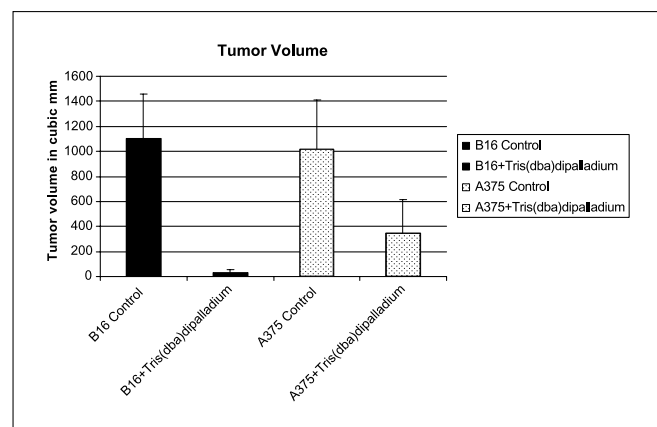


Fig. 5. Effect of Tris DBA in melanomas *in vivo*. Nude mice were injected with 1 million B16 or A375 cells and received i.p. injection with Tris DBA and vehicle control. Animals were euthanized on day 15, secondary to tumor burden in the control animals. Average tumor volume (mm³) in each of the B16 and A375 treated and control groups. Bars, SE. Differences are significant ($P < 0.05$).

total of six rounds of injections were given over a period of 2 weeks, after which the mice were sacrificed due to overwhelming tumor burden in the control group. Animals were euthanized on day 15, secondary to tumor burden in the control animals. Graph represents average tumor volume (mm³) in each of the two groups with controls (Fig. 5) with bars as SE.

Results

Tris DBA inhibits melanoma proliferation. Tris DBA was used to treat at five different concentrations on B16 and A375 cells.¹ Treatment with Tris DBA for 24 h significantly reduced the number of viable cells. A 10 µg/mL concentration of Tris DBA resulted in a 99% decrease in B16 cell count (Fig. 1A) and a 96% decrease in A375 cell count (Fig. 1B).

Tris DBA inhibits activation of MAPK, Akt, Stat-3, and phospho-S6 kinase in melanoma cells. To examine this effect, we tested 10 µg/mL Tris DBA on MAPK and Akt signaling pathway using B16 melanoma cells. Using a time-course pattern, we found that it inhibited the phosphorylated forms of both downstream (Fig. 2A). We further tested Tris DBA on human melanoma cell line and found that it inhibits phosphorylated forms of S6 kinase downstream (Fig. 2B) and down-regulates phospho-Stat-3 at shorter time intervals (1 and 4 h) than after 24 h of treatment (Fig. 2C).

Inhibition of human NMT-1 activity and NMT-1 expression by Tris DBA. Gene array was done on A375 melanoma cells treated with Tris-DBA palladium compared with control cells. Among the genes that were strongly down-regulated as a result of treatment was NMT-1. We confirmed this down-regulation by RT-PCR (Fig. 3A).

When purified human NMT was incubated in the presence of various concentrations of Tris DBA, the human NMT was inhibited in a concentration-dependent manner, with maximal inhibition at a concentration of 2.5 ± 0.97 µmol/L and half-maximal inhibition at 1.0 ± 0.26 µmol/L (Fig. 3A). Further testing of 10 µg/mL Tris DBA on NMT-1 expression on A375 cells, we found an 80% decrease compared with control (Fig. 3B and C). Thus, Tris DBA is a novel and potent inhibitor of NMT-1 activity.

Tris DBA inhibits VEGF expression in murine and human melanoma cells *in vitro*. We tested 10 µg/mL Tris DBA on VEGF expression on B16 cells and found a 60% decrease in VEGF expression compared with control. We further tested 10 µg/mL of the same compound on A375 cells and found an 80% decrease compared with control (Fig. 4).

Tris DBA inhibits B16 and A375 melanoma growth *in vivo*. To determine if compounds that inhibit VEGF, phosphorylated forms of MAPK, Akt, Stat 3, and p70 S6 kinase *in vitro* would affect melanoma formation *in vivo*, we injected 1 million B16 cells s.c. into six nude mice and 1 million A375 cells s.c. into six nude mice. I.p. treatment with Tris DBA resulted in a 97% decreased tumor volume compared with control when using the B16 murine melanoma model. In the A375 human melanoma model, there was a 65% reduction in tumor volumes compared with control (Fig. 5). Neither local nor systemic toxicity was observed in any of the nude mice as a result of treatment.

Discussion

Melanoma is a common solid tumor notorious for its high rate of metastasis and resistance to chemotherapy and radiation. Several factors may account for the resistance of melanoma to current therapies. First, melanomas are derived from melanocytes, specialized neural crest cells that are specialized to produce melanin. The production of melanin results in the generation of toxic reactive oxygen species and cytotoxic phenol derivatives; thus, melanocytes are equipped with mechanisms to resist these insults. Recently, the microphthalmia gene (MITF), which is a master transcriptional switch of melanocytes, has been shown to possess antiapoptotic activity and is found in metastatic lesions at a high frequency (29, 30). Second, multiple signaling pathways are activated in melanoma. B-raf is mutated in many melanomas, resulting in constitutive activation of MAPK signaling (1, 29). N-ras is also mutated frequently in melanoma, resulting in activation of MAPK, PI3K/Akt signaling, and S6 kinase activation (30, 31). Although B-raf and constitutive MAPK activation is sufficient to cause transformation of melanocytes into melanoma (3, 4), other signal transduction events are frequently observed in B-raf mutant melanomas, such as loss of the tumor suppressor PTEN (31, 32). The consequences of PTEN loss is activation of PI3K/Akt activation.

Multiple regimens have been tried for the treatment of locally advanced and metastatic melanoma. Initial trials several decades ago used agents such as hydroxyurea, and more recent agents used against melanoma include dacarbazine and platinum-based therapies including cisplatin and carboplatin. Other therapies, including biochemotherapy, have included interleukin-2 infusion and infusion of lymphocytes, which are present in melanoma lesions and have been expanded *ex vivo* (33). All of these therapies have had modest success in a minority of patients, but with significant toxicity, including pulmonary leak syndrome (34–38). Currently, IFN-α is employed in high-risk patients, and prolonged therapy results in a 10% long-term survival benefit.

Targeted therapies have been attempted in melanoma. Sorafenib was developed as a B-raf inhibitor based on the observation that B-raf mutation is common in melanoma.

However, results from initial trials of sorafenib in melanoma have been disappointing (14). Everolimus has also been tried against human melanoma and has not been successful as a single agent (39). Current knowledge of signaling may provide an explanation of why previous therapies have failed. PI3K activation has been shown to mediate against extrinsic pathways of apoptosis, which include apoptosis due to TRAIL, tumor necrosis factor- α , and IFNs (10). Monotherapies of these cytokines may be frustrated in the face of PI3K activation. Similarly, apoptosis induced by tumor-infiltrating lymphocytes may be frustrated by PI3K activation. PI3K also activates VEGF expression; in addition to stimulating angiogenesis, VEGF inhibits dendritic cell function, impairing immune responses to melanoma (40–45).

Targeting MAPK as monotherapy in melanoma is clearly insufficient to eliminate melanoma in most patients. MAPK is activated in a majority of human melanomas, including those that lack B-raf mutation (3). In a previous study of human melanomas, we showed that a subset of advanced melanomas had decreased MAPK activation, implying that additional signaling pathways are operative *in vivo* (3). Further support of this hypothesis is our previous finding that treatment of EBV-induced Burkitt's lymphomas with antioxidants resulted in compensatory MAPK activation (17). It is likely that

treatment of melanoma patients with sorafenib results in compensatory activation of non-MAPK pathways. Similarly, mammalian target of rapamycin inhibition due to rapamycin and derivatives has been shown to result in compensatory Akt activation (46). Tris DBA has the advantage that it inhibits several pathways required for melanoma tumorigenesis, including MAPK activation, PI3K/Akt activation, Stat-3 activation, and S6 kinase activation, and down-regulates NMT-1 at the level of enzyme activity and the level of mRNA. Down-regulation of these pathways may lead to diminished transcription of NMT-1. Although no drug is likely to be completely effective as monotherapy in melanoma, Tris DBA is well tolerated systemically in mice and has a novel profile of action compared with other clinically used chemotherapeutic agents. Its ability to inhibit PI3K activation may enhance the activity of cytokines, which require Akt inactivation for optimal activity, and may enhance the activity of other chemotherapeutic agents. Our studies provide a rationale for the further investigation of Tris DBA in the treatment of malignant melanoma.

Disclosure of Potential Conflicts of Interest

J.L. Arbiser has a patent on Tris (dibenzylideneacetone) dipalladium.

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