Cancer Therapy: Preclinical

The Novel SMAC Mimetic Birinapant Exhibits Potent Activity against Human Melanoma Cells

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Abstract

Purpose: Inhibitor of apoptosis proteins (IAP) promote cancer cell survival and confer resistance to therapy. We report on the ability of second mitochondria-derived activator of caspases mimetic, birinapant, which acts as antagonist to cIAP1 and cIAP2, to restore the sensitivity to apoptotic stimuli such as TNF-α in melanomas.

Experimental Design: Seventeen melanoma cell lines, representing five major genetic subgroups of cutaneous melanoma, were treated with birinapant as a single agent or in combination with TNF-α. Effects on cell viability, target inhibition, and initiation of apoptosis were assessed and findings were validated in 2-dimensional (2D), 3D spheroid, and in vivo xenograft models.

Results: When birinapant was combined with TNF-α, strong combination activity, that is, neither compound was effective individually but the combination was highly effective, was observed in 12 of 18 cell lines. This response was conserved in spheroid models, whereas in vivo birinapant inhibited tumor growth without adding TNF-α in in vitro resistant cell lines. Birinapant combined with TNF-α inhibited the growth of a melanoma cell line with acquired resistance to BRAF inhibition to the same extent as in the parental cell line.

Conclusions: Birinapant in combination with TNF-α exhibits a strong antimelanoma effect in vitro. Birinapant as a single agent shows in vivo antitumor activity, even if cells are resistant to single agent therapy in vitro. Birinapant in combination with TNF-α is effective in a melanoma cell line with acquired resistance to BRAF inhibitors. Clin Cancer Res; 19(7); 1784–94. ©2013 AACR.

Introduction

Treatment options for metastasized melanoma, a disease with a low 5-year survival rate, have improved remarkably in the last 2 years. After a decades-long period, in which the gold standard remained dacarbazine chemotherapy with modest response rates (1), two new therapies have been U.S. Food and Drug Administration-approved recently (2–4) and several are currently in late-stage clinical development (5). Among small-molecule inhibitors, the majority targets 2 major pathways: the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. Therapy through modulating T-cell responses (4) is also highly promising, but unfortunately for all new therapies, most patients eventually progress.

Antiapoptotic mechanisms are also frequently at work in melanoma (6) and an attractive target for therapeutic intervention. For example, targeting the B-cell lymphoma 2 (Bcl-2) family of antiapoptotic proteins using Bcl-2 antisense (7) and Bcl-2 homology domain 3 (BH3) mimetics (8, 9) has also been promising preclinically, but will require additional studies to fully realize their potential in clinical trials.

One of the factors responsible for the difficulties in engaging the apoptotic cascade efficiently in melanoma is the upregulation of conserved inhibitor of apoptosis proteins (IAP; refs. 10, 11). IAPs are a family of proteins defined by the presence of baculoviral IAP repeats. The best described members are XIAP, cIAP1, cIAP2, ML-IAP, and survivin. These proteins are associated with chemoresistance and poor outcome in many cancer types [reviewed in ref. (12)]. IAPs themselves are controlled by the second mitochondria-derived activator of caspases (SMAC). SMAC is released from mitochondria upon onset of apoptosis (13) and binds directly to IAPs leading to their degradation (14, 15). A number of IAP inhibitors, designed to function as SMAC mimetics, have shown preclinical activity in a variety...
of cancer types (16–19). In contrast to XIAP, cIAP1 and cIAP2 can bind to caspases but lack binding domains capable of inhibiting caspases (20). Previous studies have shown that synthetic IAP antagonists are able to induce apoptosis in tumor cells primarily through inhibition of cIAP1 and or cIAP2, which results in changes in TNF-α signaling and caspase-8 activation (21). Moreover, SMAC mimetics can perturb NF-κB signaling downstream of TNFR (22).

Birinapant is a novel dimeric SMAC mimetic designed to specifically target cIAP1 and cIAP2 for degradation, resulting in a switch in TNFR signaling; activation of TNFR upon binding of TNF-α in the presence of cIAP1 and cIAP2 leads to NF-κB activation and increased cell proliferation. In contrast, following cIAP1 and cIAP2 inhibition, TNF-α signaling leads to activation of caspase-8 and induction of apoptosis (12).

Immune cell infiltrates are often found in melanoma lesions, leading to chronic inflammation and (among other factors) elevated levels of TNF-α (23, 24). TNF-α can then be used by melanoma cells to promote cell growth, invasion, and metastasis (25). Binding of TNF-α to its receptor leads to the activation of TNFR complex-I, a membrane-localized activator of the canonical NF-κB pathway composed of TNFRSF1A associated via death domain (TRADD), the ubiquitin ligases TRAF2, TRAF5, cIAP1, and cIAP2, and the protein kinase RIPK1 (26). This leads to increased cell proliferation via an ubiquitination cascade and downstream activation of NF-κB. After SMAC mimic-induced degradation of cIAP1 and cIAP2, binding of TNF-α to its receptor leads to the formation of a death complex and activation of caspases. This results in a reversal of the role of TNF-α from promoting proliferation to inducing cell death (12).

In the present study, birinapant in combination with TNF-α led to a reduction of viability in the majority of 17 melanoma cell lines tested. This effect was dependent on TNF-α.

Our laboratory has previously published on melanoma cell lines with acquired resistance to BRAF inhibitors (27). Notably, birinapant in combination with TNF-α elicited the same antitumor effect in a parental and BRAF inhibitor-resistant cell line. This suggests a potential use of birinapant for the treatment of patients with BRAF inhibitor-refractory melanoma.

Materials and Methods

Cell lines and reagents

Human melanoma cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% FBS and grown at 37°C in 5% CO2. All cell lines were periodically authenticated by DNA finger printing using Coriell’s microsatellite kit and tested for mycoplasma by PCR.

Genomic DNA from samples were analyzed for mutations in BRAF, CDK4, CTNNB1, FBX04, KIT, MEK1, MEK2, MET, and NRAS by the nucleotide extension assay using the iPLEX platform (Sequenom, Inc; ref. 28).

Birinapant was provided by TetraLogic Pharmaceuticals. Birinapant is a novel bivalent, selective small-molecule peptidomimetic of the Smac tetrapeptide that binds with high affinity to multiple members of the IAP family including XIAP and cIAP1. The dissociation constant (Kd) for XIAP and cIAP-1 was determined to be 45 and <1 nmol/L, respectively (29).

Birinapant was developed by TetraLogic Pharmaceuticals and designed on the basis of the 4 N-terminal amino acids of Smac/DIABLO (Ala-Val-Pro-Ile) and caspase-9 (Ala-Thr-Pro-Phe); ref. 30).

Human recombinant TNF-α was obtained from Invitrogen. TNF-α monoclonal antibody (mAb) MAB610 was obtained from R&D Systems.

cis-Diamineplatinum(II) dichloride (cisplatin) was obtained from Sigma-Aldrich.

Immunoblot analysis

For immunoblot analyses, adherent cells were washed with cold PBS containing 100 μmol/L Na3VO4, scraped, collected by centrifugation, and quick-frozen in dry ice before lysis. Xenograft tumors were snap frozen in liquid nitrogen immediately after harvesting. Tumor chunks were ground on liquid N2 using a MM2 mixer mill (Retsch). Cells and powder were lysed and equal amounts of protein (10–40 μg) was subjected to SDS-PAGE and proteins transferred onto PVDF membranes (Immobilon). Antibodies used were: cIAP1, AF8181; cIAP2, AF8171 (both R & D systems); PARP, 7150; GAPDH, 32233 (both Santa Cruz biotechnology); ERK, sc-271270, pERK, sc 7976, 4882 (Cell Signaling); RIP1, 51-6559GR, RAC1, 610651 (both BD Biosciences); ER, sc-25270, pER, sc 7976, 4882 (Cell Signaling); XIAP, ADI-AAM-050 (ENZO); NFKB2 p100- p52, 3031 (both Cell Signaling); Caspase-8, 9746; NF-kBp65, 3031 (both Cell Signaling); XIAP, ADI-AAM-050 (ENZO); NFkB2 p100- p52, 4882 (Cell Signaling); RIP1, 51-6559GR, RAC1, 610651 (both BD Biosciences); ERK, sc-25270, pER, sc 7976, 4882 (both Santa Cruz biotechnology). Membranes were probed with primary antibodies overnight at 4°C, then further incubated with Alexa Fluor-labeled secondary antibodies.
In vitro drug sensitivity assays

For monolayer cell culture assays, cells were allowed to attach for 24 hours and subsequently incubated with birinapant and/or TNF-α for 24 or 72 hours. CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) assay (Promega) was conducted according to the manufacturer’s description. For cell-cycle analysis, melanoma cells were fixed in 70% ethyl alcohol and stained with propidium iodide. Samples were subsequently analyzed with an EPICS XL (Beckman-Coulter) apparatus. Annexin V staining was conducted with an Annexin V allophycocyanin conjugate (Invitrogen) according to the manufacturer’s description. Briefly, cells were treated with dimethyl sulfoxide control, birinapant, and/or TNF-α for 24 hours. Resuspended cells were washed and incubated with the conjugate for 15 minutes and Annexin-binding buffer was added. Samples were subsequently analyzed with an EPICS XL (Beckman-Coulter) apparatus.

451Lu and WM1366 melanoma cells were treated with birinapant 1 μmol/L in combination with TNF-α 1 ng/mL. Cells were then incubated for 72 hours in the presence or absence of Z-VDAP-FMK (R & D Systems) a pan-caspase inhibitor. Proliferation was assessed using the MTS assay.

451Lu and WM1366 melanoma cells were treated with birinapant 1 μmol/L in combination with TNF-α 1 ng/mL. Cells were then incubated for 72 hours in the presence or absence of Necrostatin-1 (N9037, Sigma) a RIP1 kinase inhibitor. Proliferation was assessed using the MTS assay.

RIPK1 gene expression was evaluated using commercially available gene expression assays from Life Technologies. Briefly, RNA was isolated using Qiagen RNeasy kit and RNA was quantified and normalized for addition to cDNA reaction. cDNA was created using cDNA kit from Life Technologies. cDNA was amplified with cDNA kit from Life Technologies (4368814) and real-time PCR was carried out for RIPK1 (assay ID Hs00169407_m1) and GUSB (assay ID Hs99999908_m1) as a housekeeping gene. For analysis, the relative quantity of RIPK1 to GUSB was calculated.

For 3-dimensional (3D) melanoma spheroids assays, cells were prepared as described previously (31, 32). Collagen-embedded spheroids were incubated with reagents for 72 hours. Spheroids were imaged using a Leica TCS SP2 confocal microscope after addition of a live/dead viability assay kit using the fluorescent dyes calcein AM and ethidium homodimer-1 (Invitrogen). To assess viability, spheroids were grown in a 96-well format, and allowed to grow in the presence of birinapant and/or TNF-α for 72 hours, then AlamarBlue (Invitrogen) was added. Fluorescence was measured using an Envision Xcite plate reader (Perkin Elmer). All experiments were carried out in triplicate.

To measure TNF-α levels in cell culture supernatants, cells were grown for 3 days to 70% confluence. Supernatants were collected and 200 μL were tested in triplicate following the manufacturer’s instructions using the BD OptEIA Human TNF-α ELISA Kit II (BD).

Xenograft studies

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care and Use Committee of the National Cancer Institute. Male nude mice (nu/nu) purchased from Charles River Laboratories were used in all experiments. Animals were sacrificed and tumors were harvested at 4 time points after the last treatment.

To test birinapant as a single agent and calculate the half maximal inhibitory concentration (IC50) values in this cell line panel, cells were treated with birinapant at 1, 10, 100,
and 1,000 nmol/L for 72 hours and cell viability was assessed through the MTS assay. Only one cell line (WM9, BRAFV600E) out of 17 showed a response to birinapant (Fig. 1, gray bars, TL32711). However, when birinapant was combined with TNF-α (1 ng/mL), the majority of cell lines showed birinapant IC50 values in the low nanomolar range (Fig. 1, black bars). This response was independent of the cell line genetic background. All cell lines were resistant to TNF-α alone (Supplementary Fig. S1).

Three distinct response patterns following birinapant addition were observed. For subsequent experiments, 4 cell lines were therefore selected on the basis of their response to birinapant in combination with TNF-α: 1205Lu, resistant to the combination therapy; WM9, sensitive to birinapant as a single agent; and 2 cell lines sensitive to birinapant only in combination with TNF-α: 451Lu (BRAFV600E) and WM1366 (NRASQ61L; Fig. 2A).

cIAP1 and cIAP2 downregulation was observed in all response types, but apoptosis occurred only in sensitive cell lines

Immunoblot analysis showed inhibition of the birinapant target protein cIAP1 in all 4 cell lines. Although cIAP2 was upregulated in the 1205Lu and WM1366 cell lines upon treatment with TNF-α, and was again degraded in the combination with birinapant. No change in the levels of XIAP could be observed in WM9, sensitive to birinapant as a single agent; and 2 cell lines sensitive to birinapant only in combination with TNF-α: 451Lu (BRAFV600E) and WM1366 (NRASQ61L; Fig. 2A).

To confirm that birinapant resistance was not caused by failure of target degradation, we conducted immunoblots for cIAP1 and XIAP in a cohort of birinapant-TNF-α combination therapy–resistant cell lines and found cIAP1 combination of birinapant and TNF-α. The p52 band of NF-κB2 was not detectable. No change in the protein levels of RIP1 kinase, which was found at similar levels in all 4 cell lines, could be observed upon treatment with birinapant or TNF-α alone, but RIP1 was depleted in 3 of 4 cell lines when both agents were combined (Fig. 2B).

We then confirmed the induction of apoptosis seen at the protein level through cell-cycle analysis by propidium iodide staining. A significant increase in sub-G1 fractions, indicative of apoptosis, was seen in WM9 cells when treated with birinapant alone; in 451Lu and WM1366 cells only when birinapant was combined with TNF-α; but not in 1205Lu cells, even after combination treatment (Fig. 2C). To confirm the induction of apoptosis seen in the cell-cycle analysis, Annexin V staining was conducted. The relative increase in Annexin V–positive cells seen with birinapant alone only in WM9 and with birinapant in combination with TNF-α in WM9, 451Lu, and WM1366, but not in 1205Lu cells, could confirm the pattern of apoptosis seen by cell-cycle analysis (Fig. 2D).

To investigate a possible role of MAPK and RAC1 signaling in resistance to birinapant, phosphorylation status of ERK1/2 and protein levels of RAC1 upon treatment with birinapant at different time points in birinapant-sensitive and -resistant cell lines were assessed. We could observe a marked decrease in phosphorylation of ERK1/2 only at 24 hours and this decrease was seen in a birinapant-sensitive as well as a birinapant-resistant cohort of cell lines. RAC1 protein was found at similar levels in both sensitive and resistant cohorts and there was no significant change upon birinapant treatment (Supplementary Fig. S2).

An immunoblot conducted in the 451Lu cell line showed that birinapant could cause sustained cIAP1 protein degradation within one hour at a dose of 100 nmol/L, whereas XIAP levels were not affected (Fig. 3A). To confirm that birinapant resistance was not caused by failure of target degradation, we conducted immunoblots for cIAP1 and XIAP in a cohort of birinapant-TNF-α combination therapy–resistant cell lines and found cIAP1...
protein levels to be depressed to similar levels as seen in
the birinapant-sensitive cell line 451Lu (Supplementary
Fig. S3).
Next, we investigated whether the observed cell death
with birinapant at a dose of complete cIAP1 inhibition in
combination with TNF-α was caspase dependent. Two
combination-sensitive cell lines (451Lu and WM1366) were
treated with the combination of birinapant and TNF-α with
or without addition of Z-VAD-FMK, a pan-caspase inhibitor.
In both cell lines, the inhibition of caspases led to a complete
restoration of proliferation (Fig. 3B). In a similar experi-
ment, we then further explored the role of RIP1 kinase, an
essential part of the caspase-initiating complex activated by
TNF-α. By treating the 2 cell lines as above, now in the
absence or presence of necrostatin-1, a RIP1 kinase inhib-
itor, we could again reverse the effect of birinapant and TNF-
α and restore cell proliferation (Fig. 3C).
As loss of RIP1 expression could therefore be a possible
mechanism of resistance to birinapant, we assessed RIP1
expression in the whole panel of cell lines. However, no loss
or decrease in RIP1 expression in the 6 birinapant-resistant
cell lines compared with the sensitive group could be
observed. This was also true after stimulation with TNF-α
1 ng/ml (Fig. 3D).

Figure 2. Effect of birinapant alone
or in combination with TNF-α. Four
cell lines showing varying
responses to birinapant as seen in
the whole panel of cell lines
assessed: combination resistant
(1205Lu), single-agent-sensitive
(WM9), and combination-sensitive
(451Lu and WM1366). A, dose-
response curves after 72-hour
incubation with birinapant
(TL32711) at indicated doses alone
or in combination with TNF-α 1 ng/
ml using the MTS assay. For the
cell lines 451Lu and WM1366:
P < 0.05 for doses ≥10 nmol/L.
Data are represented as mean of 3
or more experiments ± SEM. B,
immunoblots showing the same
four cell lines after 24 hours
incubation with birinapant
1 μmol/L, TNF-α 1 ng/mL, or the
combination of both, compared
with untreated controls. Levels of
target IAP proteins, cleaved PARP,
NF-κB p65, NF-κB p100- p52,
and RIP1 were assessed. GAPDH
was included to ensure equal
loading. C, relative number of cells
in the sub-G0 fraction as assessed
by propidium iodide cell-cycle
analysis after 24-hour incubation
with birinapant 1 μmol/L, TNF-α
1ng/ml, or combination of both.
Data are represented as mean ±
SEM (n = 3 biologic replicates). D,
relative number of Annexin V–
positive cells as assessed by flow
cytometry after 24-hour incubation
with birinapant 1 μmol/L, TNF-α
1 ng/ml, or combination of both.
Data are represented as mean ±
SEM (n = 3 biologic replicates).
Three-dimensional spheroid models confirmed responses seen in adherent cell cultures

Cells grown in 3D spheroid cultures were previously shown to have altered drug response profiles compared with adherent cell cultures (34), and are expected to more accurately predict \textit{in vivo} efficacy due to similar architecture and microenvironmental signals (35, 36). The 4 previously selected cell lines were grown as 3D spheroids in a collagen matrix and treated with birinapant alone or in combination with TNF-\( \alpha \). A live/dead fluorescent cell stain was used to visually assess treatment effects using confocal microscopy (Fig. 4A): Spheroids of the birinapant single-agent–sensitive cell line, WM9, did indeed show an extensive reduction in live cells after addition of birinapant, but not after addition of TNF-\( \alpha \) alone. The combination-sensitive cell lines, 451Lu and WM1366, retained the same response patterns in 3D cultures: both showed a marked decrease in live cells and increase in dead cells only after treatment with birinapant in combination with TNF-\( \alpha \). In addition, the cell line that was completely resistant to the combination treatment in adherent cell culture, 1205Lu, showed only slight growth retardation when grown as spheroids in the presence of birinapant in combination with TNF-\( \alpha \).

To objectively quantify viability in this model, we assessed metabolic activity of spheroids after treatment with birinapant in combination with TNF-\( \alpha \) using Alamar Blue. The viability results mirrored the responses seen in the Live/Dead assay: a near total loss of viability in WM9, a dramatic decrease in viability in the combination-sensitive cell lines (451Lu, WM1366), and only a slight reduction of viability in the 1205Lu cell line (Fig. 4B).

Birinapant inhibits tumor growth in melanoma xenotransplantation models as a single agent

To investigate whether birinapant could inhibit melanoma tumor growth in an \textit{in vivo} setting as a single agent, 2 cell lines were selected for xenotransplantation experiments: both were \textit{in vitro} birinapant single-agent–resistant, but...
451Lu did respond in vitro to the combination of birinapant with TNF-\(\alpha\), whereas 1205Lu did not respond to the combination treatment in vitro. Both cell lines were inoculated subcutaneously in NUDE mice and allowed to form palpable tumors before being randomized into vehicle control and birinapant treatment groups. During 3 weeks of dosing, birinapant showed an antitumor effect in both models, although the effect in the in vitro combination-sensitive cell line was more sustained with abrogation of tumor growth in the birinapant-treated animals. In contrast, 1205Lu tumors showed a marked slowing of tumor growth, but not abrogation of tumors (Fig. 5A).

In a subsequent in vivo experiment, we then went on to confirm birinapant target inhibition in both models by immunoblot of tumor lysates. Animals were again inoculated with both xenograft models and tumors allowed to form. Animals were then pretreated twice in an interval of 48 hours and tumors were harvested 3, 6, 12, and 24 hours after the second dosing. Compared with vehicle control, cIAP1 protein was reduced to low levels 3 hours later, and this effect was sustained for 24 hours in both models (Fig 5B). Staining for activated caspase-3 in biopsies of the same tumors showed a modest increase in apoptotic cells in the birinapant-treated animals compared with vehicle control, 24 hours after treatment (Fig. 5C).

To further investigate the combination activity between birinapant and TNF-\(\alpha\) in vitro, the 4 cell lines previously selected on the basis of their response profiles were again used. All 4 cell lines had comparable levels of TNF-\(\alpha\) in cell culture supernatants (Supplementary Fig. S4) and adding high levels of TNF-\(\alpha\) alone to all 4 cell lines had no effect on cell proliferation (Supplementary Fig. S5). The one cell line sensitive to birinapant as a single agent, WM9, provided an opportunity to further investigate the role of TNF-\(\alpha\) in this setting. We therefore added a TNF-\(\alpha\)-blocking monoclonal antibody to WM9 cell cultures before treatment with birinapant. By gradually adding endogenous TNF-\(\alpha\) in the supernatant with increasing doses of the antibody, it was possible to completely abrogate the effect of birinapant in a dose-dependent manner (Fig. 6A). This indicates that while no addition of exogenous TNF-\(\alpha\) was necessary to observe a birinapant effect in this cell line, the observed antitumor effect was still dependent on endogenous TNF-\(\alpha\).

Additional evidence on the causative role of TNF-\(\alpha\) on the effect of birinapant was provided by varying the schedule of combining birinapant with TNF-\(\alpha\): a combination-sensitive cell line treated with birinapant for 36 hours and subsequently incubated with TNF-\(\alpha\) for 36 hours, showed significant growth inhibition. Conversely, when treated first with TNF-\(\alpha\) for 36 hours and then with birinapant for 36 hours, cells were significantly less sensitive (Fig. 6B). Therefore, the combination activity between birinapant and TNF-\(\alpha\) is schedule dependent. This corroborates the hypothesis that birinapant-mediated IAP inhibition has to occur before the activation of TNFR to induce an antitumor effect.

Previously, our group has generated human melanoma cell lines with acquired resistance to BRAF inhibitors (27). The mechanism of resistance in these cell lines was RAF isoform switching and increased IGF-1R/PI3K signaling. We compared the cell lines 451Lu (BRAFV600E, BRAF inhibitor sensitive) and 451Lu-BR (BRAF inhibitor resistant) in regard to birinapant response. Cell viability was not affected after treatment with birinapant alone in neither the parental nor the resistant cell line. When birinapant was combined with TNF-\(\alpha\), however, both cell lines showed a strong response that was identical between the sensitive and the resistant cell line (Fig. 6C).

Next, we investigated whether birinapant could sensitize melanoma cells to chemotherapy. For this experiment, 2 birinapant single-agent–resistant cell lines were treated with cisplatin, a cytotoxic drug, with or without addition of birinapant. Cisplatin as a single agent reduced viability in...
both melanoma cell lines (mean of both cell lines) at increasing doses up to 20 μmol/L. Birinapant-mediated cIAP1 degradation in combination with cisplatin significantly ($P < 0.05$) enhanced sensitivity to cisplatin in both cell lines (Fig. 6D).

Discussion

A delicate balance between inducers and inhibitors of apoptosis exists in any given cell (37). In cancer cells, including melanoma, this equilibrium is often skewed in favor of survival, with IAPs being predominant (38, 39). Committing melanoma cells to apoptosis, which would be an ideal outcome for most therapies, therefore requires additional stimuli. Inhibition of IAPs for one can restore the balance between survival and death signals, thereby facilitating programmed cell death in melanoma.

Chronic inflammation in the tumor microenvironment of melanoma lesions often leads to elevated levels of TNF-α, at least in part, provided by the tumor-infiltrating immune cells such as macrophages (23–25). Levels of tumor-infiltrating macrophages have been shown to be associated with aggressive disease (40). All melanoma cell lines tested were resistant to treatment with exogenous TNF-α as a single agent. This was in line with previous clinical experiences, where minimal antitumor effect and significant toxicity was observed (41, 42). Birinapant-mediated downregulation of cIAP1 by itself did not induce any antitumor effect in vitro. As neither compound was effective alone, but both were highly effective in combination, this satisfies the definition of "coalism" according to the so-called Saariselkä agreement (43, 44). This was confirmed on a subset of cell lines to be apoptosis-mediated and dependent on caspases and RIPK1 activity.

Independent of mutational background (we assessed melanoma cell lines from major genetic subgroups), 2 patterns of responses emerged: cell lines that were either exquisitely sensitive or remarkably resistant to birinapant in combination with TNF-α at low or high doses of both compounds respectively. We confirmed downregulation of cIAP1 target protein in resistant and sensitive cell lines by Western blot analysis, but only the sensitive cell lines showed PARP cleavage, indicative of apoptosis. This observation could also be shown by increased sub-G1 cell fractions and increased Annexin V–positive cells, indicating an increase in apoptotic cells, in the combination-sensitive cell lines.
As a subgroup of melanoma cell lines of all genetic backgrounds tested was found to be resistant to birinapant in combination with TNF-α, we investigated whether levels of phosphorylated ERK 1/2 or RAC1 were increased after birinapant treatment in these cell lines. These were recently described to play a role in resistance to SMAC mimetics (45, 46). Although we observed a decrease in ERK1/2 activation after 24 hours of birinapant treatment in most cell lines tested, we could not find any differential regulation of these 2 proteins in the resistant cell lines as compared with the sensitive subset. Another possible mechanism of resistance to SMAC mimetics is loss of RIP1 expression. We observed the effect of birinapant to be dependent on RIP1 kinase in concordance with previously published reports on compounds from the same class (47). As there was no difference in RIP1 expression between birinapant-resistant and -sensitive cell lines in the panel used in this study, RIP1 expression is likely not a suitable biomarker to predict melanoma response to birinapant. Therefore, further investigation to define a biomarker for this subgroup is warranted, as this would be highly useful in the clinical application of birinapant.

Our observations remained consistent across increasingly complex models from monolayer culture, to 3D matrix cultures, to human melanoma xenograft models. While the 3D spheroid models closely mirrored the effects seen in adherent monolayer culture, the in vivo xenotransplantation experiment results were reflecting the complexity of the in vitro setting. While in vitro 451Lu cells responded only to TNF-α stimulation experiment results were reflecting the complexity of the in vivo setting. While in vitro 451Lu cells responded only to TNF-α stimulation, we investigated whether levels of phosphorylated ERK 1/2 or RAC1 were increased after birinapant treatment in these cell lines. These were recently described to play a role in resistance to SMAC mimetics (45, 46). Although we observed a decrease in ERK1/2 activation after 24 hours of birinapant treatment in most cell lines tested, we could not find any differential regulation of these 2 proteins in the resistant cell lines as compared with the sensitive subset. Another possible mechanism of resistance to SMAC mimetics is loss of RIP1 expression. We observed the effect of birinapant to be dependent on RIP1 kinase in concordance with previously published reports on compounds from the same class (47). As there was no difference in RIP1 expression between birinapant-resistant and -sensitive cell lines in the panel used in this study, RIP1 expression is likely not a suitable biomarker to predict melanoma response to birinapant. Therefore, further investigation to define a biomarker for this subgroup is warranted, as this would be highly useful in the clinical application of birinapant.

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Birinapant was effective as a single agent in vitro only in one of the 17 cell lines tested. We therefore investigated the role of TNF-α signaling on the birinapant effect in this cell line. Blocking endogenous TNF-α in the
supernatant completely abrogated the effect of birinapant in a dose-dependent fashion, thereby showing the dependency of birinapant on concurrent TNF-α stimulation in vitro.

This dependency was furthermore schedule-specific, as we could show in a cell line sensitive to birinapant only in combination with TNF-α. The effect of the combination therapy was preserved when sequentially added in the following order: first birinapant, with downregulation of cIAP1 and cIAP2, and subsequent TNF-α, with activation of the TNFR complex 2 and the apoptotic cascade. Adding these 2 compounds in the reverse order diminished their effectiveness significantly.

With the prospect of increased numbers of patients with melanoma acquiring resistance to BRAF inhibitors, the question whether birinapant might be a feasible second-line therapy for such cases was explored. Indeed, a cell line with acquired resistance to BRAF inhibition did not alter its sensitivity profile to birinapant when compared with the parental cell line. This suggests birinapant as a possible second-line therapy in patients with acquired resistance to BRAF inhibitors.

Although targeted therapy with small molecules is showing impressive results in melanoma therapy, cytotoxic agents are still playing a major role in this disease when patients are either not eligible for kinase inhibitors and immunotherapy, or have relapsed on these drugs. We have therefore investigated the role of birinapant in sensitizing melanoma to cisplatin, a DNA-damaging agent. Although the results seen in this study are encouraging, more combinations and schedules will have to be explored as these studies are currently ongoing.

Disclosure of Potential Conflicts of Interest

E.M. Neiman is employed (other than primary affiliation; e.g., consulting) in TetraLogic Pharmaceuticals as a Research Scientist. M. McKinlay has ownership interest (including patents) in TetraLogic Common Stock. No potential conflicts of interest were disclosed by the other authors.

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