Small Molecule Therapeutics

Birinapant (TL32711), a Bivalent SMAC Mimetic, Targets TRAF2-Associated cIAPs, Abrogates TNF-Induced NF-κB Activation, and Is Active in Patient-Derived Xenograft Models

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Abstract

The acquisition of apoptosis resistance is a fundamental event in cancer development. Among the mechanisms used by cancer cells to evade apoptosis is the dysregulation of inhibitor of apoptosis (IAP) proteins. The activity of the IAPs is regulated by endogenous IAP antagonists such as SMAC (also termed DIABLO). Antagonism of IAP proteins by SMAC occurs via binding of the N-terminal tetrapeptide (AVPI) of SMAC to selected BIR domains of the IAPs. Small molecule compounds that mimic the AVPI motif of SMAC have been designed to overcome IAP-mediated apoptosis resistance of cancer cells. Here, we report the preclinical characterization of birinapant (TL32711), a bivalent SMAC-mimetic compound currently in clinical trials for the treatment of cancer. Birinapant bound to the BIR3 domains of cIAP1, cIAP2, XIAP, and the BIR domain of ML-IAP in vitro and induced the autoubiquitylation and proteasomal degradation of cIAP1 and cIAP2 in intact cells, which resulted in formation of a RIPK1:caspase-8 complex, caspase-8 activation, and induction of tumor cell death. Birinapant preferentially targeted the TRAF2-associated cIAP1 and cIAP2 with subsequent inhibition of TNF-induced NF-κB activation. The activity of a variety of chemotherapeutic cancer drugs was potentiated by birinapant both in a TNF-dependent or TNF-independent manner. Tumor growth in multiple primary patient-derived xenotransplant models was inhibited by birinapant at well-tolerated doses. These results support the therapeutic combination of birinapant with multiple chemotherapies, in particular, those therapies that can induce TNF secretion. Mol Cancer Ther; 13(4); 867–79. ©2014 AACR.

Introduction

Apoptosis or programmed cell death is a genetically encoded process that results in the clearance of damaged cells without inducing an inflammatory response and can be initiated by either intrinsic or extrinsic cellular cues. Apoptotic signaling culminates in the activation of a family of cysteine proteases with aspartate specificity (termed caspases), which cleaves key protein substrates ensuring that the cell is dismantled in a controlled manner. Resistance to apoptosis is a fundamental property of cancer (1). The acquisition of genetic lesions, which dysregulate the execution of apoptosis, is known to cooperate with other cellular defects such as oncogene activation leading to tumor initiation and progression (1, 2). In addition, the emergence of drug resistance has also been attributed, in part, to defects in apoptosis (3, 4). The development of drugs aimed at regulation of the apoptotic signaling of cancer cells represents a new paradigm in cancer treatment and several of these agents have entered clinical trials.

The inhibitor of apoptosis (IAP) proteins are a highly conserved family of signaling regulators that play key roles in cell death as well as immunity, inflammation, and cell division (5, 6). Originally discovered for their cytoprotective effect in baculovirus-infected insect cells, the IAP proteins have been conserved throughout evolution. The Baculovirus IAP Repeat (BIR) domain is the defining feature shared by all IAP proteins and facilitates specific interactions with other proteins. Some family members...
also possess a C-terminal really interesting new gene (RING) domain with E3 ubiquitin ligase activity. There are 8 human IAPs with XIAP being the only direct inhibitor of the proteolytic activity of caspases (7). The cellular IAPs, cIAP1, and cIAP2 (referred collectively as cIAP1/2) play key roles in the promotion of survival signaling induced by members of the TNF superfamily of cell surface receptors. Upon ligand binding, cIAP1/2 are recruited to the membrane-bound TNF receptor 1 (TNFR1) complex via the TNF receptor-associated factor (TRAP) family of proteins. In contrast, cIAP1/2 function as ubiquitin E3 ligases, which catalyze the attachment of ubiquitin or polyubiquitin chains through the action of their C-terminal RING domains. These ubiquitylation events serve to either provide a scaffold for the further assembly of protein complexes and subsequent signal transduction or target-specific proteins for proteasome-mediated degradation (8).

The second mitochondria-derived activator of caspases (SMAC) also referred to as the direct IAP binding protein with low pI (DIABLO) is an endogenous antagonist of IAP proteins. SMAC is released from the mitochondrial compartment in response to apoptotic stimuli and neutralizes IAP proteins thus allowing sustained caspase activity and execution of the apoptotic process. The contact between IAPs and SMAC is mediated through the N-terminal AVPI tetrapeptide of SMAC, which binds within a groove on the IAP BIR domain. The design of small molecules that mimic the IAP binding motif of SMAC and pharmacologically inhibit IAP protein function has been described (9).

The IAP proteins are frequently dysregulated in many cancers and thus have been suggested as contributing to chemoresistance and treatment failure. DNA amplification of the cIAP1 and cIAP2 genes (BIRC2 and BIRC3, respectively), located on chromosome 11q22-q23, has been observed in human lung cancers (10), liver carcinomas (11), oral squamous cell carcinomas (12, 13), medulloblastomas (14), glioblastomas (15), and pancreatic cancers (16). Frequent dysregulation of IAP proteins has also been observed at the protein level in multiple cancer cell lines and tumor samples (17, 18). Consequently, the IAP proteins have been pursued as anticancer drug targets. Various approaches have been utilized to target IAP proteins, including antisense oligonucleotides (19) and small molecules with specificity toward BIR domains of XIAP, survivin, ML-IAP, and cIAP1/2 (9).

Here we report the preclinical activity of birinapant (TL32711), a biindole-based bivalent SMAC mimetic. We show that birinapant binds with high affinity to the isolated BIR3 domains of cIAP1, cIAP2, and XIAP and the single BIR domain of ML-IAP and rapidly degrades TRAF2-bound cIAP1 and cIAP2 thereby inhibiting TNF-mediated NF-kB activation. In addition, birinapant promotes caspase-8:RIPK1 complex formation in response to TNF stimulation, resulting in the activation of downstream caspases. Moreover, birinapant treatment afforded synergistic cytotoxicity of several widely used chemotherapeutic agents. In murine xenograft models of ovarian and colorectal cancers and melanoma patient-derived tumors, birinapant displayed antitumor activity as a single agent at well-tolerated doses. These data suggest that birinapant targets IAP proteins to trigger an apoptotic response both in vitro and in vivo and has potential application in multiple tumor malignancies.

Materials and Methods

Reagents, plasmids, and tissue culture

pGEX-2T plasmids with N-terminal glutathione-S-transferase (GST)-tagged XIAP-BIR3 (amino acids 238–358) and cIAP1 BIR3 (amino acids 255–364) were obtained from Yigong Shi (Tsinghua University, China). The ML-IAP BIR domain was cloned into a pGEX-6p-2 vector and the cIAP2 BIR3 domain was cloned into a pET28a vector. The NF-kB luciferase reporter plasmid was from Stratagene. The cDNA encoding hemagglutinin (HA)-tagged enhanced green fluorescence protein (EGFP) was cloned into the N-termini of human cIAP1 and cIAP2 in the mammalian expression plasmid pcDNA3 and designated as HA2x-EGFP-cIAP1 and HA2xEGFP-cIAP2, respectively. Cell lines were obtained from various sources (American Type Culture Collection; Fox Chase Cancer Center, Leibniz-Institut DSMZ-Deutsches Sammlung von Mikroorganismen und Zellkulturen GmbH) and maintained in appropriate medium.

Establishment of stable cells

A375 cells were transfected with HA2xEGFP-cIAP1 and HA2xEGFP-cIAP2 linearized with Pvu I by using Lipofectamin2000, G418, restriction enzymes, protein A/G agarose, and a NuPAGE system were purchased from Invitrogen. Birinapant (TL32711) and biotin-labeled birinapant were synthesized at TetraLogic Pharmaceuticals. Other reagents included AbuRPFK(5-Fluoroacetamido)-S-Fam)-NH2 peptide (Biomer Technologies), anti-cIAP1, -cIAP2, and -ML-IAP antibodies were from R&D. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and -PARP antibodies were from Santa Cruz Biotechnology, anti-XIAP antibody (Stressgen), anti-β-actin antibody was from Cell Signaling, anti-RIPK1 antibody was from BD, anti-caspase-8 and TRAF2 antibodies were from both Santa Cruz Biotechnology and Cell Signaling.

Coimmunoprecipitation and Western blot analysis

For immunoprecipitation of TRAF2 protein complex, cells were collected and lysed with buffer-A consisting of 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L sodium chloride, 10% glycerol, 2 mmol/L EDTA, 50 mmol/L sodium fluoride, 25 mmol/L β-glycerophosphate, 0.2 mmol/L...
sodium vanadate, 10 mmol/L sodium pyrophosphate, 2 mmol/L DTT, 0.5% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche). Cleared total cell lysate (2 mg) was incubated with anti-TRAF2 antibody overnight at 4°C. The immunoprotein complex was purified by using Protein A/G agarose beads and subjected to Western blot analysis with indicated antibodies. Immunoprecipitation of ripoptosome, complex-I, and complex-II were essentially carried out as described (20, 21). Protein samples were applied to NuPAGE gel, followed by the electrotransfer to Immobilon-FL membranes (Millipore). Membranes were incubated with blocking buffer (LI-COR Bioscience, Inc.). Detection of proteins on the membrane was performed with a standard procedure using primary antibodies indicated in the figures and their appropriate secondary antibodies conjugated to Alexa Fluor 680 or 800 IRDye. Membranes were scanned and analyzed by Odyssey infrared imaging system (LI-COR Biosciences, Inc.).

Fluorescence polarization assay
The binding affinities of compounds to the purified BIR3 domains of cIAP1, cIAP2, and XIAP, and the single ML-IAP BIR domain were determined as described previously (22) and are reported as dissociation constant ($K_d$) values. The $K_d$ values of competitive inhibitors were calculated using the equation described in ref. 22 using IC$_{50}$ values, the $K_d$ value of the protein, and the concentrations of the protein and fluorescence polarization (FP) peptide in the competition assay.

Measurement of caspase-3 activity in cell lysates
MDA-MB-231 breast carcinoma cells were harvested by trypsinization and collected by centrifugation at 1,000 × g for 10 minutes at room temperature. Cell pellet was washed one time by resuspending in 5 mL hypotonic lysis buffer (20 mmol/L HEPES, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L MgCl$_2$, 1.0 mmol/L EDTA, 1.0 mmol/L DTT) and recollected by centrifugation. Pellet was next resuspended in 1 volume of hypotonic lysis buffer supplemented with a complete protease inhibitor tablet and allowed to swell on ice for 30 minutes. Cells were disrupted by approximately 50 passages through a 27-gauge needle. Lysis was monitored by light microscopy. Lysate was approximately 50 passages through a 27-gauge needle.

Measurement of caspase activity in intact cells
MDA-MB-231 cells were seeded in 96-well plates at an approximate density of 20,000 cells per well and allowed to adhere overnight. The next day, birinapant was added at various concentrations, and the plates were incubated at 37°C and 5% CO$_2$ for an additional 24 hours. Following the 24 hours incubation, Caspase-Glo assay (Promega) was performed according to manufacturer’s instructions. Luminescence was read on a Perkin-Elmer Victor$^2$V multilabel reader.

GFP-cIAP degradation assay
A375 GFP-cIAP1 and GFP-cIAP2 cells plated in a 96-well plate were treated with various concentrations of birinapant as indicated in the figures for either 2 hours or 24 hours. After the incubation, cells were collected by trypsinization and suspended in PBS. A total of 10^6 cells were analyzed using a FACSscan (Becton Dickinson). GFP fluorescence was detected using 488 nm excitation and 530 nm emission wavelengths.

Cell viability assay
Viability was determined in 96-well plates by MTT assay. Briefly, cells were seeded in 96-well plates at an approximate density of 5,000 to 10,000 cells per well and allowed to adhere overnight. The next day, test agents were added and the plates were incubated at 37°C and 5% CO$_2$ for an additional 72 hours. Following the 72 hours incubation, 50 µL of 5 mg/mL MTT reagent was added to each well (final concentration, 1 mg/mL). The IC$_{50}$ was derived by nonlinear regression analysis using GraphPad Prism 4.

Xenograft methods
Female athymic nude (nu/nu) mice (Harlan) between 5 to 8 weeks of age were housed on irradiated corncob bedding (TecKlad) in individual HEPA-ventilated cages (Sealsafe Plus, Techniplast) on a 12 hours light–dark cycle at 21°C to 23°C and 40% to 60% humidity. Animals were fed water ad libitum (reverse osmosis, 2 ppm Cl$_2$) and an irradiated standard rodent diet (Teklad 2919) consisting of 18% protein, 9% fat, and 34% fiber. Low-passage patient-derived xenograft models representing human ovarian cancer, melanoma, and colorectal cancer were implanted unilaterally on the right flank with tumor fragments harvested from 2 to 4 host animals. Prestudy tumor volumes were recorded for each experiment beginning approximately 1 week before its estimated start date. When tumors reached approximately 122 to 208 mm$^3$, animals were matched by tumor volume into treatment and control groups and dosing initiated (Day 0); animals were tagged and followed individually throughout the experiment. Birinapant was formulated in 12.5% Captisol in sterile water and adjusted to pH 4.0 using concentrated hydrochloric acid. Dosing for vehicle and birinapant began on Day 0; animals in all groups were dosed by weight (0.01 mL per gram; 10 mL/kg). Control animals in colorectal studies are untreated. Animals were dosed by intraperitoneal injection 3 times a day, as indicated.
Beginning on Day 0, animals were observed daily and weighed twice weekly (data not shown) using a digital scale. Beginning on Day 0, tumor dimensions were measured twice weekly by digital caliper (Fowler Ultra-Cal IV) and data were recorded for each group; tumor volume was calculated using the formula: \( TV = \text{width}^2 \times \text{length} \times 0.52 \).

**Results**

**Birinapant binds to multiple IAPs with high affinity**

To evaluate the binding affinity of birinapant (Fig. 1A) to the BIR domains of IAP proteins, an FP assay using the purified BIR3 domains of XIAP, cIAP1, and cIAP2, and the single BIR domain of ML-IAP was performed. The \( K_d \) values of birinapant for selected IAP protein BIR domains have recently been published elsewhere (23) and are reproduced in Supplementary Table S1. Birinapant showed comparable or greater binding affinity to these selected BIR domains when compared with the native SMAC N-terminal tetrapeptide (AVPI). The data demonstrated that birinapant bound to these BIR domains in a concentration-dependent manner (Fig. 1B). The direct interaction of birinapant with native XIAP, cIAP1, cIAP2, and ML-IAP proteins was confirmed by using a pulldown assay with biotin-labeled birinapant (Supplementary Fig. S1) and the total cell lysate from either IGROV-1 or SK-MEL-28 tumor cells; pretreatment of IGROV-1 cells with TNF induced the expression of cIAP2, which was detected in this assay. XIAP, cIAP1, cIAP2, and both the \( \alpha \)- and \( \beta \)-isoforms of ML-IAP proteins associated with biotinylated birinapant (B-B), but not with control (Ctr) beads, under the experimental condition (Fig. 1C). In addition, the neutralizing effect of birinapant treatment toward XIAP-mediated caspase inhibition was evaluated in a caspase de-repression assay. Cytosolic fractions prepared from MDA-MB-231 breast cancer cells were incubated with cytochrome \( c \) and dATP in the absence or presence of birinapant. Caspase-3 activity was increased following birinapant treatment in a concentration-dependent manner (Fig. 1D). This result suggested that in addition to...
binding to XIAP, birinapant was able to displace activated caspases from XIAP sequestration.

**Birinapant induces degradation of cIAP1 and cIAP2 in cells**

To evaluate the ability of birinapant to degrade cIAP1 and cIAP2 in intact cells, we established cell lines that stably expressed GFP-tagged cIAP1 (GFP-cIAP1) or GFP-cIAP2. This allowed for the quantitative determination of IC50 values for the degradation of each cIAP protein by monitoring GFP fluorescence by flow cytometry. Following 2-hour birinapant treatment, IC50 values for GFP-cIAP1 and GFP-cIAP2 were 17 ± 11 nmol/L and 108 ± 46 nmol/L, respectively (Supplementary Fig. S2A). We confirmed that both endogenous cIAP1 and cIAP2 were degraded synchronously with their GFP-tagged counterparts by western blot analysis (Supplementary Fig. S2B). As described previously, pretreatment with TNF was used to induce the expression of endogenous cIAP2 in the A375 cell line to facilitate this analysis.

**Birinapant was active in a variety of cancer cell lines**

The activity of birinapant was tested as a single agent and in combination with TNF and TRAIL in a panel of 111 cell lines representing multiple tumor types (Supplementary Table S2). Eighteen of the 111 cell lines (18/111) were sensitive to birinapant as a single agent, with IC50 values <1 μmol/L. For evaluation of the activity of birinapant in combination with TNF or TRAIL, cell lines were treated in a matrix pattern consisting of a range of concentrations of both birinapant and TNF or TRAIL (Supplementary Fig. S3). Data were analyzed using the MacSynergy II program (24). Combinations resulting in synergy volumes >100 μmol/L2% were considered synergistic and are also indicated in Supplementary Table S2. The addition of TNF or TRAIL resulted in the sensitization to birinapant of approximately 45% of the single agent birinapant-resistant cell lines. Several of these cell lines were also resistant to both TNF and/or TRAIL as single agents, highlighting the synergistic nature of these combinations. In addition, no normal cells tested (HUVEC, MRC-5) were sensitive to birinapant alone or in combination with either TNF or TRAIL (data not shown). Taken together, these results illustrate that approximately 60% of cell lines were sensitive to birinapant as a single agent or in combination with TNF or TRAIL.

It has been shown that the single-agent activity of SMAC mimetics is dependent upon autocrine secretion of TNF (25–30). Consistent with these findings, birinapant-sensitive tumor cell lines secreted TNF following birinapant treatment whereas no TNF secretion was observed in birinapant-resistant tumor cell lines (Supplementary Fig. S4). To confirm that the autocrine production of TNF was responsible for the cytotoxic effect of birinapant, pretreatment of the birinapant-sensitive cell lines SK-OV-3 and EVSA-T with a TNF neutralizing antibody reduced birinapant-mediated cytotoxicity (Supplementary Fig. S5).

The activation of caspases and induction of cell death following birinapant treatment occurred below the drug concentration required for complete degradation of total cIAP2

The rapid loss of both cIAP1 and cIAP2 in tumor cells following treatment with certain SMAC mimetics has been associated with tumor cell death in sensitive cell lines (28–30). However, in some birinapant-sensitive cancer cell lines, we observed that the induction of cell death following birinapant treatment occurred below the drug concentration required for a detectable decrease in total cIAP2 (Fig. 2). For example, in the MDA-MB-231 breast cancer cell line, 1 nmol/L birinapant treatment resulted in >50% cIAP1 loss, without any observed effect on total cIAP2 levels (Fig. 2A), together with activation of caspases and cell death induction (Fig. 2C and D). At 10 nmol/L birinapant treatment, cIAP1 and cIAP2 loss was approximately 90% and 40%, respectively (Fig. 2B). Moreover, Western blot analysis of birinapant-treated MDA-MB-231 cells indicated a dose-dependent loss of cIAP1 whereas cIAP2 levels remained relatively stable until caspase-3-mediated proteolysis of PARP—a measure of apoptosis induction—became evident at 10 nmol/L birinapant (Fig. 2E).

**Birinapant preferentially induces the degradation of the TRAF2-associated pool of cIAPs**

Cellular IAP2 levels are controlled, at least in part, by the E3 ligase activity of cIAP1 (31). The apparent dissociation of cIAP2 loss and tumor cell death induction suggested that either cIAP1 alone or a specific subset of cIAP proteins might instead be the physiologic targets of birinapant. We therefore investigated the action of birinapant on those cIAP proteins, which are specifically associated with TNFR1 signaling. The TNF receptor–associated factor 2 (TRAF2) is a recognized cIAP binding partner that recruits both cIAP1 and cIAP2 to TNFR1 following TNF stimulation (32–34). A375 cells stably expressing GFP-cIAP1 or GFP-cIAP2 were utilized for communoprecipitation with an anti-TRAF2 antibody owing to the high expression level of both GFP-cIAP constructs (infrar). Following treatment with 100 nmol/L birinapant, endogenous TRAF2-bound cIAP1 and TRAF2-bound GFP-cIAP1 were synchronously degraded with similar kinetics, which accurately reflected total cIAP1 loss (Fig. 3A, top left). Birinapant also induced the degradation of TRAF2-bound GFP-cIAP2 in 2 independent experiments (Fig. 3A, top right and middle). Interestingly, the majority of non–TRAF2-bound GFP-cIAP2 remained intact. Quantitation of the second Western blot analysis confirmed that the TRAF2-bound pool of cIAP2 was, in fact, degraded preferentially to the non–TRAF2-bound pool (Fig. 3A, bottom). Notably, endogenous cIAP2 was poorly detected owing to its low expression level. To confirm these observations using endogenous cIAP2, we pretreated HeLa cells with TNF (10 ng/mL) to induce the expression of endogenous cIAP2 followed by treatment with a range of birinapant doses (Supplementary Fig. S6A). Consistent with the previous results, TRAF2-bound cIAP2
was reduced following birinapant treatment. By analyzing the TRAF2 immunoprecipitation Western blots, we estimated that the TRAF2-bound cIAP1 in HeLa cells was approximately 25% of the total cIAP1 (data not shown). In a similar fashion, treatment of HeLa cells with 10 nmol/L of birinapant induced the degradation of TRAF2-bound cIAP1 with slightly faster kinetics than total cIAP1, including non–TRAF2-bound cIAP1, and quantitation of this Western blot analysis reflected these observations (Fig. 3A, left middle and bottom). Like cIAP2, we analyzed the effects on cIAP1 levels in HeLa cells using a dose range of birinapant (Supplementary Fig. S6B). Owing to the observable levels of endogenous cIAP1, the HeLa cells were not pretreated with TNF. Consistently, the TRAF2-bound pool of cIAP1 was degraded with slightly greater efficiency than the total cIAP1 following birinapant treatment.

These results suggested that birinapant was preferentially targeting the TRAF2-bound cIAPs to elicit tumor cell death. In addition, cIAP1 was more sensitive to birinapant-induced degradation than cIAP2, which may reflect an inherent difference between these 2 proteins toward SMAC-mimetic treatment (35). To exclude the possibility that birinapant was displacing cIAP1 or cIAP2 from TRAF2, similar TRAF2 pulldown experiments were performed in the presence of the proteasome inhibitor bortezomib. Following treatment with birinapant plus bortezomib, both cIAP1 and cIAP2 remained associated with TRAF2 upon pulldown analysis (data not shown). This demonstrated that birinapant was not disrupting the association of TRAF2 with either cIAP1 or cIAP2 but was instead inducing their proteasome-mediated degradation.

TRAF2-deficient or cIAP1/cIAP2 double knockout mouse fibroblast cells are unable to activate NF-κB response to TNF stimulation (34, 36). To further confirm that birinapant induced the preferential degradation of TRAF2-bound cIAPs, we used an NF-κB promoter-driven luciferase assay to evaluate birinapant treatment on NF-κB activity. Pretreatment of HeLa cells that stably harbor the NF-κB-luciferase reporter gene with birinapant blocked TNF-mediated NF-κB activity in a concentration-dependent manner (Fig. 3B). TNF treatment of these cells caused a rapid (10 minutes) loss of the endogenous inhibitor of NF-κB α (IκBα), which could be abrogated by birinapant pretreatment (Fig. 3C). In A375 cells stably overexpressing GFP-cIAP1 or GFP-cIAP2, the TNF-mediated loss of IκBα was also blocked by pretreatment with birinapant (Supplementary Fig. S7). Taken together, these results demonstrated that birinapant treatment resulted in the loss of TRAF2-bound cIAP1/2 and the subsequent...
inhibition of both TNF-induced IκBα degradation and NF-κB activation.

**Treatment with birinapant leads to formation of a RIPK1:caspase-8 protein complex and increases caspase-8 activation through TNFR1 and TRAIL receptors**

To investigate the molecular mechanisms of cell death induction by birinapant, we examined the interaction of the receptor-interacting protein kinase 1 (RIPK1), another TNFR1-associated protein, with caspase-8. Recent reports have described the formation of a complex, named the ripoptosome, composed of RIPK1, caspase-8, and the Fas-associated death domain (FADD), that is generated following the loss of both cIAP1 and cIAP2 and the further loss of RIPK1 ubiquitylation (20, 21). In the birinapant-sensitive cell lines SK-OV-3 and EVSA-T, birinapant increased RIPK1:caspase-8 complex formation relative to untreated cells (Fig. 4A, lanes 4 and 5, respectively). Necrostatin 1 (Nec-1), a RIPK1 inhibitor (37), reduced RIPK1:caspase-8 complex formation in SK-OV-3 cells, albeit to a lesser extent (Fig. 4A, lanes 6 and 7, respectively). In contrast, the birinapant-resistant cell lines IGROV1, OVCAR-3, T-24, and HCT116, displayed reduced RIPK1:caspase-8 complex formation in response to birinapant treatment (Fig. 4A). More importantly, the RIPK1:caspase-8 complex formation and subsequent caspase-8 activation in birinapant-sensitive cells
was attenuated by a neutralizing anti-TNF antibody, suggesting that autocrine TNF secretion played a role in promoting RIPK1:caspase-8 formation in these birinapant-sensitive cells (Fig. 4B). Consistent with a role of TNF in ripoptosome formation, the addition of exogenous TNF to the birinapant-resistant A375 or HeLa cells enhanced the formation of the RIPK1:caspase-8 complex in the presence of birinapant (Fig. 4C, top and bottom, lane 7). The RIPK1:caspase-8 complex formation was transient. This is consistent with reports that RIPK1 is a target of active caspase-8 (38) and thus as caspase-8 is activated, RIPK1 is cleaved, and the RIPK1:caspase-8 complex is destabilized (Fig. 4C, top and bottom, lanes 8 and 9). Of note, the upregulation of cIAP2 by TNF was reduced in the presence of birinapant (Fig. 4C, lanes 5 and 9), which may contribute to the enhanced apoptosis induction by birinapant/TNF cotreatment. In addition, birinapant enhanced (ca. 10-fold) the activation of caspase-8 by TRAIL in A375 cells (Fig. 4D). These results suggested that birinapant promoted the intracellular cell death complex formation, which activated caspase-8 and sensitized tumor cells to TNF and TRAIL receptor–mediated cell killing.

**Birinapant can potentiate the cytotoxicity of certain chemotherapeutic drugs in vitro**

Birinapant increased the potency of several chemotherapeutic agents, including SN-38 (the active metabolite of irinotecan), gemcitabine, and 5-azacytidine (Fig. 5A). This property of birinapant seemed to be independent of p53.
status in the cell lines examined (data not shown). Given
the demonstrated role of autocrine TNF signaling for the
single-agent activity of birinapant, we inquired whether
TNF signaling was also contributing to the observed
synergy with certain chemotherapeutic drugs (Fig. 5B).
Pretreatment of cells with a neutralizing anti-TNF anti-
body resulted in the inhibition of tumor cell death induced
by the combination of birinapant plus 5-azacytidine in
HL-60 cells or by the combination of birinapant plus either
SN-38 or gemcitabine in OVCAR-3 cells. In contrast,
pretreatment with the anti-TNF antibody had no effect
on cell death induction by the combination of birinapant
plus gemcitabine in the HT-1376 bladder cancer cell line,
suggesting that TNF-independent mechanisms may also
be operative using birinapant/chemotherapy combina-
tions (39). Apart from SN-38 and the hypomethylating
nucleoside analogs gemcitabine and 5-azacytidine, the
cytotoxic activity of selected chemotherapeutic drugs,
including pemetrexed, vemurafenib, bendamustine, and
sorafenib, was not potentiated by birinapant cotreatment
(data not shown).

Activity of birinapant in patient-derived xenograft
models of ovarian and colorectal cancers and
melanoma

The antitumor activity of birinapant was assessed in vivo using low-passage, patient-derived xenotransplant
models of ovarian cancer, colorectal cancer, and melano-
ma (Fig. 6). Intraperitoneal administration of birinapant
(30 mg/kg) every third day (×5) resulted in inhibition of
tumor growth; treatment was limited to 5 doses. There
was no evidence of toxicity or body weight loss over
the course of treatment. In total, birinapant was tested
in 50 patient-derived xenotransplant models, including

Figure 5. Birinapant potentiates the activity of multiple
chemotherapeutic drugs in vitro. A, activity of gemcitabine, SN-38, 5-
azacytidine (SAC), birinapant, and combination treatments in
OVCAR3, HT-1376, and HL-60 cell lines. For combination treatment,
various concentrations of birinapant were combined with
10 nmol/L gemcitabine in HT-1376
and OVCAR-3, 3 μmol/L 5AC in
HL-60 cells, and 10 nmol/L SN-38
in OVCAR-3. Viability was
measured by MTT assay following
72 hours exposure. Data, mean ±
SD. Experiments were performed
3 times. B, TNF-dependent and
independent cell death induction
by birinapant and
chemotherapeutics. Cells were
pretreated with anti-TNF antibody
1 hour before the combination
treatment as indicated above.
Viability was measured by MTT
assay following 72 hours exposure.
Data, mean ± SD. Experiments
were performed 3 times.
ovarian and colorectal cancers and melanoma, and activity was observed in approximately one third of the models tested (data not shown). These studies highlight the efficacy and tolerability of birinapant in preclinical models.

Discussion

One hallmark of cancer cells is a compromised ability to undergo apoptosis. Therefore, strategies targeting cell death regulators such as the IAP family of proteins with the goal of overcoming resistance to apoptosis have therapeutic potential for the development of new classes of anticancer drugs.

Unlike XIAP, the cIAPs are not direct inhibitors of caspases; however, cIAPs play an important role in inhibiting the TNF-mediated extrinsic cell death pathway and enabling the activation of TNF-induced NF-κB pro-survival signaling (6, 25, 40). In this study, we show that birinapant, a biindole-based bivalent SMAC mimic, is able to induce the degradation of specific pools of cIAPs while leaving others intact. In addition, birinapant inhibits TNF-mediated NF-κB activity, induces cell death as a single agent and in combination with TNF or TRAIL in a variety of cancer cell lines, and potentiates the activity of chemotherapeutic drugs in both TNF-dependent and independent manners. Birinapant was also active in primary patient-derived xenograft models.

Western blot analysis of birinapant-induced cIAP1 and cIAP2 degradation in total protein lysates as well as the analysis of the degradation of GFP-tagged cIAP1 and cIAP2 in intact cells suggested that birinapant was capable of inducing apoptosis at concentrations that resulted in the complete degradation of cIAP1 but only partial degradation of cIAP2. This result was in conflict with multiple reports describing the redundant functions of cIAP1 and cIAP2 within the TNFR1 complex as well as the requirement of antagonizing both cIAP1 and cIAP2 for inducing TNF-dependent cell death by SMAC mimetics (41). Reasoning that distinct pools of cIAP1 and cIAP2 may be preferentially targeted by birinapant, we examined the effects of birinapant on TRAF2-associated cIAPs in order to determine if they were preferentially targeted.

Figure 6. Birinapant exhibits single-agent activity in patient-derived xenograft models of ovarian and colorectal cancer and melanoma at a well-tolerated dose. Birinapant activity in low-passage, patient-derived ovarian cancer (A), colorectal cancer (B), and melanoma (C). Vehicle control (○) or birinapant (30 mg/kg; ●) dosed intraperitoneally 3 times a day as indicated (△).
to understand these seemingly contradictory results. We found that a characteristic feature of birinapant was the preferential degradation of TRAF2-bound cIAP1 and TRAF2-bound cIAP2.

Recruitment of cIAP1 or cIAP2 to the TNFR1 complex by TRAF2 is necessary for TNF-mediated canonical NF-κB activation (34). Both cIAP1 and cIAP2, via their RING domain-mediated E3 ligase activity, can transfer ubiquitin or poly-ubiquitin chains (i.e., ubiquitylation) to RIPK1, which allows for the recruitment of the IKK complex (IKKα/IKKβ/IKKγ) to TNFR1 thus enabling the initiation of TNF-induced NF-κB activation. In the absence of TRAF2 or cIAPs, this ubiquitylated RIPK1-dependent complex fails to assemble and, hence, TNF-mediated NF-κB signaling is blocked and apoptosis is initiated.

The specific mechanisms underlying the regulation of the TRAF2-bound and non–TRAF2-bound cIAP1 and cIAP2 by birinapant are unknown. It was recently demonstrated that a bivalent SMAC mimetic, BV6, induced the autoubiquitination and degradation of the single BIR domain-containing protein, ML-IAP, whereas a related monovalent SMAC mimetic had no effect. This result suggested that BIR domain crosslinking may have a mechanistic role in bivalent SMAC-mimetic–induced E3 ligase activity (42). It is therefore possible that a similar cIAP-BIR3 domain crosslinking may be responsible for the selective degradation of TRAF2-bound cIAPs by birinapant.

It has also been reported that cIAP2 is a substrate for cIAP1-mediated ubiquitylation and proteasomal degradation and that cIAP1 is necessary for the efficient SMAC-mimetic–induced degradation of cIAP2 (43, 44). In addition, following SMAC-mimetic treatment, cIAP2 levels are stabilized possibly because of prolonged suppression of cIAP1 E3 ligase activity (44, 45). We found that birinapant degrades both TRAF2-bound and non–TRAF2-bound cIAP1.

Although TRAF2-bound cIAP2 is subject to birinapant-mediated degradation, non–TRAF2-bound cIAP2 is partly spared from birinapant-induced degradation. In contrast, treatment with the pan-IAP antagonist, Compound A, induced the degradation of TRAF2-bound and non–TRAF2-bound pools of both cIAP1 and cIAP2, indicating that different SMAC mimetics can elicit selective effects on these 2 proteins (46). To affect the ubiquitylation/degradation of TRAF2-bound cIAP2 while sparing non–TRAF2-bound cIAP2, birinapant may selectively stabilize the homodimeric TRAF2-bound cIAP2 E3 ligase complex or, alternatively, a heterodimeric TRAF2-bound cIAP1–cIAP2 E3 complex might be engaged. Heterodimeric E3 ligases such as BRCA:BARD and MDM2:MDMX have been reported (47, 48). Alternatively, different SMAC-mimetic–ligated homodimeric cIAP1 E3 ligases may offer unique ubiquitin transfer kinetics, which allows non–TRAF2-bound cIAP2 to partially escape birinapant-mediated ubiquitylation whereas a Compound A–derived cIAP1 E3 ligase would offer no selectivity.

Following TNF binding, the cIAPs ubiquitinate RIPK1 allowing the assembly of a TNFR1 signaling complex composed of the IKK (IKKα/IKKβ/IKKγ) and TAK/TAB complexes. Recent reports have described the spontaneous formation of the ripoptosome, composed of RIPK1, caspase-8, and the FADD, generated via loss of cIAP1 and cIAP2 (20, 21). In the tumor cell lines tested, birinapant treatment induced cell death despite sparing of non–TRAF2-bound cIAP2. This suggested that RIPK1 was not a substrate for non–TRAF2-bound cIAP2 and thus non–TRAF2-bound cIAP2 does not seem to contribute to apoptosis resistance.

The ability to potentiate the cytotoxicity of mechanistically distinct chemotherapeutic drugs is relevant to the clinical development of birinapant. Drugs such as irinotecan and gemcitabine are important components of clinical anticancer regimens (49). We observed that TNF contributes to the activity of birinapant in combination with SN-38 (the active metabolite of irinotecan), gemcitabine, and 5-azacytidine in certain tumor cell lines, which is consistent with other reports of SMAC-mimetic use in combination with chemotherapeutic drugs (50). These observations, as well as the ability of TNF to broadly sensitize cell lines to birinapant, support the clinical paradigm of combining birinapant with agents capable of increasing TNF levels within the tumor microenvironment. TNF-independent mechanisms of SMAC-mimetic–mediated synergy with chemotherapeutics have also been reported in lung cancer cell lines (39). Consistent with these observations, we have observed that TNF neutralization does not block birinapant-mediated potentiation of all chemotherapeutic agents, suggesting multiple proapoptotic mechanisms of interaction.

The primary patient-derived xenotransplant studies highlight the activity and tolerability of birinapant in vivo. These models, which were developed from patient-derived tumor explants and passaged in vivo, are considered to more accurately reflect the original tumor than cell–line–derived xenograft models. As described in this article, significant antitumor efficacy was observed at concentrations of birinapant that are devoid of overt toxicity to the animals, which highlight the safety and efficacy of birinapant in preclinical models. Furthermore, the results described in this article support the clinical investigation of birinapant both as a single agent as well as in combination with multiple chemotherapies.

Disclosure of Potential Conflicts of Interest

M.A. Graham has ownership interest (including patents) in TetraLogic Pharmaceuticals. D. Weng has ownership interest (including patents) in TetraLogic Pharmaceuticals Corp. Y. Shi is a scientific advisory board member. M.A. McKinlay has ownership interest (including patents) in TetraLogic Pharma. S.K. Chunduru has ownership interest (including patents) in TetraLogic. No potential conflicts of interest were disclosed by the other authors.

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References


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Correction: Birinapant (TL32711), a Bivalent SMAC Mimetic, Targets TRAF2-Associated cIAPs, Abrogates TNF-Induced NF-κB Activation, and Is Active in Patient-Derived Xenograft Models

In this article (Mol Can Ther 2014 April; 13:867–79), which was published in the April 2014 issue of Molecular Cancer Therapeutics (1), the authors regret that dosing is incorrectly stated as “3 times a day” in the legend for Figure 6 and in the xenograft section of the Materials and Methods. The correct dosing is “every three days.” In addition, the authors acknowledge that the titles of the xenograft models in Fig. 6 incorrectly read as “PDX” followed by a number. They should instead be titled “ST” followed by the number.

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