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Dual Inactivation of Akt and ERK by TIC10 Signals Foxo3a Nuclear Translocation, TRAIL Gene Induction, and Potent Antitumor Effects

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Abstract

Recombinant tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is an antitumor protein that is in clinical trials as a potential anticancer therapy but suffers from drug properties

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Author contributions: J.E.A. and W.S.E.-D. designed all the experiments. J.E.A. conducted the experiments and wrote the manuscript. G.S.W. constructed the plasmids used in the high-throughput screening that identified TIC10. G.K. and P.A.M. conducted the high-throughput screening for TRAIL-inducing compounds. J.E.A. and L.P. validated hits from the screen. G.S.W. and J.-Y.Z. made the MDA-MB-231 shTRAIL and control cell lines. J.E.A. and A.S.P. conducted the intracranial xenograft experiments. N.G.D. constructed and validated the myr-Akt HCT116 cell line. E.M. provided freshly resected colon cancer tissue. W.W. constructed the H460 cell line expressing DR5 DD-EGFP. K.A.S. assisted with the design and execution of ChIP assays. D.T.D. conducted flow cytometry analysis. W.S.E.-D. supervised the experiments and contributed as senior author including editing of the manuscript and responsibility for oversight of conduct of the research.

Competing interests: W.S.E.-D. is a co-founder and chief scientific advisor of Oncoceutics Inc., a biotech company focused on developing novel small-molecule anticancer therapies targeting p53-deficient tumors.

Data and materials availability: We received TIC10 from the NCI Developmental Therapeutics Program. The microarray data for TIC10 have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE34194).

SUPPLEMENTARY MATERIALS

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that may limit efficacy such as short serum half-life, stability, cost, and biodistribution, particularly with respect to the brain. To overcome such limitations, we identified TRAIL-inducing compound 10 (TIC10), a potent, orally active, and stable small molecule that transcriptionally induces TRAIL in a p53-independent manner and crosses the blood-brain barrier. TIC10 induces a sustained up-regulation of TRAIL in tumors and normal cells that may contribute to the demonstrable antitumor activity of TIC10. TIC10 inactivates kinases Akt and extracellular signal-regulated kinase (ERK), leading to the translocation of Foxo3a into the nucleus, where it binds to the TRAIL promoter to up-regulate gene transcription. TIC10 is an efficacious antitumor therapeutic agent that acts on tumor cells and their micro-environment to enhance the concentrations of the endogenous tumor suppressor TRAIL.

INTRODUCTION

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a powerful inducer of apoptosis in a wide range of human cancer cell lines via proapoptotic death receptor 4 (DR4; TRAIL-R1) (1) and death receptor 5 (DR5; TRAIL-R2) (2, 3) at the cell surface through engagement of the extrinsic or intrinsic apoptotic pathways (4). TRAIL serves as a tumor suppressor during immune surveillance, but this antitumor mechanism is lost during cancer progression. The ability of TRAIL to initiate apoptosis selectively in cancer cells has led to ongoing clinical trials with recombinant TRAIL and the longer-lived TRAIL receptor agonist antibodies, which target either of TRAIL's two proapoptotic death receptors (5–11). Variants of recombinant TRAIL and other protein-based therapeutics continue to be developed to recapitulate the anti-tumor efficacy of endogenous TRAIL and to improve its stability (12, 13). Mesenchymal stem cells overexpressing TRAIL have recently been explored to improve the biodistribution of TRAIL to allow for its use in glioma (14).

Although current TRAIL-based therapies are costly to produce for clinical applications and may be limited by stability and/or biodistribution, endogenous TRAIL is a robust and selective tumor suppressor and naturally lends itself as a drug target to restore antitumor immunity. We hypothesized that up-regulation of TRAIL expression by a small molecule would lead to potent antitumor effects and improve the biodistribution and pharmacokinetic properties of TRAIL by increasing its half-life as well as its concentration within the tumor microenvironment.

The transcription factors p53 (15) and Foxo3a (16), which typically serve as tumor suppressors (17), positively regulate the TRAIL gene. In our search for TRAIL-inducing compounds that up-regulate the TRAIL gene, we explicitly selected those that do not rely on p53 because p53 is frequently inactivated in late-stage cancers, which causes resistance to many standard-of-care therapies such as 5-fluorouracil and doxorubicin (18). Among FOXO family members, Foxo3a transcriptionally regulates the TRAIL gene through a region of the promoter that is downstream of the p53 regulatory site that we previously identified (15). Foxo3a is primarily regulated by control of its localization through phosphorylation events that dock the transcription factor to cytoplasmic 14-3-3 proteins and inactivate it (19). This phosphorylation is carried out by a number of kinases involved in prosurvival signaling, such as I κ B kinase (IKK), serum- and glucocorticoid-induced kinase (SGK), Akt, and

extracellular signal-regulated kinase (ERK) (19, 20). In summary, Foxo3a is normally sequestered in the cytoplasm by growth factor/prosurvival signaling pathways, which prevents its ability to activate the TRAIL gene. Thus, modulation of Foxo3a by targeting upstream prosurvival signaling pathways that have well-established roles and are highly conserved in cancer could allow therapeutic up-regulation of the TRAIL gene.

RESULTS

TIC10 stimulates tumor cell production of TRAIL

To overcome limitations of recombinant TRAIL, we screened the National Cancer Institute (NCI) Diversity Set II for small molecules capable of up-regulating endogenous TRAIL gene transcription to pharmacologically increase tumor and host TRAIL protein production. A cell-based bioluminescence reporter screen conducted in TRAIL-resistant Bax-null HCT116 human colon cancer cells harboring a TRAIL gene promoter luciferase reporter yielded the small-molecule TIC10 as a TRAIL-inducing compound (Fig. 1A and fig. S1). TIC10 caused a dose-dependent increase in TRAIL mRNA (Fig. 1B) and induced TRAIL protein localization on the cell surface of several cancer cell lines in a p53-independent manner (Fig. 1C). A time-course analysis found that TRAIL was localized to the cell surface as a late event but that this induction could be sustained even after removal of TIC10 from the media (Fig. 1, D and E). Thus, TIC10 exposure led to a significant ($P < 0.05$) and sustained presence of TRAIL on the cell surface of cancer cells.

TIC10 induces TRAIL-mediated apoptosis

TIC10 had broad-spectrum activity against multiple malignancies in vitro (fig. S2A) and induced an increase in sub-G₁ DNA content suggestive of cell death in TRAIL-sensitive HCT116 p53^{-/-} cells, but did not alter the cell cycle profiles of normal fibroblasts at equivalent doses (Fig. 2A). Similarly, TIC10 decreased the clonogenic survival of cancer cell lines and spared normal fibroblasts (Fig. 2, B and D). We also found that TIC10 increased the percentage of sub-G₁ DNA in cancer cells in a p53-independent and Bax-dependent manner, as we previously reported for TRAIL-mediated apoptosis (21) (Fig. 2E). As expected for apoptotic cell death, TIC10 activated caspase-3 (Fig. 2F) and increased sub-G₁ DNA content; this effect was significantly ($P < 0.05$) inhibited by co-incubation with the pan-caspase apoptosis inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) (Fig. 2G). TIC10-induced apoptosis appeared to be specifically mediated by TRAIL, as indicated by inhibition of TIC10-induced cytotoxicity after stable knockdown of TRAIL by short hairpin RNA (shRNA) (Fig. 2H and fig. S2B). Additional evidence for the requirement of TRAIL in TIC10-induced tumor cell death was observed after disruption of the DR5 death domain that modulates proapoptotic TRAIL signaling (Fig. 2I) and through experimental sequestration of TRAIL by use of a blocking antibody (fig. S2C). We also tested the activity of TIC10 on freshly resected colon tumor cells (a mucinous adenocarcinoma resected from an 85-year-old female patient) and found that TIC10 induced TRAIL and had potent cytotoxic effects, unlike 5-fluorouracil (fig. S2, D and E). In addition, we found that the cytotoxic activity of TIC10 was thermally stable, unlike that of TRAIL (Fig. 2J), supporting our central hypothesis. Together, these data demonstrate that TRAIL and TRAIL signaling play a critical role in TIC10-mediated apoptosis.

TIC10 is a TRAIL-dependent antitumor agent in vivo

We found that TIC10 and TRAIL treatment caused tumor regression in the HCT116 p53^{-/-} xenograft to a comparable extent when both are administered as multiple doses (Fig. 3A). Single-dose experiments in HCT116 wild-type (Fig. 3B) and RKO (Fig. 3C) human colon cancer xenograft-bearing mice corroborated the antitumor activity of TIC10. TIC10 also induced regression of MDA-MB-231 human triple-negative breast cancer xenografts, whereas TRAIL-treated tumors progressed; the effect of TIC10 was significantly ($P < 0.005$) inhibited by stable knockdown of TRAIL (Fig. 3D and fig. S3A). It is difficult to directly compare the potency of a small molecule and a biological agent in vivo. Nevertheless, this result directly demonstrated that the antitumor activity of TIC10 is superior to that of TRAIL when administered as single doses under these experimental conditions and acts, at least in part, through TRAIL produced by tumor cells. In DLD-1 colon cancer xenografts, TIC10 induced tumor stasis at 1 week after treatment, whereas TRAIL-treated tumors progressed after a single dose (fig. S3B). A single dose of TIC10 also induced a sustained regression of the SW480 xenograft and was equally effective when delivered by intraperitoneal or oral route, suggesting favorable oral bioavailability for TIC10 (fig. S3C). Titration of a single oral dose of TIC10 in the HCT116 xenograft model revealed sustained antitumor efficacy at 25 mg/kg (Fig. 3E). The lack of apparent toxicity at multiple doses delivered at fourfold above this therapeutic dose in previous xenografts, along with no adverse effects on body weight or liver histology (fig. S3, D to F), suggested that TIC10 has a wide therapeutic window. Exposure to oral TIC10 at 25 mg/kg weekly for 4 weeks in immunocompetent mice did not cause any changes in selected serum chemistry markers (table S1). We applied the same oral dosing schedule to Eμ-myc transgenic mice, which spontaneously develop meta-static lymphoma from weeks 9 to 12 of age, and found that TIC10 significantly ($P = 0.00789$) prolonged the survival of these mice by 4 weeks (Fig. 3F).

We then searched for cooperative combinations of TIC10 with approved chemotherapeutic agents. Among the tested chemotherapies, we observed potential in vitro synergy between TIC10 and the taxanes paclitaxel and docetaxel (fig. S4, A to D). The combination of TIC10 and either of these taxanes cooperated to yield sustained cures for 10 days in the H460 non-small cell lung cancer xenograft (fig. S4, E to H). TIC10 also cooperated with bevacizumab when both were given once a week in a metastatic orthotopic mouse model of p53-deficient colorectal cancer. The combination reduced tumor burden at the primary cecal tumor and decreased spread to distal metastatic sites including the lung, liver, lymph nodes, and peritoneum (fig. S4, I and J). TIC10 alone and in combination with bevacizumab was well tolerated and caused no significant changes in body weight at the endpoint of this multidose regimen (fig. S4K).

TIC10 causes tumor-specific cell death by TRAIL-mediated direct and bystander effects

Immunohistochemical (IHC) analysis of TIC10-treated tumors revealed increased amounts of TRAIL and cleaved caspase-8, the initiator caspase involved in TRAIL-mediated apoptosis. Histological analysis and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining further confirmed that TIC10 induced apoptosis in the treated tumors (Fig. 4A). We also found that TIC10 induces TRAIL

not only in the tumor but also in the stromal fibroblasts bordering the tumor (Fig. 4B). Quantification of TRAIL transcript revealed that TIC10 strongly increased TRAIL gene transcription in tumor xenografts at 48 to 108 hours after a single dose, which far exceeds the serum half-life of recombinant TRAIL (~30 min) (22) and matches the in vitro kinetics of TIC10 (Figs. 1D and 4C). Metabolic analysis of TIC10 in vitro indicated the presence of amine modifications that were not time-dependent (table S2). Metabolic analysis of serum TIC10 isolated from mice revealed a potential metabolite indicative of oxidation and glucuronide conjugation that was evident only at 8 hours after administration (table S3). Pharmacokinetic analysis revealed that TIC10 is quickly distributed and has a plasma half-life of ~6.5 hours (fig. S5 and table S4). Together, this suggests that TIC10 itself has a longer half-life than recombinant TRAIL and that the effects of TIC10 are temporally sustained for days in vivo and in vitro.

IHC analysis of normal tissues in TIC10-treated non-tumor-bearing mice revealed that TRAIL is up-regulated at the protein level in the brain, kidney, and spleen of mice without apparent toxicity as determined by histology (Fig. 4D). TRAIL up-regulation in response to TIC10 was not noted in other tissues including the liver at any time point (Fig. 4D).

We tested the effects of TIC10 on normal fibroblasts and found that TIC10 selectively induced apoptosis in p53-deficient tumor cells but not in normal fibroblasts in coculture experiments (Fig. 4E). TIC10 did induce a significant ($P < 0.05$) though modest amount of TRAIL on the surface of normal fibroblasts (Fig. 4F). Transplanting normal fibroblasts that were pre-incubated with TIC10 into a coculture with p53-deficient colon cancer cells resulted in a modest but significant ($P < 0.05$) increase in TRAIL-specific cell death of the cancer cell subpopulation (Fig. 4G). Together, these data indicate that TIC10 has a favorable therapeutic index and induces TRAIL in both tumor and normal cells, contributing to the antitumor efficacy of TIC10 through direct as well as bystander mechanisms.

TIC10 is an effective antitumor agent against orthotopic human glioblastoma multiforme tumors

The sustained induction of TRAIL in brain tissue suggested that the TIC10 small molecule can cross the intact blood-brain barrier, providing a clear advantage over a large protein such as TRAIL. We explored the possibility that TIC10 may serve as an antitumor agent against brain tumors. We first tested the activity of TIC10 in glioblastoma multiforme (GBM) cell lines and found that TIC10 induced TRAIL (Fig. 5A) and had a p53-independent GI_{50} (concentration that results in 50% growth inhibition) in the low micromolar range that is comparable with other cancer cell lines (Fig. 5B). We also found that TIC10 had substantial cytotoxic effects on freshly resected primary human GBM cells that are temozolomide-resistant and were previously irradiated. The tumor specimen was a grade IV glioblastoma with oligodendroglial component taken from a 38-year-old female patient who had undergone previous cytoreductive surgery and radiation (Fig. 5C). We tested TIC10 in preclinical models of GBM as a mono-agent and in combination with bevacizumab on the basis of its favorable combination in the intracerebral xenograft model (fig. S4, I to K). TIC10 exerted p53-independent cytotoxicity against a panel of GBM cell lines, including temozolomide-resistant GBM cell lines such as T98G, and induced a sustained regression of

subcutaneous T98G xeno-grafts to an extent similar to that of bevacizumab when given as a single oral dose (Fig. 5D). Furthermore, we found that a single dose of TIC10 doubled the overall survival of mice as a monoagent in an aggressive intracranial xenograft of human GBM with the SF767 cell line. It also cooperated with bevacizumab to triple the duration of survival of such brain tumor-bearing mice (Fig. 5, E and F, and table S5).

TIC10-induced TRAIL up-regulation is Foxo3a-dependent

We investigated gene expression profiles in TIC10-treated HCT116 p53^{-/-} cells, revealing transcriptional changes in target genes of the FOXO family of transcription factors (Fig. 6A). This family includes Foxo3a, which has been previously shown to regulate the TRAIL gene promoter (23). We found that the FOXO target gene TRAIL receptor DR5 is up-regulated by TIC10 in tumors and several cancer cell lines and, to a much lesser extent, in normal cells (fig. S6, A to C). Regulation of FOXO family members is often achieved by changes in sub-cellular localization, such that transcriptional activity is correlated with nuclear localization. We found that among FOXO family members, Foxo3a (but not Foxo1a) undergoes a nuclear translocation in response to TIC10 (Fig. 6, B and C, and fig. S6, D and E). This is accompanied by a dose-dependent increase in the amount of Foxo3a localized to the TRAIL promoter as shown by a chromatin immunoprecipitation (ChIP) assay (Fig. 6D). Transient knockdown of Foxo3a and Foxo1 revealed that Foxo3a specifically mediates TIC10-induced TRAIL up-regulation (Fig. 6E). Stable knockdown of Foxo3a significantly inhibited TIC10-induced up-regulation of TRAIL production and subsequent tumor cell death (Fig. 6F and fig. S6F). Furthermore, overexpression of a plasmid encoding for a mutant Foxo3a lacking its DNA binding domain blunted the transcriptional induction of TRAIL in response to TIC10 (fig. S6G). Stable knockdown of Foxo3a in tumor cells also significantly ($P < 0.05$) inhibited the antitumor activity of TIC10 and TIC10-induced hallmarks of TRAIL-mediated apoptosis in tumors in vivo (Fig. 6, G and H).

Dual inactivation of Akt and ERK by TIC10 cooperatively induces TRAIL

We explored TIC10-induced changes in previously described regulators of Foxo3a, such as IKK (24, 25), Akt (26), and ERK (20, 27) (Fig. 7A). We found that both pAkt and pERK are down-regulated by TIC10 treatment in a dose-dependent manner. This decrease in pAkt and pERK is accompanied by dephosphorylation of the sites they phosphorylate on Foxo3a. TIC10 also caused a down-regulation of the total expression of ERK (fig. S7A). We found that ERK mRNA was unaffected, whereas the protein stability of ERK decreased in response to TIC10 (fig. S7, B and C). The inhibition of Akt and ERK activity is indirect, as confirmed by in vitro kinase activity assays (fig. S7D). We found that Akt myristoylation can counteract the cytotoxic response to TIC10, including TRAIL up-regulation and the nuclear translation of Foxo3a, and that overactivating Akt can suppress even basal expression of TRAIL (fig. S7, E to G). A time-course analysis revealed that TIC10-induced inactivation of Akt and ERK occurs after 48 hours, in concert with the kinetics of dephosphorylation of Foxo3a and TRAIL up-regulation (Fig. 7B and fig. S7H). These effects were also observed in TIC10-treated xenograft tumors in vivo (Fig. 7C) and in several human cancer cell lines of different tumor types with diverse genetic alterations (Fig. 7D).

We hypothesized that dual inhibition of the phosphoinositide 3-kinase/Akt and the mitogen-activated protein kinase (MAPK) pathways will cooperatively lead to the nuclear translocation of Foxo3a and ensuing TRAIL up-regulation. We found that A6730 and U0126 monoethanolate, which are inhibitors of Akt1/2 (28) and MEK (mitogen-activated or extracellular signal-regulated protein kinase kinase) (29), respectively, cooperatively induce Foxo3a-dependent TRAIL up-regulation and lead to TRAIL-mediated cell death (Fig. 7E and fig. S7, I and K). These observations were corroborated by small interfering RNA (siRNA) experiments, revealing that ERK and Akt can be inhibited to cooperatively up-regulate TRAIL gene expression (Fig. 7, F and G, and fig. S7L).

We next examined the effects of TIC10 on the MAPK signaling pathway. TIC10 does not affect the phosphorylation or expression of epidermal growth factor receptor (EGFR), B-Raf, or C-Raf (Fig. 7, H and I, and fig. S8, A to D). However, the phosphorylation of MEK and ERK is abolished in response to TIC10 even when the drug is washed out, unlike the EGFR inhibitor gefitinib. The dephosphorylation of MEK and ERK in response to TIC10 was not affected by treatment with okadaic acid, which inhibits phosphatases (fig. S8E). These findings suggest that TIC10 indirectly inhibits the MAPK signaling pathway at the level of MEK and its downstream targets. Together, these data indicate that TIC10 causes a dual inactivation of Akt and ERK, which leads to the translocation of their mutual substrate Foxo3a into the nucleus, where it transcriptionally induces the TRAIL gene to activate cell death and antitumor effects in vivo (Fig. 8).

DISCUSSION

Our findings demonstrate that TIC10 is a safe and orally active anti-tumor agent that has potent cancer-specific cytotoxicity through sustained stimulation of the endogenous TRAIL tumor suppressor by normal and tumor tissues, including the brain. TIC10 induces TRAIL in a Foxo3a-dependent manner, which also up-regulates TRAIL death receptor DR5 among other targets, potentially allowing for sensitization of some TRAIL-resistant tumor cells. Our observations demonstrate that pharmacological activation of Foxo3a is a powerful antitumor strategy that requires TRAIL as a proapoptotic effector target gene, and that Foxo3a activation through dual inhibition of Akt and ERK is achievable by the single small-molecule TIC10. The induction of TRAIL caused by TIC10 is sustained in both tumor and host cells, and in vitro evidence suggests that these normal host cells contribute to TIC10-induced cancer cell death through a bystander effect.

TIC10 has broad-spectrum activity that we demonstrated in primary patient samples and cell lines resistant to conventional therapies. This bodes well for the clinical utility of TIC10, as its activity does not rely exclusively on molecules that are commonly altered in cancer, such as EGFR, Her2, KRAS, p53, or PTEN. This is in agreement with a previous study describing the utility of Foxo3a activation as an anti-cancer mechanism that targets cancer cells that are resistant to therapies inhibiting upstream regulators of MAPK signaling (30). The elucidation of the mechanism of TIC10 yields important information for the clinical translation of this molecule, as it allows for the identification of resistance mechanisms such as overactivated Akt, which we demonstrate directly. Additionally, it suggests that pERK,

pAkt, Foxo3a localization and phosphorylation, as well as cell surface and serum TRAIL can be tracked to monitor the effectiveness of TIC10 in clinical trials.

The use of small molecules to induce an endogenous gene in lieu of administering a recombinant protein is a promising therapeutic approach. Our study demonstrates that limitations of a therapeutic recombinant protein such as TRAIL, including biodistribution and pharmacokinetic properties, can be effectively overcome through the use of a small molecule. Furthermore, a secreted protein such as TRAIL may be a particularly well-suited target for such a strategy because normal cells at sites outside the tumor can produce soluble or cell-bound TRAIL protein that may contribute to the therapeutic response via a bystander mechanism. TRAIL-mediated bystander effects with normal cells have previously been reported in response to radiation and may represent a promising therapeutic opportunity to augment efficacy (31). This strategy has the potential to produce large quantities of antitumor proteins as the trillions of cells in the human body universally harbor the potential to produce endogenous proteins in a temporally sustained fashion.

The bystander protein production strategy must be used carefully because normal cells must maintain their normal function and the target protein must be nontoxic to the normal cells, as is the case for TRAIL (32). Additionally, the pleiotropic effects of the small molecule must also be considered because they have the potential to cause toxicity or increase efficacy through cooperative mechanisms, such as the concomitant DR5 up-regulation observed in tumor cells in response to TIC10.

One limitation of this study is that although we demonstrate that TIC10 requires TRAIL for its apoptotic activity, other targets of Akt, MEK, and ERK may contribute to its substantial antitumor activity. Previous reports have described several FOXO-induced changes in genes that mediate TRAIL sensitivity, including DR5 up-regulation as well as down-regulation of FLIP (33, 34), although we did not observe the latter in our gene expression profiling of changes in response to TIC10. Thus, the increased spectrum of activity and antitumor effects gained with TIC10 relative to TRAIL treatment cannot be exclusively ascribed to the induction of only the TRAIL gene by TIC10. More studies will be needed to further elucidate the mechanism of action of TIC10, including its direct binding target. Medicinal chemistry with TIC10 may be explored to clarify structure-activity relationships and to identify a more potent compound that maintains the wide therapeutic window of TIC10. Another limitation of this study is that while the safety of TIC10 was evaluated in vitro and in vivo, the identification of adverse events and a complete safety study in nonrodent species will need to be executed before clinical testing. Finally, the efficacy comparisons between TRAIL and TIC10 in this study are confounded by difficulties associated with comparing the potency of biological agents and small molecules. Whereas the preclinical activity of TIC10 is promising and outperforms TRAIL in some preclinical studies, controlled comparisons of clinical activity are needed to truly assess the superiority of the compound.

Small molecules have targeted other members of the FOXO family, for example, to induce the nuclear translocation of Foxo1 (35). The identification of Foxo3a as the transcription factor and TRAIL as the apoptotic effector for TIC10-induced therapeutic effects has implications that extend beyond the small molecule itself. Our observations strongly argue

that TRAIL plays an essential role in the apoptotic response induced by Foxo3a, which is a promising drug target and accessible cellular mechanism for inducing the TRAIL gene. The conservation of the Foxo3a-dependent mechanism of TIC10 in the presence of diverse upstream oncogenic alterations holds promising therapeutic potential. The Akt- and ERK-dependent mechanism of TIC10 is in full agreement with a recent report supporting that the dual inactivation of Akt and MAPK signaling pathways in mutant KRAS cancer cells is a potent and cooperative strategy (36). Our observations suggest that the Foxo3a/TRAIL axis may play a critical role in apoptosis associated with dual inhibition of Akt and ERK, and that the combination of approved antitumor agents targeting indirectly these two pathways should be explored in an effort to gain Foxo3a/TRAIL-dependent activity. Our findings also highlight TRAIL as an important target of Foxo3a and are in congruence with a recent report arguing that Foxo3a-dependent TRAIL is responsible for HIV-induced apoptosis of memory B cells (37). The observation that Foxo3a rather than Foxo1, which has been previously targeted by small molecules (35), specifically regulates the TIC10-induced response lends credence to the notion that different members of the FOXO family of transcription factors play distinct roles.

The induction of TRAIL by histone deacetylase inhibitors such as vorinostat has been reported (38). In addition, there are several other TRAIL-based therapies that are being investigated, such as the TRAIL receptor agonist antibodies lexatumumab and mapatumumab, proteins engineered to mimic the proapoptotic effects of TRAIL on the death receptors (13), and adenovirus-delivered TRAIL (39). We posit that the mechanism of TIC10, its spectrum of activity, and drug properties make TIC10 a promising alternative with some advantages. The requirement of TRAIL for activity, bystander effects, bio-availability, biodistribution, stability, and concomitant up-regulation of DR5 by TIC10 cumulatively position TIC10 as an attractive therapeutic approach aimed at using the apoptotic potential of TRAIL with demonstrable advantages over currently available TRAIL pathway-based therapies.

TIC10 is an anticancer therapy that provides an excellent opportunity for modulation of host antitumor responses and the pharma-cokinetic properties of an endogenous antitumor protein. Moreover, the pharmacological induction of TRAIL in the brain demonstrates a therapeutic approach in brain malignancies otherwise refractory to current therapies and an alternative solution for the general limitations of exogenous protein delivery. TIC10 offers an effective cancer therapeutic strategy that uses both normal and tumor cells to endogenously produce an antitumor agent via conserved signaling. The modulation of pharmacokinetic properties of endogenous TRAIL as a tumor-suppressive agent by a small molecule such as TIC10 suggests that exploration of pharmacological induction/ pharmacokinetic tuning of other endogenous antitumor proteins is feasible and warranted. The molecular mechanism of TIC10 also suggests that the antitumor effects of Foxo3a and the TRAIL receptor pathway can be harnessed through the dual inhibition of Akt and ERK, which should be explored with targeted agents in clinical development.

MATERIALS AND METHODS

Reagents and cell-based assays

All cell lines were obtained from the American Type Culture Collection, except for the HCT116 Bax^{-/-} and HCT116 p53^{-/-} cells (gifts from B. Vogelstein, Johns Hopkins University) and GBM cell lines (provided by A. Mintz, Wake Forest University). Lentiviral infection was performed with MDA-MB-231 cells and TRAIL shRNA or vector and HCT116 with Foxo3a shRNA or vector purchased from Sigma-Aldrich. H460 DR5 DD-EGFP cells were constructed with complementary DNA coding for a DR5 fragment without a death domain by inserting amino acids 1 to 298 of the human DR5 gene into the pEGFP-N1 vector to express a DR5(1–298) fusion protein. The fusion construct was transfected into H460 cells with Lipofectamine 2000 (Invitrogen) and selected with G418. Positive clones were verified by fluorescence microscopy and Western blot analysis. TIC10 (NSC350625) was obtained from the NCI Developmental Therapeutic Program. A6730 and U0126 monoethanolate were obtained from Sigma. Purified, recombinant TRAIL was produced as previously described (40). The RIK-2 antibody (Santa Cruz Biotechnology) was used at 1 µg/ml and zVAD-fmk (Promega) was used at 20 µM.

Flow cytometry and cell death assays

Floating and adherent cells were analyzed on a Coulter-Beckman Elite Epics cytometer. For surface TRAIL experiments, adherent cells were harvested by brief trypsinization, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, incubated with an anti-TRAIL antibody (Abcam, ab2435) at 1:250 overnight, washed and incubated with anti-rabbit Alexa Fluor 488 (Invitrogen) for 30 min, and analyzed. Cells were gated on forward and side scatter to eliminate debris and dead cells from the analysis. Surface TRAIL data are expressed as median fluorescence intensity relative to that of control samples unless indicated otherwise. Surface DR5 was analyzed similarly with an antibody from Imgenex. For sub-G₁ content and cell cycle profile analysis, all cells were pelleted and ethanol-fixed, followed by staining with propidium iodide (Sigma) in the presence of RNase. Cell viability assays were carried out in 96-well black-walled clear-bottom plates with CellTiter-Glo (Promega) according to the manufacturer's protocols.

Colony formation assays

The indicated cell lines were plated at 500 cells per well and allowed to adhere, and then treated the next day in fresh complete medium. At 3 days after treatment, the medium was replaced with drug-free medium and cells were propagated for 10 days, with fresh medium given once every 3 days. At the end of the 10-day period, cells were washed in PBS, fixed with methanol, stained with Coomassie blue, rinsed, and dried for quantification.

Western blot analysis

Western blot analysis was conducted as previously described (41) with NuPAGE 4 to 12% bis-tris gel and visualized with SuperSignal West Femto (Thermo Scientific) and x-ray film. Densitometry was performed with NIH ImageJ. Nuclear and cytoplasmic extracts were prepared with a cytoplasmic lysis buffer (10 mM Hepes, 10 mM KCl, 2 mM MgCl₂, 1 mM

dithiothreitol) followed by a nuclear lysis buffer (20 mM Hepes, 420 mM NaCl, 1.5 mM MgCl₂, 250 μM EDTA, 25% glycerol). For all lysis buffers, fresh protease inhibitor (Roche) and 1 mM sodium orthovanadate were added immediately before use.

Primary specimens

All primary patient specimens were obtained in accordance with the Institutional Review Board at Penn State Hershey Medical Center under approved protocols. Samples were received immediately after resection, manually digested in complete Dulbecco's modified Eagle's medium (DMEM), filtered with a 100-μm nylon mesh, and plated at 2×10^5 cells/ml in complete DMEM for the described experiments.

In vivo studies

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the Pennsylvania State University College of Medicine. For subcutaneous xenografts, 4- to 6-week-old female athymic nu/nu mice (Charles River Laboratories) were inoculated with 1×10^6 cells (2.5×10^6 for T98G) of the indicated cell lines in each rear flank as a 200-μl suspension of 1:1 Matrigel (BD)/PBS. All subcutaneous tumors were allowed to establish for 1 to 4 weeks after injection until reaching a volume of ~125 mm³ before treatment initiation. Other experimental details are outlined in Supplementary Materials.

ChIP assays

ChIP assays were carried out as previously described for the TRAIL promoter (38) with a ChIP-grade antibody for Foxo3a (Abcam) or an equivalent concentration of rabbit immunoglobulin G (SouthernBiotech) as a nonspecific control.

Statistical analyses

For pairwise comparisons, we analyzed the data using Student's two-tailed *t* test in Excel (Microsoft). Log-rank statistical analysis was performed with a Web-based script that interfaces with the statistical package R (<http://bioinf.wehi.edu.au/software/russell/logrank/>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

TIC10 is a small molecule that induces TRAIL independent of p53. **(A)** Chemical structure of TIC10. **(B)** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of TRAIL mRNA concentrations in HCT116 p53^{-/-} cells (48 hours, $n = 4$). **(C)** Surface TRAIL induced by TIC10 in a panel of cancer cells (10 μM, 72 hours, $n = 3$). **(D)** Surface TRAIL in HCT116 p53^{-/-} cells after TIC10 treatment at the indicated conditions and time points after treatment ($n = 3$). **(E)** HCT116 p53^{-/-} TRAIL surface levels measured by flow cytometry at 72 hours after TIC10 treatment initiation (5 μM, $n = 3$). Cells were treated with TIC10 or dimethyl sulfoxide (DMSO) control for the indicated time of preincubation and then changed to a drug-free medium for the remaining period until analysis at 72 hours. Error bars indicate SD of replicates. * $P < 0.05$ between the indicated condition and controls.

Fig. 2.

TIC10 induces TRAIL-mediated apoptosis in vitro. (A) Cell cycle profiles of HCT116 p53^{-/-} and HFF cells treated with TIC10 (5 μM, 72 hours, $n = 3$). (B and C) Representative wells (B) and quantification of colony formation assays (C) of cancer cells per well with or without TIC10 (10 μM, 72 hours, $n = 3$). (D) Parallel experiments as in (C) but with HFF cells that were counted at the 72-hour endpoint ($n = 3$). (E) Percentage of sub-G₁ DNA (fragmented DNA indicative of cells undergoing apoptosis) in HCT116 wild-type (WT), p53^{-/-}, and Bax^{-/-} cells after treatment with DMSO, TIC10 (1, 5, or 10 μM), or rhTRAIL (25 ng/ml) (72 hours, $n = 3$). (F) Cleaved caspase-3 (CC3) in HCT116 p53^{-/-} cells assayed by immunofluorescence (left panel, 5 μM TIC10, caspase-3 shown in green) or Western blot analysis (right panel) treated with DMSO or TIC10 (1, 2.5, 5, and 10 μM) for 72 hours. (G) Percentage of sub-G₁ DNA in TIC10-treated cancer cells with or without preincubation with zVAD-fmk (10 μM, 72 hours, $n = 3$). (H) Percentage of sub-G₁ DNA in MDA-MB-231 cells with stable knockdown of TRAIL by shRNA treated with TIC10 (1, 5, and 10 μM) for 72 hours ($n = 3$). (I) Percentage of sub-G₁ DNA in H460 cells with endogenous DR5 or overexpressing a DR5 construct (DR5 DD) with its death domain replaced by enhanced green fluorescent protein (EGFP) (10 μM TIC10, 72 hours, $n = 3$). (J) Ability of TIC10 (5 μM) or TRAIL (25 ng/ml) to reduce cell viability in HCT116 cells after a 1-hour preincubation at the indicated temperatures (72 hours, $n = 3$). Error bars indicate SD of replicates. * $P < 0.05$ compared to control unless otherwise indicated.

Fig. 3.

TIC10 is a TRAIL-dependent antitumor agent in vivo. (A) Relative tumor size in mice bearing a HCT116 p53^{-/-} xenograft and treated with three doses of TIC10 (intraperitoneal), TRAIL (intravenous), or vehicle (intraperitoneal) administered on days 0, 3, and 6 as indicated by gray vertical bars ($n = 10$). (B) Bioluminescence imaging of luciferase-expressing HCT116 p53^{-/-} xenografts that received a single intraperitoneal injection of TIC10 or vehicle ($n = 6$). (C) Relative tumor size in RKO xenografts treated with a single dose of TIC10 (intraperitoneal), TRAIL (intravenous), or vehicle (intraperitoneal; $n = 10$). Right panel shows near-infrared images of sample mice from each cohort on day 13 after treatment and 3 days after injection with AngioSense 680. (D) Box and whisker plot of tumor volume on day 9 after treatment initiation in MDA-MB-231 xenografts expressing vector or shTRAIL and treated with single doses of TIC10 (intraperitoneal), TRAIL (intravenous), or vehicle (DMSO, intraperitoneal) ($n = 8$). (E) TIC10 or vehicle administered as a single oral dose in the HCT116 xenograft ($n = 6$). (F) Overall survival of Eμ-myc mice treated during weeks 9 to 12 with weekly single oral dose of TIC10 (25 mg/kg). Right panel shows hematoxylin and eosin (H&E) staining of axillary lymph nodes from Eμ-myc and WT C57/B6 mice at 14 weeks of age. P value was determined by log-rank test. For relative tumor volume plots, tumor size is expressed relative to the tumor size on day 0, which is defined as the day of treatment initiation. Error bars indicate SD of replicates. * $P < 0.05$, ** $P < 0.005$, compared to control unless otherwise indicated.

Fig. 4.

TIC10 induces TRAIL in tumor and normal cells. **(A)** H&E and IHC analysis of HCT116 p53^{-/-} xenograft tumors at 2 and 7 days after a single dose of TIC10 on day 0 [100 mg/kg, in-traperitoneally (ip)]. **(B)** H&E and IHC analysis for TRAIL at the border between tumor and stromal fibroblasts from HCT116 p53^{-/-} xenograft tumors after treatment with TIC10 (100 mg/kg, ip) or vehicle on day 2 after treatment. The yellow dashed lines indicate stromal fibroblasts, and the black dashed lines indicate the tumor. **(C)** qRT-PCR analysis of TRAIL transcript in HCT116 p53^{-/-} xenograft tumors in athymic nude mice after a single dose of DMSO or TIC10 [25 mg/kg, intravenously (iv)] (*n* = 4). **(D)** Histological and TRAIL IHC analysis of normal tissue in athymic nude mice after TIC10 administration on day 0 (100 mg/kg, iv). **(E)** Coculture of HCT116 p53^{-/-} and HFF cells labeled as red and green, respectively. TIC10 (10 μM)- or DMSO-treated wells are shown immediately before

treatment or 3 days after treatment. The bottom two panels were taken at the 3-day endpoint after being counterstained with Hoechst. **(F)** Surface TRAIL analysis of HFF cells after TIC10 treatment (0, 2.5, 5, or 10 μM from left to right) (72 hours, $n = 3$). Surface TRAIL is shown relative to cells that were not treated with TIC10. **(G)** Percentage of sub-G₁ DNA in a coculture of HCT116 p53^{-/-} cells and pretreated HFFs (24 hours, $n = 3$). HFF pretreatment consisted of a 72-hour incubation with TIC10 (10 μM) or DMSO. Coculture was in the presence or absence of the TRAIL-blocking antibody RIK-2. Scale bars, 100 μm . Error bars indicate SD of replicates. * $P < 0.05$, compared to control unless otherwise indicated.

Fig. 5.

TIC10 is effective as an antitumor agent in GBM. **(A)** Surface TRAIL in GBM cell lines after incubation with TIC10 (5 μ M, 72 hours, n = 3). **(B)** GI_{50} values extrapolated from cell viability assays of the indicated GBM cell lines at 72 hours after treatment with TIC10 or DMSO (n = 3). **(C)** Cell viability assay of freshly resected glioblastoma tissue treated with DMSO, TIC10, or temozolomide (TMZ, 10 μ M) (72 hours, n = 3). **(D)** Relative tumor size (compared to day 0) in a subcutaneous xenograft of T98G in mice treated with a single dose of vehicle, TIC10 (30 mg/kg, orally), or bevacizumab (10 mg/kg, iv) on day 0 (n = 8). **(E)** Overall survival of mice harboring SF767 intracranial tumors treated with a single oral dose of vehicle (n = 8), TIC10 (25 mg/kg, n = 7), bevacizumab (bev) (10 mg/kg, iv, n = 6), or TIC10 and bevacizumab (n = 7) at 2 weeks after implantation. **(F)** Bioluminescence imaging of a sample control mouse and a TIC10-treated mouse bearing an intracranial xenograft of SF767 glioblastoma. The bioluminescence scale bar on the right applies to all images in the panel. * P < 0.05 between the indicated condition and control.

Fig. 6.

TIC10-induced TRAIL and cytotoxicity are Foxo3a-dependent. **(A)** Transcriptional changes associated with FOXO signaling from gene expression profiling of HCT116 p53^{-/-} cells at 48 hours after TIC10 treatment (10 μM) normalized to DMSO-treated cells (n = 3). *P* < 0.05 between DMSO and TIC10 treatment groups for all of these changes. **(B)** Immunofluorescence of Foxo3a in HCT116 cells with and without TIC10 treatment (48 hours, 10 μM). **(C)** Western blot analysis of whole-cell lysates (W) and cytoplasmic (C) and nuclear (N) extracts from HCT116 cells treated with DMSO or TIC10 (48 hours, 10 μM). β-Actin and lamin B1 are shown as cytoplasmic and nuclear loading controls, respectively. **(D)** ChIP assay for TIC10-induced translocation of Foxo3a to the TRAIL promoter at 48 hours after TIC10 treatment in HCT116 p53^{-/-} cells (0, 2.5, 5, or 10 μM from left to right). **(E)** Flow cy-tometric analysis of cell surface TRAIL induced by TIC10 (10 μM) with or without transient knockdown of Foxo1 and/or Foxo3a in HCT116 p53^{-/-} cells with siRNA (72 hours, n = 3). The cell surface TRAIL ratio refers to the amount of surface TRAIL in treated cells relative to that in cells that were not treated with TIC10. Confirmation of knockdown is shown by Western blot analysis (right). **(F)** Percentage of sub-G₁ DNA in HCT116 cells with or without stable knockdown of Foxo3a and with or without TIC10 treatment (10 μM, 72 hours, n = 3). **(G)** Tumor volume of HCT116 xenograft with or without stable knockdown of Foxo3a by shRNA after a single oral dose of vehicle or TIC10 (25 mg/kg) on day 0 (n = 10). **(H)** IHC analysis and TUNEL staining of HCT116 tumors with or without stable knockdown of Foxo3a 3 days after a single dose of TIC10 (25 mg/kg, orally). Scale bars, 100 μm. Error bars indicate SD of replicates. **P* < 0.05, compared to control unless otherwise indicated.

Fig. 7.

TIC10 inactivates Akt and ERK to induce TRAIL through Foxo3a. **(A)** Western blot analysis of HCT116 p53^{-/-} cells treated with TIC10 (2.5, 5, and 10 μM) for 72 hours. **(B)** Time course of TIC10-induced effects determined by den-sitometry of Western blot analysis of HCT116 p53^{-/-} cells treated with TIC10 (5 μM) or DMSO ($n = 3$). Data are expressed relative to the control sample for each time point and normalized to Ran as a loading control. TRAIL was quantified by flow cytometry as a parallel experiment ($n = 3$). **(C)** Western blot analysis of HCT116 p53^{-/-} xenograft tumors in athymic nude mice after a single dose of DMSO or TIC10 (25 mg/kg, iv) **(D)** Western blot analysis of TIC10-induced effects on Foxo3a in DLD-1 human colon cancer, MDA-MB-468 human breast cancer, and T98G human GBM cell lines (10 μM, 72 hours). **(E)** Flow cytometric analysis of TRAIL in HCT116 p53^{-/-} cells after incubation with 10 μM A6730 (Akt inhibitor), U0126 monoethanolate (MEK inhibitor), or both (48 hours), with or without stable knockdown of Foxo3a ($n = 3$). **(F)** qRT-PCR analysis of TRAIL mRNA at 48 hours after transient knockdown of Akt and/or ERK in HCT116 p53^{-/-} cells ($n = 3$). For siERK and siAkt combination, $P < 0.05$ compared to all other conditions. **(G)** Confirmation of Akt and ERK knockdown by Western blot analysis. **(H)** Western blot analysis of B-Raf/MEK/ERK signaling in HCT116 cells treated with TIC10 at the indicated concentrations (48 hours). **(I)** Western blot analysis of Raf expression and phosphorylation in HCT116 cells treated with TIC10 at the indicated concentrations (48 hours). Error bars indicate SD of replicates. * $P < 0.05$, compared to control unless otherwise indicated.

Fig. 8. TIC10 up-regulates TRAIL through inhibition of Akt and MEK/ERK and activation of Foxo3a. TIC10 causes inactivation of the prosurvival kinases MEK, ERK, and Akt. ERK and Akt normally phosphorylate Foxo3a at S²⁵³ and S²⁹⁴, respectively. These phosphorylation events create docking sites for 14-3-3 proteins that bind Foxo3a and sequester it in the cytoplasm, thereby inhibiting its activity as a transcription factor. Through its actions on Akt and ERK, TIC10 inhibits Foxo3a phosphorylation, which allows Foxo3a to translocate to the nucleus and bind to the TRAIL promoter that harbors a FOXO binding site. This binding stimulates TRAIL gene transcription and translation, increasing the amount of TRAIL on the cell surface.