The New Antitumor Drug ABTL0812 Inhibits the Akt/mTORC1 Axis by Upregulating Tribbles-3 Pseudokinase

Tatiana Erazo1, Mar Lorente2,3, Anna López-Plana4, Pau Muñoz-Guardiola1,5, Patricia Fernández-Nogueira4, José A. García-Martínez2, Paloma Bragado4, Gemma Fuster4, María Salazar2, Jordi Espadaler5, Javier Hernández-Losa6, Jose Ramon Bayascas7, Marc Cortal5, Laura Vidal8, Pedro Gascón4,8, Maríana Gómez-Ferreria5, José Alfón5, Guillermo Velasco2,3, Carles Domènech5, and Jose M. Lizcano1

Abstract

Purpose: ABTL0812 is a novel first-in-class, small molecule which showed antiproliferative effect on tumor cells in phenotypic assays. Here we describe the mechanism of action of this antitumor drug, which is currently in clinical development.

Experimental Design: We investigated the effect of ABTL0812 on cancer cell death, proliferation, and modulation of intracellular signaling pathways, using human lung (A549) and pancreatic (MiaPaCa-2) cancer cells and tumor xenografts. To identify cellular targets, we performed in silico high-throughput screening comparing ABTL0812 chemical structure against ChEMBL15 database.

Results: ABTL0812 inhibited Akt/mTORC1 axis, resulting in impaired cancer cell proliferation and autophagy-mediated cell death. In silico screening led us to identify PPARs, PPARα and PPARγ as the cellular targets of ABTL0812. We showed that ABTL0812 activates both PPAR receptors, resulting in upregulation of Tribbles-3 pseudokinase (TRIB3) gene expression. Upregulated TRIB3 binds cellular Akt, preventing its activation by upstream kinases, resulting in Akt inhibition and suppression of the Akt/mTORC1 axis. Pharmacologic inhibition of PPARα/γ or TRIB3 silencing prevented ABTL0812-induced cell death. ABTL0812 treatment induced Akt inhibition in cancer cells, tumor xenografts, and peripheral blood mononuclear cells from patients enrolled in phase I/II first-in-human clinical trial.

Conclusions: ABTL0812 has a unique and novel mechanism of action, that defines a new and drugable cellular route that links PPARs to Akt/mTORC1 axis, where TRIB3 pseudokinase plays a central role. Activation of this route (PPARα/γ-TRIB3-Akt-mTORC1) leads to autophagy-mediated cancer cell death. Given the low toxicity and high tolerability of ABTL0812, our results support further development of ABTL0812 as a promising anticancer therapy. Clin Cancer Res; 22(10); 2508–19. ©2015 AACR.

Introduction

The PI3K/AKT/mTORC1 (mTOR complex-1) signaling pathway drives signals from ligand-stimulated receptor tyrosine kinases (RTK) to proteins that control cell metabolism, growth, size, survival, and angiogenesis (1). In response to growth or survival factors, phosphorylated RTKs activate PI3K, which generates the phosphatidylinositol 3,4,5-trisphosphate (PIP3) second messenger and allows the recruitment of protein kinase Akt to the cellular membrane. Akt binds PIP3 through its PH domain, resulting in a conformational change that enables its activation through phosphorylation of two critical residues: Thr308 at the T-loop of the kinase domain by the phosphoinositide-dependent protein kinase-1 (PDK1), and Ser473 at the C-terminal hydrophobic motif by mTOR complex-2 (mTORC2; refs. 2, 3). Active Akt phosphorylates a plethora of substrates that regulate cell survival and metabolism, and also cell growth through subsequent activation of mTORC1. Akt phosphorylation of PRAS40 and TSC2 leads to activation of mTORC1, which promotes protein translation and synthesis by phosphorylating ribosomal S6-kinase (S6K) and the elongation factor 4EBP-1 (4).

Human cancer is very often associated with activation of the PI3K/Akt/mTORC1 pathway, mainly due to overexpression or
Translational Relevance

Hyperactivation of the Akt/mTORC1 axis is commonly observed in many human cancers and blocking this pathway is an important anticancer strategy. Inhibition of Akt is a valid target for anticancer therapy and several AKT inhibitors are under clinical development. We present ABTL0812, a first-in-class small molecule with a unique mechanism of action. In cells and tumor xenografts models, ABTL0812 induces upregulation of TRIB3 pseudokinase. TRIB3 binds Akt, preventing its activation by upstream kinases and resulting in an effective inhibition of the Akt/mTORC1 axis and in autophagy-mediated cancer cell death. ABTL0812 is currently in phase I/II first-in-human clinical trial in patients with advance solid tumors. Preliminary results show that ABTL0812 also induces Akt inhibition in humans. These evidences, together with a very low toxicity and high tolerability, support further development of ABTL0812.

activating mutations of RTKs and PI3K, to deletions/mutations of the PIP3 phosphatase PTEN and to amplification of Akt (3, 5). These types of tumors are hypersensitive to inhibition of each of the components of the PI3K/Akt/mTORC1 axis, and therefore major efforts have been taken to develop inhibitors of this signaling pathway (6). Akt acts as a central node of this pathway, and hyperactivated Akt is a common feature observed in human solid tumors (3, 5). Therefore, Akt has been proposed as an interesting target in cancer therapy. Two classes of Akt inhibitors are now in clinical development: ATP-competitive inhibitors (such as GSK690693) which target active Akt, and allosteric inhibitors (such as MK-2206 and perifosine, currently in phase II and phase III clinical trials, respectively) that by binding the PH domain of Akt prevent its activation by upstream kinases (3, 7).

Here we present ABTL0812, a first-in-class molecule with antitumor activity which is currently in phase I/II clinical trials in patients with advanced solid tumors. We provide evidences showing that ABTL0812 inhibits Akt phosphorylation by upregulating the expression of TRIB3, a pseudokinase that binds and inhibits Akt, leading to mTORC1 inhibition and autophagy-mediated cell death.

Methods

Cell culture, viability assay, transfection, and lysis

Human cancer cell lines were purchased from ATCC (2008; ATCC-authorization by isoenzymes analysis). Atg5-/- and Atg5+/+ T-large antigen–transformed MEFs were donated by Dr. Mizushima (Tokyo Medical University, Tokyo, Japan). TRB3+/+ and TRB3-/- RasV12/E1A–transformed Mouse embryonic fibroblasts (MEF) have been described before (8). Cell lines were cultured as recommended and transfected using Lipofectamine-2000. Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium Blue) reduction assay (9). Cells were lysed in ice-cold RIPA buffer supplemented with 1 mmol/L sodium-orthovanadate, 50 mmol/L NaF, and 5 mmol/L sodium-pyrophosphate, sonicated and stored at –20°C.

Immunoblotting and immunoprecipitation

Immunoblot analyses were performed following standard procedures as described (10) and antibodies listed in the Supplementary Table S1. Akt was immunoprecipitated following the protocol previously described (11).

Transmission electron microscopy

Cells treated with ABTL0812 or vehicle (ethanol) were processed as described before (12). Pellets were embedded in Eponate-12TM resin (Ted Pella). Ultrathin (70-nm thick) sections were contrasted with uranyl-acetate and lead citrate solutions, and visualized in transmission electron microscope (Jeol JEM-1400) equipped with a CCD- GATAN-ES1000W Erlangshen camera.

Xenograft models

For A549 model, athymic female nude mice (n = 5 per group) were injected subcutaneously with 5 × 10⁶ cells in each flank. When tumors reached 80 to 100 mm³, mice were randomly distributed into treatment groups and administered the corresponding treatments. ABTL0812 was administered by oral gavage at 120 mg/kg every day. Docetaxel was administered by intraperitoneal route at 15 mg/kg once a week. MiaPaCa-2 cells were injected in athymic male mice (n = 5 per group) with 10 × 10⁶ cells in one flank. When tumors reached 80 to 160 mm³, mice were randomly distributed and treated with vehicle or ABTL0812 120 mg/kg by oral gavage five times per week. Tumor volumes were measured as (length × width²)/2, twice a week. All procedures involving animal were performed with the approval of the Hospital Clinic Animal Experimentation Committee, according to Spanish official regulations.

Pharmacokinetics of ABTL0812 in mice and rats

ABTL0812 was orally (gavage, 100 mg/kg) and intravenously (10 mg/kg) administered to male CD-1 mice. ABTL0812 was quantified in plasma by validated LC/MS-MS methods and pharmacokinetic parameters were calculated with WinNonlin software.

Clinical trial and isolation of PBMC from patients

ABTL0812 is currently in clinical evaluation in a phase I/II trial in patients with advanced solid tumors. This study was approved by the local Ethics Committee of the Hospital Clinic Barcelona and the Spanish Agency of Medicines. The trial is registered at www.clinicaltrials.gov (NCT02201823). ABTL0812 was administered daily by the oral route at a dose of 1,000 mg twice daily for a period of 28 days. Blood samples for biomarker study were obtained by venipuncture before and after ABTL0812 treatment. PBMC were isolated by Ficoll (GE-Healthcare) density centrifugation, lysed in RIPA buffer, sonicated, and stored at –20°C.

Statistical analysis

All in vitro data were assessed using one-way ANOVA followed by Bonferroni multiple comparison test. Tumor volumes of mice were compared using the ANOVA followed by t test. Statistical significance between the groups was assessed with the log-rank test (GraphPad). Levels of statistical significance were set at P ≤ 0.05.

Results

ABTL0812 has anticancer activity in vitro and in vivo

A novel class of chemically modified lipid-derived small molecules was selected on the basis of two phenotypic assays: antiproliferative effect on tumor cells and low toxicity after high doses
Figure 1.
ABTLO812 induces cancer cell death in vitro and reduces tumor growth in human lung and pancreatic xenografts. A, chemical structure of ABTLO812. B, ABTLO812 cell viability assays in human cancer cell lines. IC50 values were obtained in MTT assays after exposure to ABTLO812 for 48 hours. Values are the mean ± SD of three separate determinations. (Continued on the following page.)
Inhibition of Akt/mTORC1 Axis by TRIB3 Pseudokinase

ABTL0812 was selected for further preclinical development. The formula for ABTL0812 is 2-hydroxyisobutyric acid (Fig. 1A).

ABTL0812 reduced viability of different human cancer cell lines tested with IC_{50} values of 20 to 60 μmol/L (Fig. 1B). Real-time cell analyses further demonstrated the antiproliferative effect of ABTL0812 (Supplementary Fig. S1). ABTL0812 also induced cell death, which was confirmed by propidium iodide staining (Supplementary Fig. S2). Importantly, ABTL0812 induced death in cancer cells but not in normal cells. Compared with LN-18 glioblastoma cells, primary astrocytes were resistant to ABTL0812 treatment at the highest ABTL0812 concentration tested (200 μmol/L; Fig. 1C).

To address ABTL0812 pharmacokinetics, we administered the compound to mice by oral and intravenous routes. ABTL0812 showed an excellent bioavailability by oral route (F: 103%), with rapid oral absorption (t_{max}: 30 minutes), high pick concentrations (C_{max}: 2.0 μg/mL), and wide volume of distribution (Vss: 10.4 L/kg). Clearance was also high (Cl: 82 mL/minute/kg). Overall, the compound was well tolerated and animals appeared healthy with no clinical signs of distress, local or systemic toxicity.

To test the efficacy of ABTL0812 in inhibiting tumor growth in vivo, we used human lung and pancreatic xenograft models. A549 human lung cancer xenografts of nude mice were treated with ABTL0812 or the standard-of-care docetaxel. ABTL0812 inhibited tumor progression with an efficacy similar to docetaxel (Fig. 1D). Moreover, ABTL0812 lacked the toxic effects observed for docetaxel, as shown by body weight measurements. Similarly, ABTL0812 also inhibited the growth of MiaPaCa-2 human pancreatic cancer xenografts in nude mice, without affecting body weight (Fig. 1E).

ABTL0812 induces autophagy-mediated cell death

Next, we investigated the type of cell death induced by ABTL0812 in A549 and MiaPaCa-2 cells. ABTL0812 did not induce nuclear fragmentation/conondensation, or caspase-3 activation (Supplementary Fig. S3). Treatment with staurosorine resulted in typical features of apoptosis, showing that A549 and MiaPaCa-2 cells are apoptosis competent. These results indicate that ABTL0812 fails to induce apoptosis. In turn, several evidences indicated that ABTL0812-induced cell death is mediated by autophagy.

Two of the hallmarks of autophagy are the conversion of the soluble form of LC3 to a lipidated form associated to autophagosomes (LC3-II), and the elimination of autophagosome cargo proteins such as p62 (13). In A549 and MiaPaCa-2 cells, ABTL0812 induced the appearance of LC3-II and a decrease on p62 protein in a concentration-dependent manner (Fig. 2A). We also observed the occurrence of LC3-positive dots in cells treated with ABTL0812, which indicates association to autophagosomes (Supplementary Fig. S4). Electron microscopy analysis of ABTL0812-treated A549 and MiaPaCa-2 cells revealed the presence of double membrane vacular structures with the morphologic features of autophagosomes (Fig. 2B). Moreover, ABTL0812 also induced hallmarks of autophagy in vivo, as tumors from A549 xenograft mice treated with ABTL0812 showed an increase in LC3-II levels, compared with mice treated with vehicle (Fig. 2C).

Next, we investigated the role of autophagy in the cell death induced by ABTL0812. Pharmacologic inhibition of autophagy was achieved by treating cells with a combination of the lysosomal protease inhibitors E64d and Pepstatin-A (PA) that block the final step of autolysosomal degradation. Treatment of cells with E64d+PA prevented ABTL0812-induced cell death and resulted in an enhancement of LC3-II accumulation in cells treated with ABTL0812 (Fig. 2D), indicating that ABTL0812 induces dynamic autophagy in cancer cells. Finally, we evaluated the activity of ABTL0812 in oncogene-transformed embryonic fibroblasts derived from Ag5+/− (autophagy-deficient) mice. Ag5 is an essential protein for autophagosome formation (14). Transformed Ag5+/− MEf cells were more resistant to ABTL0812-induced cell death than transformed Ag5+/- MEf cells, and did not activate autophagy in response to ABTL0812 treatment (Fig. 2E). Taken together, these results show that autophagy mediates ABTL0812-induced cancer cell death.

ABTL0812 inhibits the Akt/mTOR axis

Autophagy induction is often consequential to the inhibition of mTOR signaling, as mTORC1 acts as a central regulator in autophagy induction (15). We therefore investigated whether ABTL0812-induced autophagy and cancer cell death occurred via mTORC1 inhibition, by measuring levels of activation of components of the Akt/mTORC1 signaling pathway. A549 and MiaPaCa-2 cells showed measurable phosphorylation levels for all the proteins of Akt/mTORC1 axis in basal conditions. ABTL0812 treatment reduced phosphorylation of ribosomal-S6 kinase and of ribosomal-S6 protein (a well-established S6k substrate) in a concentration-dependent manner (Fig. 3).

Protein kinase Akt modulates the activation of mTORC1 by phosphorylating and inactivating the TSC2 and PRAS40 proteins that act as repressors of mTORC1 activity (1). Thus, Akt inhibition results in impaired mTORC1 activity and activation of autophagy. ABTL0812 induced Akt inhibition in A549 and MiaPaCa-2 cells in a dose-dependent manner, by reducing phosphorylation of the two critical residues involved in Akt activation, Thr308 and Ser473. Consequently, ABTL0812 also reduced phosphorylation of the Akt substrates PRAS40 and TSC2 (Fig. 3). These results show that ABTL0812 induced inhibition of the Akt/mTORC1 axis in cancer cells.

ABTL0812 binds and induces PPARα and PPARγ transcriptional activities, which mediate in ABTL0812-induced cancer cell death

To identify the molecular targets of ABTL0812, we performed high-throughput screening comparing ABTL0812 chemical structure against the ChEMBL15 database (https://www.ebi.ac.uk/chembl/), following the similar property and active analog principles (16). This approach allowed the identification of several ABTL0812 target candidates, including PPARα and PPARγ.

www.aacrjournals.org Clin Cancer Res; 22(10) May 15, 2016 2511

Published OnlineFirst December 15, 2015; DOI: 10.1158/1078-0432.CCR-15-1808
ABTL0812 induces autophagy in vitro and in vivo. Autophagy mediates ABTL0812-induced cancer cell death. A, cells were treated with 50 to 200 μmol/L ABTL0812 for 12 hours, lysed and levels of the autophagy-marker proteins LC3 and p62 visualized by immunoblotting. B, representative electron microscopy microphotographs showing autophagosomes (right) in cells treated with 50 μmol/L ABTL0812 for 10 hours. C, total protein extracted from A549 xenograft tumors (Fig. 1C) were analyzed by immunoblotting for expression of LC3. Figure shows a representative analysis from vehicle (n = 4) and treated (n = 5) tumors. LC3-II levels were normalized with actin and estimated in densitometric units. D, A549 (white columns) or MiaPaCa-2 (black columns) cells were preincubated with vehicle or a combination of lysosomal protease inhibitors E64d (10 μmol/L) and Pepstatin-A (PA, 10 μg/mL), before treatment with 50 μmol/L ABTL0812 for 24 hours. Cell viability was determined by MTT assay. Each value is the mean ± SD of three different experiments. *P < 0.05; **P < 0.001 from ABTL0812 treated cells. Cells were lysed and LC3 lipidation was visualized by immunoblotting. E, Atg5+/+ and Atg5−/− MEFs were treated with vehicle or ABTL0812 and cell viability determined 24 hours later. Values are the mean ± SD of three different experiments. *P < 0.05; **P < 0.001 from Atg5−/− MEFs.
Inhibition of Akt/mTORC1 Axis by TRIB3 Pseudokinase

ABTL0812 activates PPARα- and PPARγ-dependent expression of TRIB3

Koo and colleagues showed that PPARs regulate the expression of the pseudokinase Tribbles 3 (TRIB3), through binding a PPAR-responsive element sequence within the TRIB3 promoter (18). Other authors have shown that TRIB3 can interact with Akt, preventing its activation by upstream kinases and resulting in an inactive form of Akt (8, 19). Thus, we hypothesized that ABTL0812 could inhibit Akt and downstream mTORC1 by upregulating TRIB3 via PPARα/γ activation. In A549 and MiaPaCa-2 cells, ABTL0812 induced a quick and robust increase in TRIB3 protein levels after 12 hours of treatment (Fig. 5A), that correlated with an increase in gene transcription (Fig. 5B) and qPCR-measured TRIB3 mRNA levels (Fig. 5C). Although we cannot discard an effect of ABTL0812 on TRIB3 stability, these results indicate that ABTL0812 upregulates TRIB3 gene transcription. Furthermore, treatment with PPARα/γ or PPARγ inhibitors GW6471 and GW9662, respectively, completely abolished the ABTL0812-induced upregulation of TRIB3 gene expression (Fig. 5C). Interestingly, treatment with either PPARα or PPARγ agonists resulted in increased TRIB3 expression and reduced Akt phosphorylation (Fig. 5D). However, it was necessary to combine treatment with both agonists to obtain similar levels of inhibition of Akt (phospho-PRAS40) and mTORC1 activities (phospho-S6K and phospho-S6) than those induced by ABTL0812. Altogether, our results demonstrate that ABTL0812 induces TRIB3 gene expression via activation of PPARα and PPARγ activities.

ABTL0812 inhibits Akt and mTORC1 via TRIB3

To investigate the role of upregulated TRIB3 on Akt activation, we overexpressed increasing amounts of TRIB3 in A549 and MiaPaCa-2 cells. We observed reduced phosphorylation of Akt and Akt substrate PRAS40 that inversely correlated with TRIB3 expression (Fig. 6A). This was probably due to a TRIB3–Akt physical interaction, because ABTL0812 treatment increased the amount of Akt coimmunoprecipitated with TRIB3 (Fig. 6B). Moreover, unlike wild-type transformed MEF, TRIB3-deficient MEF cells did not show Akt (Ser73) or mTORC1 (pS6) inhibition, or induction of autophagy (LC3-II marker) in response to ABTL0812 treatment (Fig. 6C). Cell viability assays showed that TRIB3-deficient MEFs are resistant to ABTL0812-induced cell death, unlike wild-type transformed MEFs (Fig. 6D). These results support a pivotal role of TRIB3 on the impairment of the Akt/mTORC1 axis and on the autophagy-mediated cancer cell death observed in response to ABTL0812 treatment.

ABTL0812 induces TRIB3 upregulation and Akt inhibition in vivo

Finally, we performed cell viability assays in the presence of PPARα/γ antagonists. In A549 and MiaPaCa-2 cells, preincubation with PPARα antagonist GW6471 or PPARγ antagonist GW9662 prevented cell death induced by ABTL0812 (Fig. 4C), indicating that PPAR receptors mediate in ABTL0812-induced cell death.

Of note, a possible interaction with PPARβ/δ was not found. Putative ABTL0812-binding molecules identified in this screening were further investigated through docking analysis. Figure 4A shows the molecular model of ABTL0812 into the ligand-binding pocket of PPARγ. In this model, ABTL0812 may bind the PPARγ ligand-binding domain (LBD) through its carboxylate group, which forms hydrogen bonds with the side of key amino acid residues (Ser289, His323, Tyr473, and His449), as it has been shown for other PPARγ agonists such as rosiglitazone (17). The 2-hydroxyl group of ABTL0812 may also form hydrogen bonds with residues Cys285 and Ser289, which are also conserved in the PPARα LBD. This molecular model proposes that ABTL0812 might establish interactions similar to the specific PPARγ agonist rosiglitazone, thus predicting that ABTL0812 could act as PPARα agonist. Indeed, in vitro radioligand displacement assays using purified proteins showed that ABTL0812 binds PPARα and PPARγ ligand-binding pockets with Ki values of 7.1 μmol/L and 4.7 μmol/L, respectively (Supplementary Fig. S5).

Next, we investigated the functional relevance of this interaction, performing luciferase-based gene reporter assays in cells transfected with a PPAR-response element (PPRE) fused to luciferase construct. In A549 and MiaPaCa-2, ABTL0812 induced approximately 5-fold PPAR transcriptional activity, similarly to the specific agonists WY-14643 (PPARα) or rosiglitazone (PPARγ; Supplementary Fig. S6). To determine whether ABTL0812-induced PPARα activation was mediated by the PPAR isoforms α and/or γ, we performed analogous gene reporter assays in cells transiently transfected with plasmids encoding each isoform. ABTL0812-induced PPARα transcriptional activity was higher in cells overexpressing PPARα or PPARγ proteins, compared with those having the endogenous proteins only (Fig. 4B), suggesting that ABTL0812 functionally interacts with both receptors in cells.

www.aacrjournals.org
and inhibition of Akt phosphorylation (Ser473) in A549 and MiaPaCa-2 tumors (Fig. 6E). These data, together with that presented in Fig. 2 showing that ABTL0812 induces autophagy in A549 xenografts, suggest that the antitumor activity of ABTL0812 in vivo relies on inhibition of Akt and induction of autophagy-mediated cell death.

Akt phosphorylation is a pharmacodynamic marker in patients treated with ABTL0812. Finally, we evaluated the activity of ABTL0812 in some patients included in phase I/II clinical trials (NCT02201823), monitoring Akt phosphorylation levels in PBMCs. We collected blood samples, pretreatment and at different days of chronic treatment with 1,000 mg of ABTL0812, a dose that was safe and well tolerated (preliminary data not shown). Our preliminary results show that treatment with ABTL0812 resulted in a marked reduction on Akt phosphorylation in PBMCs from 3 patients (Fig. 6F). We could not detect TRIB3 protein in PBMCs, using commercially available anti-TRIB3 antibodies. However, parallel qPCR analysis showed a significant increase in TRIB3 mRNA levels in human PBMCs treated in vitro with ABTL0812 (Supplementary Fig. S7), thus suggesting that ABTL0812 also induces Akt inhibition in humans through upregulation of TRIB3.

Discussion

Here, we present the mechanism of action of ABTL0812, a novel and first-in-class antitumor drug which is currently in phase I/II clinical trials in patients with advanced solid tumors. ABTL0812 is a polyunsaturated fatty acid derivative, small molecule that...
induces cell death in a broad panel of cancer cell lines, whereas it does not affect cell viability of nontumorogenic cells. ABTL0812 also inhibits tumor growth in human lung and pancreatic tumor xenografts. Herein, we elucidated a novel cell signaling pathway through which ABTL0812 induces autophagic cell death. We showed that ABTL0812 induces upregulation of the TRIB3 pseudokinase through activation of PPARα and PPARγ transcription factors. Upregulated TRIB3 binds to Akt and prevents its activation by PDK1 and mTORC2 upstream kinases, resulting in inhibition of the Akt/mTORC1 axis and, therefore, autophagy-mediated cell death (Fig. 6G).

Autophagy is an essential process that consists of selective degradation of cellular components, assuring the maintenance of cellular homeostasis via lysosomal degradation pathways (13). The role of autophagy in cancer is still far from clear, as it can act as a tumor suppressor and tumor promoter, depending on tumor type, stage, and genetic context, as well as on the duration and strength of the triggering stimuli (15, 20). Our results demonstrate that ABTL0812 induces autophagy-mediated cancer cell death in vitro and in vivo, without activating cellular apoptosis. These results are in agreement with those reporting that polyunsaturated fatty acid and derivatives exert their antiproliferative action through activation of autophagy (21–23).

Inhibition of Akt/mTORC1 Axis by TRIB3 Pseudokinase

Figure 5. ABTL0812 induces upregulation of the Akt endogenous inhibitor TRIB3 through PPARα/γ activation. A, cells treated with ABTL0812 for the indicated times were lysed, and levels of TRIB3 and actin analyzed by immunoblotting. B, cells were transfected with luciferase-TRIB3 promoter reporter and pRL-CMV-Renilla plasmids. Sixteen hours posttransfection, cells were treated with vehicle or 100 μmol/L ABTL0812. Twenty-four hours later, lysates were subjected to dual-luciferase assay. Values are the mean ± SD of three different experiments, each performed in triplicate and normalized using Renilla values. C, cells were preincubated with vehicle or PPARα antagonist GW6471 (0.2 μmol/L) and/or PPARγ antagonist GW9662 (1 μmol/L) for 2 hours, and treated for 24 hours with vehicle or 50 μmol/L ABTL0812. TRIB3 mRNA levels were monitored by RT-qPCR. Values are the mean ± SD of three different determinations, each performed in duplicate. **P < 0.001 from vehicle-treated cells. D, A549 cells were treated with PPARα agonist WY14643 (1 μmol/L) and/or PPARγ agonist rosiglitazone (1 μmol/L) for 24 hours, lysed and proteins analyzed by immunoblotting.
Figure 6.
ABTL0812-evoked TRIB3 upregulation inhibits Akt activity in vitro and in vivo. A, cells transfected with empty or increasing amounts of a plasmid encoding human-TRIB3 were lysed and levels of proteins monitored by immunoblotting. B, immunoprecipitation of Akt from MiaPaCa-2 lysates after treatment with vehicle or ABTL0812 for 24 hours. Top, levels of Akt and TRIB3 in the immunoprecipitates. C, wild-type and TRIB3-KO MEF cells were treated with ABTL0812 for 24 hours, and levels of the indicated proteins were analyzed by immunoblotting. D, wild-type and TRIB3-KO MEFs were treated with ABTL0812 for 24 hours, and cell viability measured by MTT analysis. Values are the mean ± SD of three different determinations. **P < 0.01; ***P < 0.005; ****P < 0.001 from vehicle-treated tumors. E, A549 and MiaPaCa-2 tumors were collected after sacrifice, and TRIB3 was evaluated by immunohistochemical analysis (left; scale bars: 100 μm). Levels of phospho-Akt (Ser473) were evaluated in total protein extracts, by immunoblotting (right). Histograms show the corresponding quantifications. *, P < 0.01; **, P < 0.005; ***P < 0.001 from vehicle-treated tumors. F, phospho-Akt and Akt levels in PBMCs from patients before (day 0) or after 1,000 mg bid ABTL0812 oral chronic treatment. Protein extracts were analyzed by immunoblotting for phospho-Akt-Ser473 and total Akt. Upper graphs show the quantification of levels of phospho-Akt normalized with Akt protein, estimated by densitometric analysis (day 1 = 100%). G, a model showing the mechanism of action of ABTL0812.
is frequently associated with resistance to antitumor drugs (6). As ABTL0812 is a potent inhibitor of the Akt/mTORC1 axis, its administration in combination with standard chemotherapeutic drugs might prove effective in therapy-resistant or apoptosis-refractory tumor. This has been shown for the mTORC1 inhibitor everolimus (RAD001), which sensitizes papillary thyroid cancer cells to treatment with doxorubicin via an autophagy-mediated mechanism (28).

Here we present in vitro and in vivo evidences showing that ABTL0812 binds to and activates PPARα and PPARγ transcriptional activities, and that pharmacologic inhibition of both receptors prevents ABTL0812-induced cancer cell death, suggesting that PPARα/γ are the primary molecular targets of ABTL0812 in the cell. PPARs are nuclear receptors that heterodimerize with the retinoid-X receptors to modulate gene transcription. Mammalian everolimus (RAD001), which sensitizes papillary thyroid cancer cells to treatment with doxorubicin via an autophagy-mediated mechanism (28).

Te impression of Akt/mTORC1 axis is observed in the majority of human cancers and blocking this pathway is an important anticancer strategy (5). We have shown here the activity of ABTL0812 in a panel of cancer cell lines that express high levels of phosphorylated/active Akt. Importantly, we have preliminary observed activity of ABTL0812 in patients. ABTL0812 treatment

Inhibition of Akt/mTORC1 Axis by TRIB3 Pseudokinase

www.aacijournals.org

Published OnlineFirst December 15, 2015; DOI: 10.1158/1078-0432.CCR-15-1808
impaired Akt phosphorylation in PBMCs from patients included in the ongoing phase I clinical trial (Fig. 6F). Therefore, Akt phosphorylation will be used as a surrogate pharmacodynamic biomarker, for estimating the recommended dose for the forthcoming phase II trials. We have preliminarily observed increased TRIB3 mRNA levels in human PBMCs treated with ABTL0812 (Supplementary Fig. S7), suggesting that TRIB3 qPCR analysis could also be used to monitor the biologic activity of ABTL0812 in humans. Finally, and given its unique mechanism of action, it will be important to evaluate ABTL0812 in combination with standard chemotherapeutic compounds as well as with other target therapies. We foresee ABTL0812-based therapies will contribute to improve patients’ treatment and quality of life.

Disclosure of Potential Conflicts of Interest

J. Espadaler has ownership interest (including patents) in and is a consultant/advisory board member for Ability Pharmaceuticals SL. J.M. Lizcano is a consultant/advisory board member for Ability Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M. Salazar, J. Espadaler, M. Gómez-Ferreria, J. Alfon, C. Domínech, J.M. Lizcano


Acquisition of data (provided animals, acquired and managed patients, facilities, etc.): T. Erazo, M. Lorente, P. Muñoz-Guardiola, F. Fernández-Nogueira, J.A. García-Martínez, P. Bragado, M. Salazar, J. Hernández-Losa, J.R. Bayascas, L. Vidal, G. Velasco


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Lizcano

Study supervision: P. Gascón, G. Velasco, J.M. Lizcano

Acknowledgments

The authors thank Pablo Escribá and Xavier Busquets (Universitat Illes Balears, Spain) for the preliminary identification of ABTL0812 as an anticaner agent (patent number WO/2010/106211) and Giovani Cincilla, Emiliana D’Orta, and Oscar Villacatias (Intelligent Pharma SL) for in silico analysis, Luis Botella and Javier Soto (Medalchem) for the synthesis of ABTL0812, and Washington Biotechnology Inc. for preliminary preclinical studies. The authors also thank Victor Yuste for helpful discussions, Cristina Gutierrez for tissue culture assistance, and Servei de Genòmica i Informàtica from the UAB.

Grant Support

This work was supported by grants from the Government of Catalonia (ACCIO/FINEBT1-1-0047), and from the Spanish Ministry of Economy and Competitiveness (MINECO), (CDTI/PID/JDI/20101630, Genoma España/INNOCASH/2011196, ENISA/EBI/2012/100375, INNPACTO/EPT-2012-0614-010000). J. Alfon and M. Gómez-Ferreria were funded by Torres-Quevedo grant (miNECO), and M. Cortal by an Innova grant (miNECO). Work in C. Velasco’s laboratory was supported by grants from Spanish Ministry of Economy and Competitiveness (MINECO), Fondo Europeo de desarrollo Regional (FEDER) (PII12/02248 and PI15/00339) and Fundación Mutua Madrileña (AP101042012).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 30, 2015; revised November 20, 2015; accepted November 30, 2015, published OnlineFirst December 15, 2015.

www.aacrjournals.org Clin Cancer Res; 22(10) May 15, 2016 2519

Inhibition of Akt/mTORC1 Axis by TRIB3 Pseudokinase

Published OnlineFirst December 15, 2015; DOI: 10.1158/1078-0432.CCR-15-1808

Downloaded from clincancerres.aacrjournals.org on October 23, 2017. © 2016 American Association for Cancer Research.
The New Antitumor Drug ABTL0812 Inhibits the Akt/mTORC1 Axis by Upregulating Tribbles-3 Pseudokinase

Tatiana Erazo, Mar Lorente, Anna López-Plana, et al.


Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/12/15/1078-0432.CCR-15-1808.DC1

Cited articles
This article cites 49 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/22/10/2508.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/22/10/2508.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.