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Therapeutic potential of the new TRIB3-mediated cell autophagy anticancer drug ABTL0812 in endometrial cancer

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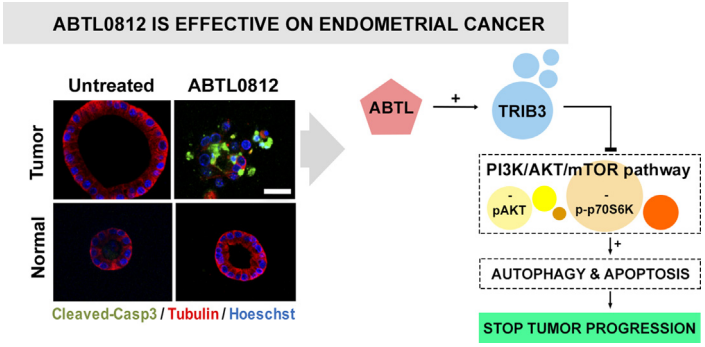
HIGHLIGHTS

- ABTL0812 is a small molecule inhibitor and is an effective therapeutic option for high-risk endometrial cancer patients.
- ABTL0812 acts by inducing TRIB3 expression, inhibiting the PI3K/AKT/mTOR axis, and promoting autophagy cell death.
- In preclinical models, ABTL0812 kills endometrial cancer cells but not healthy endometrial cells.
- ABTL0812 stops hyperplasic lesions to progress to cancer.

GRAPHICAL ABSTRACT



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ABSTRACT

Objectives. The PI3K/AKT/mTOR pathway is frequently overactivated in endometrial cancer (EC). We assessed the efficacy of ABTL0812, a novel first-in-class molecule presenting a unique mechanism of action inhibiting this pathway.

Methods. We investigated the effects of ABTL0812 on proliferation, cell death and modulation of intracellular signaling pathways in a wide panel of endometrioid and non-endometrioid cell lines, an inducible PTEN knock-

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out murine model, and two patient-derived xenograft murine models of EC. Then, TRIB3 expression was evaluated as potential ABTL0812 pharmacodynamic biomarker in a Phase 1b/2a clinical trial.

Results. ABTL0812 induced an upregulation of TRIB3 expression, resulting in the PI3K/AKT/mTOR axis inhibition and autophagy cell death induction on EC cells but not in healthy endometrial cells. ABTL0812 treatment also impaired PTEN knock-out cells to progress from hyperplasia to cancer. The therapeutic effects of ABTL0812 were demonstrated *in vivo*. ABTL0812 increased TRIB3 mRNA levels in whole blood samples of eight EC patients, demonstrating that TRIB3 mRNA could be used as a pharmacodynamic biomarker to monitor the ABTL0812 treatment.

Conclusions. ABTL0812 may represent a novel and highly effective therapeutic agent by inducing TRIB3 expression and autophagy in EC patients, including those with poorer prognosis.

1. Introduction

Endometrial cancer (EC) is the most frequent of the infiltrating tumors of the female genital tract. In 2012, the number of new cases and deaths due to EC worldwide was 319,605 and 76,160 respectively [1] and unfortunately, death rates have been increasing since then. Surgical and adjuvant treatment are the cornerstone treatment of EC patients. In order to reliably diagnose EC and guarantee the best treatment, clinicians should precisely determine the histological subtype, the grade of differentiation, and the FIGO stage. Endometrioid ECs, represents approximately 85% of all cases and is associated with a good prognosis; whilst non-endometrioid ECs, accounting for the remaining 15% of EC, mostly includes serous, carcinosarcoma, and clear cell histologies. High grade EEC and NEEC are associated with a poor prognosis of the disease [2]. The clinical setting of EC is reasonably favorable for those patients diagnosed with tumor confined to the uterus. However, the 5-year survival rate associated to patients with regional and distant dissemination declines dramatically. In those cases, the response rate to standard treatment, a combination of carboplatin and paclitaxel, is very limited [3]. This situation strongly reflects the need for new and efficient therapeutic strategies.

The PI3K/AKT/mTOR pathway is frequently overactivated in women with EC due to the loss of PTEN, mutations in the PKI3CA receptor, or AKT amplification [4]. Since Oza and collaborators [5] reported this pathway could be targeted using the mTOR inhibitor (temsirolinus), many trials have been published, targeting individual components of the pathway [6–9] or dual inhibitors targeting PI3K/mTOR simultaneously [10] showing discrepant results [11]. However, the challenge in targeting PI3K/AKT/mTOR pathway is the complex feedback loops within the signaling cascade leading to activation of compensatory pathways or shift in isoform dependency [12,13]. Treatment with new alternative inhibitors of the PI3K/AKT/mTOR pathway may result in a significant therapeutic benefit for EC patients. Considering this, ABTL0812, a new first-in-class molecule with a unique mechanism of action to inhibit the PI3K/AKT/mTOR pathway, emerge as a promising alternative for first line therapy. This compound was has a therapeutic effect on lung and pancreatic cancers [14].

In this study, we aimed to first, study the efficacy of ABTL0812 in endometrioid and non-endometrioid EC in monotherapy and in combination with the current standard of care in chemotherapy; second, assess its effect in normal and premalignant lesions of the endometrium; and third, evaluate the use of TRIB3 mRNA expression levels as a potential pharmacodynamic biomarker to monitor ABTL0812 treatment in human clinical trials.

2. Materials and methods

2.1. Cell culture

Human endometrioid EC cell lines, Ishikawa (IK), HEC-1a, and AN3CA, were purchased from American Type Culture Collection (2008; ATCC-authentication by isoenzymes analysis). Non endometrioid EC cell lines, ARK1 and ARK2, were generously donated by Dr. A Santin (Yale University, New Haven, CT, USA) and Dr. I Fidler (MD Anderson Cancer center, Houston, TX, USA) and have been characterized in previous reports [15,16]. We authenticated these cell lines by confirming serous histology *via* pathological review.

2.2. Viability assays

Cell viability was evaluated as general mitochondrial activity of the cells by assaying reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. Further details are described in supplementary materials and methods.

2.3. Western blotting

Western-blot was performed as previously described [17]. Antibodies blotted were: Anti-TRIB3 (Abcam, 50516), anti-LC3 (Cell Signaling Technology, 2775), anti-pAKTs473 (Cell Signaling Technology, 4060), anti-actin (Sigma-Aldrich, a5441), anti-Akt1 (Santa Cruz Biotechnology, 1618), anti-p-p70S6K (Cell Signaling Technology, 9205), anti-p70S6K (Cell Signaling Technology, 9202), anti-cleaved-CASP8/caspase-8 (Calbiochem, AM4 6 T), anti-cleaved-CASP3/caspase-3 (Cell Signalin Technology, 9661), anti-cleaved-CASP9/caspase-9 (Cell Signaling Technology, 9508), anti-αTubulin (Sigma-Aldrich, T5168). Signal was detected with SuperSignal West Femto Trial Kit (Thermo Scientific).

2.4. mRFP-GFP tandem fluorescent-tagged LC3B (tfLC3) autophagic flux assay

Ishikawa and ARK2 cells were transfected with tfLC3 plasmid (kindly provided by Dr. Tamotsu Yoshimori, Osaka University, Japan) and left to grow overnight before treatment with ABTL (25 μM), chloroquine (20 μM) and rapamycin (500 nM) during 24 h. After this period, cells where pictured randomly under an epifluorescence microscope and analyzed for co-localization of green and red puncta. Each channel (mRFP and EGFP) was acquired by separate scan avoiding bleed-through. We analyzed all cells using the same threshold settings across all images from each channel. Double mRFP-GFP puncta were determined using the Co-localization plugin in ImageJ. All puncta were quantified using Analyze Particle plugin in ImageJ.

2.5. Bromodeoxyuridine (BrdU) incorporation assay

For the determination of DNA and after the indicated treatments, 3D cultures were incubated with 3 ng/ml of 5-bromodeoxyuridine (BrdU, Sigma-Aldrich) during 15 h and then fixed with 4% paraformaldehyde for 20 min. Further analysis was performed as previously described [18].

2.6. Immunofluorescence

Antibodies used were: cleaved-CASP3/caspase-3 (Cell Signaling Technology, 9661) anti-tubulin (Sigma-Aldrich, T8203) and

rhodamine-conjugated phalloidin (Sigma-Aldrich, P1951). Secondary anti-mouse Alexa Fluor 546 (Invitrogen, A11005) and Alexa Fluor 488 (Invitrogen, A11029) or anti-rabbit antibodies Alexa Fluor 594 (Invitrogen, R37119) and Alexa Fluor 488 (Invitrogen, A11034). Further details are described in Supplementary material and methods.

2.7. Confocal imaging and evaluation of spheroid perimeter

Images of endometrial epithelial spheroids were captured and digitized with a confocal microscope (Fluoview FV1000, Olympus). Epithelial perimeter analysis was processed by image analysis software (ImageJ version 1.46r; NIH, Bethesda, MD, USA), generating binary images of the spheroids as previously described [18].

2.8. Total RNA extraction, reverse transcriptase-PCR and quantitative real-time

Probes used are mouse GAPDH Mm99999915_g1; mouse TRIB3 Mm00454880_m1; human GAPDH Hs03929097_g1; human TRIB3 Hs01082394_m1. Further details are described in Supplementary material and methods.

2.9. Genetically modified mouse models

The study was approved by the Ethics Committee on Animal Experiments of the University of Lleida and the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya. Floxed homozygous PTEN (C;129S4-PTENtm1Hwu/J; referred here as PTENfl/fl), Cre-ERT (B6.Cg-Tg(CAG-Cre/Esr1*5Amc/J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Cre-ERT+/−PTENfl/fl mice were bred in a mixed background (C57BL6; 129S4) by crossing PTEN fl/fl and Cre-ERT+/− mice. To obtain mice carrying both PTEN-floxed alleles (PTENfl/fl) and a single Cre-ERT (Cre-ERT+/−), Cre-ERT+/− PTENfl/+ mice were backcrossed with PTENfl/fl mice to obtain Cre-ERT+/−PTENfl/fl (referred here as Cre:ER+/−; PTENfl/fl). Confirmation of the models is detailed in supplementary materials and methods.

2.10. Isolation of endometrial epithelial cells and three-dimensional glandular cultures

The isolation and 3D culture of endometrial epithelial cells was performed using a method previously described with minor modifications [19]. Detailed information is included in supplementary materials and methods.

2.11. Immunohistochemical study

For mice and human samples, histopathological and immunohistochemical studies were performed as describes previously [20]. Antibodies used were Ki67 antibody (1:100 dilution Abcam #15580) and anti-TRIB3 (1:100 dilution Abcam #50516). Appropriate negative controls including no primary antibody were also tested. Immunohistochemical results were evaluated by following uniform pre-established criteria. Immunostaining was graded semi-quantitatively by considering the percentage and intensity of the staining. A histological score was obtained from each sample and values ranged from 0 (no immunoreaction) to 300 (maximum immunoreactivity). The score was obtained by applying the following formula, Histoscore $= 1 \times$ (% light staining) $+ 2 \times$ (% moderate staining) $+ 3 \times$ (% strong staining). To support the scoring of immunohistochemistry an automated imaging system, the ACIS III Instrument (DAKO), was also used. An intensity score, which ranged from 60 to 255, was obtained from four different areas of each sample.

2.12. Subcutaneous xenografts and treatment

Immunodeficient female athymic nude mice (age 12 weeks; weight 20–25 g) were maintained in specific pathogen free (SPF) conditions. Animals were subcutaneously injected with HEC-1A cells (1.5×10 [6]) suspended in 100 μl PBS + Matrigel (1:1) in the flank. Tumors were allowed to growth for 15 days. Mice were randomly distributed into treatment groups with 4 mice per group. Mice were treated daily with placebo or two different doses of ABTL0812 (60 mg/Kg or 120 mg/Kg) by oral gavage during 25 or 46 days. Tumors were measured twice weekly with a caliper, and volumes were calculated as (length $\times$ width [2])/2 $=$ mm^3.

2.13. Patient-derived tumor xenograft (PDX) establishment and treatment

PDX models were established using primary endometrial carcinomas tissue from patients undergoing surgery at Vall Hebron University Hospital, Barcelona, Spain; as previously described [20]. All procedures were performed according to protocols approved by the Clinical Research Ethics Committee (CEIC PR(IR)301/2013), including written informed patient consent and Animal Experimentation Ethics Committee (CEEA 65/13). PDX521 and PDX548 were generated from fresh-tumor tissue of a 57-year old endometrioid EC patient (FIGO IIIA, grade 2) and 73-year old serous EC patient (FIGO IIIC2, grade 3), respectively. None of the patients received chemo- or radiotherapy before surgery. The efficacy of drugs was evaluated in a cohort of 32 mice from F3 generation. When the tumors reached 150–200 mm^3, mice were randomized into groups, and treated with Placebo, ABTL0812 (120 mg/kg), carboplatin-paclitaxel (CBPT-PTX, 30 mg/kg and 5 mg/kg, respectively), or triple treated with ABTL0812 + CBPT-PTX. Drug dosages were chosen according to previous *in vivo* studies. Treatments were given as follow; ABTL0812 daily by oral gavage; CBPT-PTX intraperitoneally (i.p) weekly; the only specification for the double treatment is that ABTL0812 was not administered in the same day of CBPT-PTX treatment; control animals received the equivalent volume of vehicle (Placebo). PDX521 and PDX548 were treated for 20 and 47 days, respectively. PDX548 treatment with ABTL0812 was extended for 17 additional days. Tumor size was measured 3×/week with a digital caliper, and tumor volume was calculated as (length $\times$ width [2])/2 $=$ mm^3. Mice were also weighed at regular intervals and screened for treatment adverse effects. At endpoint, mice were euthanized and tumors were harvested, weighed and collected for further analyses.

2.14. Sample collection from patients in the Phase 1b/2a clinical trial

Blood samples from eight EC patients with advanced disease participating in the Phase 1b/2a (NCT03366480) were collected and analyzed at different time points during ABTL0812 treatment: 0 h, 8 h, 7 days and 28 days after the onset of treatment. At day 7, patients started the chemotherapy treatment. Further details are included in supplementary materials and methods.

2.15. Statistical analysis

The normality of the data was assessed by Kolmogorov–Smirnov test. No statistical method was used to predetermine sample size. Statistical analysis was performed with GraphPad Prism 6.0 (La Jolla, CA, USA). Differences between two groups were assessed by Student's *t*-test (unpaired or paired as needed depending on the study design). Differences between more than two groups were assessed by one-way ANOVA, followed by the Tukey's multiple comparison test or two-way ANOVA, followed by the Bonferroni *post hoc* comparison test. A $p \leq 0.05$ was considered statistically significant. All data examined are expressed as mean ± S.E.M.

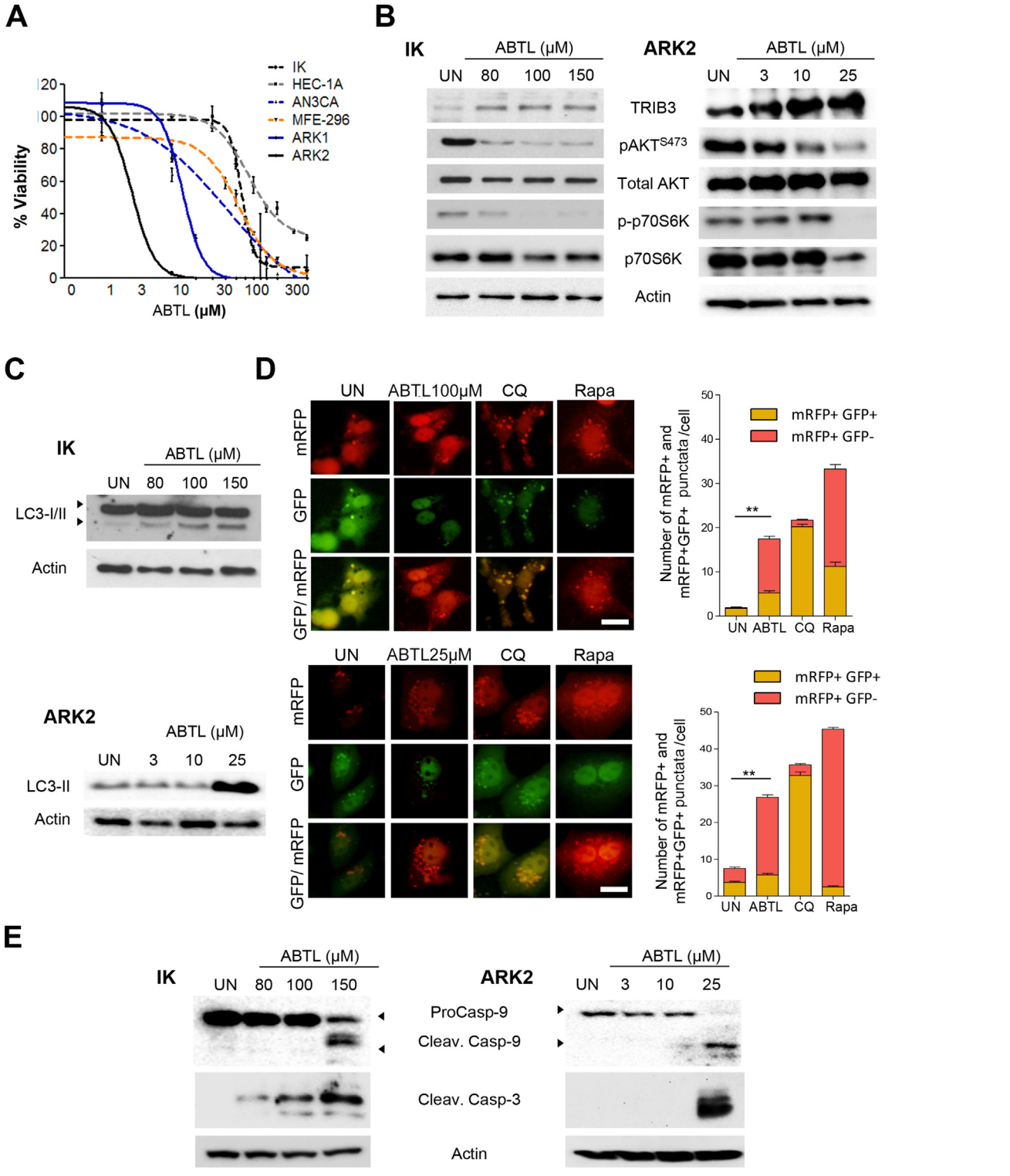

Fig. 1. ABTL0812 sensitizes endometrial carcinoma cells inducing autophagy through TRIB3 induction. A) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay of different EC cell lines treated with increasing doses of ABTL0812 during 48 h. Mean and SD values of one representative experiment is shown. B) Western blot of IK and ARK2 cells treated for 24 h with the indicated ABTL0812 doses. Protein levels of TRIB3, AKT and p70S6K were determined, actin was used as loading control. The western blot shown is representative of the three independent experiments performed. C and D) LC3B-II western blot and autophagic flux immunofluorescent analysis of IK (upper panel) and ARK2 (lower panel) cell lines treated with ABTL0812 for 24 h with the indicated doses. Immunofluorescent images and quantification represent EC cells transfected with a chimeric mRFP-GFP-LC3B probe showing mRFP (red), GFP (green) and merged mRFP and GFP (yellow) signal (puncta). Scale bar: 10 mm. EC cells were also treated with Choloroquine and Rapamycin as control for autophagic flux immunofluorescent assay. It is shown one independent assays ($n = 3$). Quantification bars represent mean and SEM. $^{**}p < 0.01$. E) Cleaved Caspase-3 and -9 protein levels determined by western blot in IK and ARK2 cell lines treated for 48 h with the indicated doses of ABTL0812. Actin was used as loading control. It is shown a representative assay (n = 3).

3. Results

3.1. ABTL0812 sensitizes endometrial carcinoma cells inducing autophagy and apoptosis through TRIB3 induction in vitro

ABTL0812, a novel class of chemically modified fatty acid-derived small molecule, has been suggested as an effective compound acting on the PI3K/AKT/mTORC1 axis [14]. Because this pathway is importantly mutated in ECs, we evaluated ABTL0812 efficacy on both endometrioid: IK, AN3CA, and HEC-1A; and non-endometrioid: ARK1 and ARK2, EC cell lines. ABTL0812 reduced viability of all cell lines, IC50 values ranging from 5.03 ± 0.12 to 36.86 ± 3.31 μmol/L (Fig. 1A and S1A). ABTL0812 sensitizes cancer cell lines though inhibition of the PI3K/AKT/mTORC1 axis, by an upregulation of TRIB3 expression and a reduction of AKT and p70s6K phosphorylation levels.

Autophagy induction is often a consequence of Akt/mTOR signaling inhibition [21,22]. Given the role of autophagy as a cellular mechanism to overcome therapeutic stress [23], we assessed whether ABTL0812 triggered a cellular autophagy response in EC. We analyzed the conversion of the soluble form of LC3 (LC3-I) to a lipidated form associated to autophagosomes (LC3-II) and we found an increase in LC3-II protein levels in IK, AN3CA, ARK1 and ARK2 cells after 24 h of ABTL0812 treatment. We further confirmed an autophagy induction by assessing the authophagic flux. To do so, we used a chimeric mRFP-GFP tandem fluorescent-tagged LC3B construct (tfLC3) [24], which under basal conditions, autophagosomes are observed as a yellow signal (merged mRFP and GFP signal). However, when autophagic conditions increase, GFP signal is gradually lost due to lysosomal enzymatic degradation activity, thereby labeling autolysosomes in red, since mRFP is resistant. Consequently, induction of autophagic flux can be analyzed by quantifying the increase in both the yellow and red signals. Our results showed that after 24 h of incubation, ABTL0812 increased the autophagic flux (red and yellow puncta), whereas chloroquine (CQ) (a lysosomal pH modifier that inhibits autophagic degradation in the lysosome) resulted in the accumulation of yellow puncta (hence autophagosomes). Rapamycin (Rapa) was used as a positive control of increased autophagy flux (Fig. 1D and S2A). These results confirmed the full induction of autophagy in all types of EC cell lines upon ABTL0812 treatment.

Next, we wanted to analyze whether autophagy was a prior event to the onset of apoptosis. Thus, we analyzed caspase-9 and -3 activation in IK and ARK2 cell lines. As shown in Fig. 1E and S2B, a 48-h treatment of ABTL0812 triggered caspase-9 and -3 cleavage, suggesting that ABTL0812 induced an autophagy-mediated apoptotic cell death.

3.2. ABTL0812 treatment impairs proliferation of endometrial carcinoma cells in vivo

To test the efficacy of ABTL0812 on tumor growth *in vivo*, we generated a xenograft murine model by subcutaneous injection of HEC-1A cell line. Mice were treated daily either with placebo or with ABTL0812 60 mg/Kg or 120 mg/Kg. The compound was well-tolerated and animals appeared healthy without body weight changes or clinical signs of distress or toxicity. Interestingly, after 30 days of treatment, we found a marked dose-dependent reduction in tumor growth on animals treated with ABTL0812 ($p < 0.001$) (Fig. 2A). Tumors treated with ABTL0812 displayed increased TRIB3 levels and LC3-II conversion at 25 and 46 days of treatment (Fig. 2B and C).

3.3. ABTL0812 treatment induces autophagy and cell death in a 3D model of precancerous endometrial glands but not in healthy endometrium

Then, we studied the effect of ABTL0812 in normal endometrium and its capacity to control the onset of the tumorigenic process. To do this, we used an inducible PTEN knock-out mice model, which carries PTEN-floxed alleles (PTEN fl/fl), and the tamoxifen-induced CRE recombinase (Cre:ER+/−) under the estrogen promoter. Thus, after the administration of one single dose of tamoxifen, PTEN is excised in PTEN fl/fl Cre:ER+/− mice and this led to a rapid and efficient development of endometrial hyperplasias and *in situ* carcinomas (EIN) *in vivo* [25]. From this model, we established 3D cultures from tamoxifen treated (TAM) or not treated (NO TAM) animals, and obtained pathogenic (*i.e.* hyperplastic glandular structures) and healthy (normal endometrial acini) endometrium models, respectively. Importantly, 3D cultures are relevant models to study EC tumorigenesis as they recapitulate endometrial acinis, mimicking architecture, cell-cell adhesion unions and cell polarity as in native tissue [26].

The 3D cultures were exposed to increasing doses of ABTL0812 for 48 h. The treatment resulted in a significant decreased of positive BrdU-incorporating cells (Fig. 3A) and glandular size (Fig. S3) in the pathogenic but not in normal endometrium. Importantly, these results suggested that ABTL0812 reduced proliferation *in vitro* specifically in pathogenic but not in healthy endometrium. The effective action of ABTL0812 was accompanied by an autophagy induction and apoptosis. As shown in Fig. 3B, we observed that ABTL0812 treatment raised levels of LC3-II expression in pathogenic glands whereas LC3-II levels remained unchanged in normal endometrium.

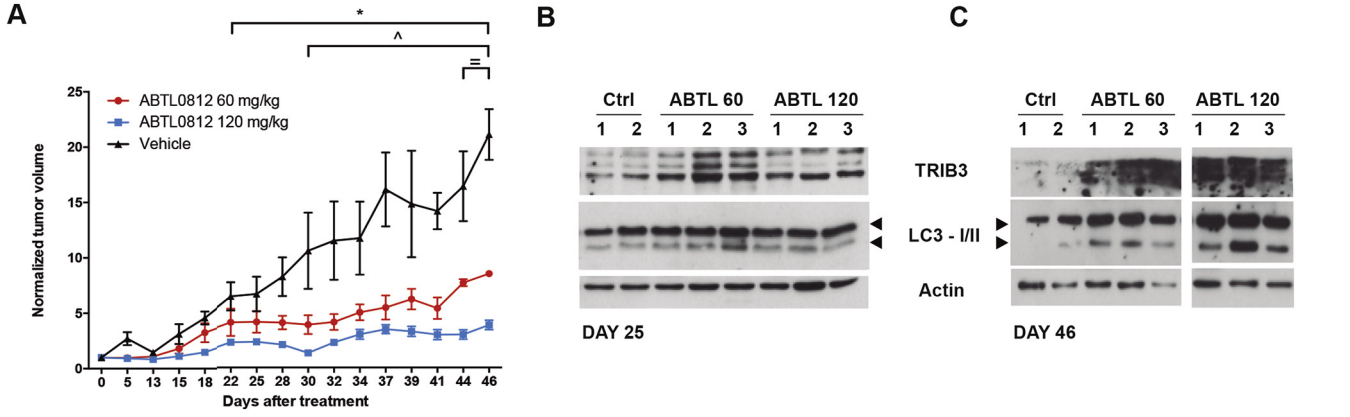

Fig. 2. ABTL0812 treatment impairs tumor growth and activates autophagy pathway in an *in vivo* xenograft model. A) Tumor growth curve of a subcutaneous murine xenograft model generated by HEC-1A cell line inoculation. Mice were treated daily either with placebo or with ABTL0812 (60 mg/Kg and 120 mg/Kg) by oral gavage for seven weeks. Each data point represents tumor volume average and SD from each group of animals. * significantly different Vehicle *vs* ABTL0812 120 mg/kg ($p \leq 0.001$), ^ significantly different Vehicle *vs* ABTL0812 60 mg/kg ($p \leq 0.001$), = significantly different ABTL0812 60 mg/kg *vs* ABTL0812 120 mg/kg ($p \leq 0.05$). B and C) Western blot from HEC-1A xenograft models. TRIB3 and LC3 I/II protein levels were determined in three different animals treated with ABTL0812 60 mg/Kg or 120 mg/Kg and two placebo mice after 25 days of treatment (B) or after 46 days of treatment (C). Actin was used as loading control.

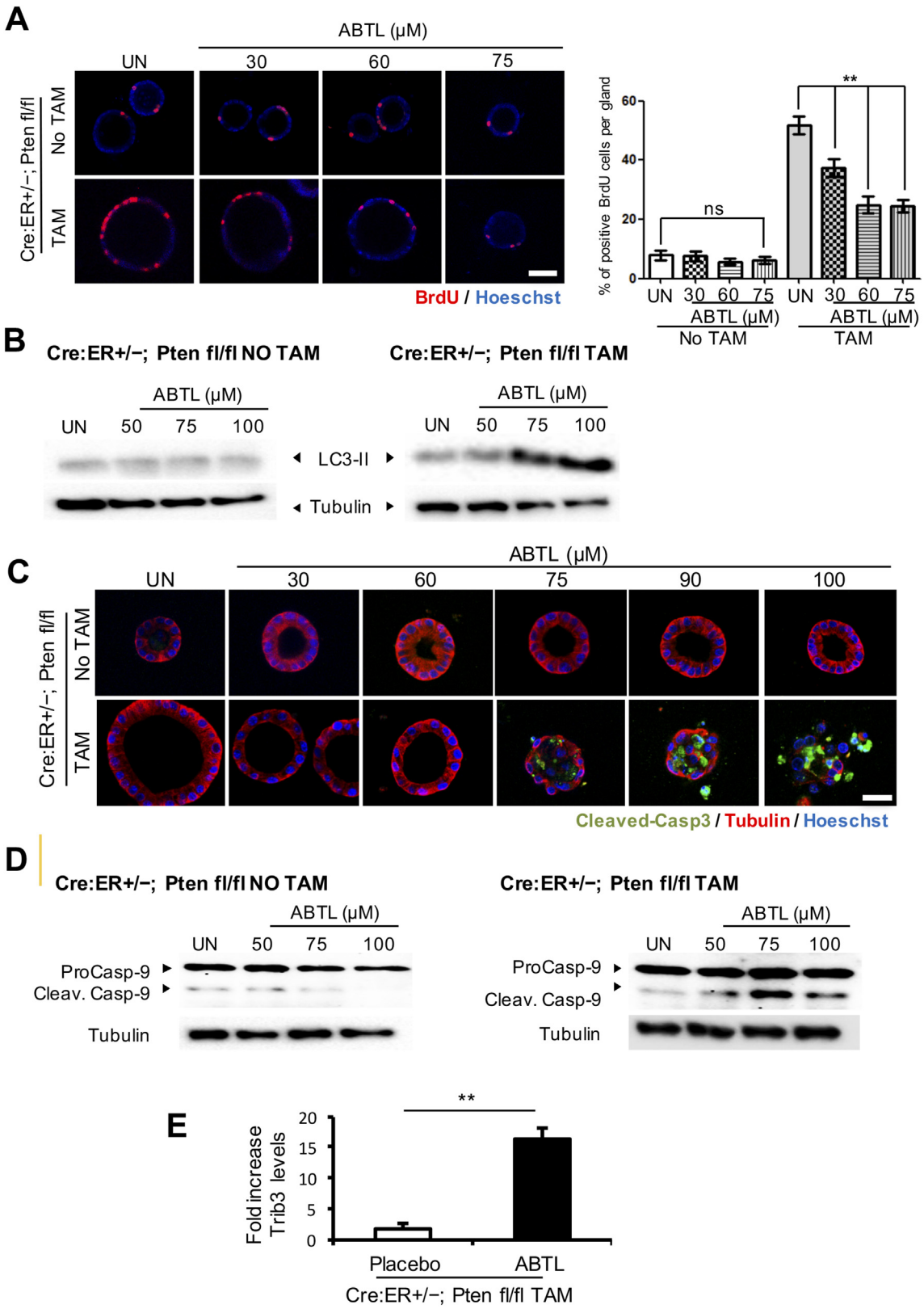

Fig. 3. ABTL0812 treatment attenuates cell growth and induces autophagic mediated cell-death on PTEN null 3D culture cells, but not in wild type cells. A) BrdU and Hoechst immunofluorescence staining images of Cre:ER+/−; Pten fl/fl 3D cultures exposed to tamoxifen (TAM) or not (NO TAM) to induce PTEN deletion and treated 7 days later with the indicated doses of ABTL during 24 h. Quantification data represent mean ± S.E.M of 3 experimental replicates. $^{**}p \leq 0.01$. Scale bar = 25 μm. B) LC3-II protein levels determined by western blot of Cre:ER+/−; Pten fl/fl 3D cultures exposed (TAM) or not (NO TAM) to tamoxifen and treated with increasing doses of ABTL0812. Tubulin was used as loading control. C) Cleaved-caspase 3 immunofluorescence of Cre:ER+/−; Pten fl/fl 3D cultures exposed (TAM) or not (NO TAM) to tamoxifen and treated with increasing doses of ABTL0812 during 48 h. Scale bar = 25 μm. D) Western blot of cleaved-caspase-9 of Cre:ER+/−; Pten fl/fl (TAM) and (NO TAM) 3D cells treated with ABTL0812 for 48 h. Tubulin protein levels were used as loading control. E) RT-qPCR analysis of TRIB3 mRNA expression from Cre:ER+/−; Pten fl/fl TAM 3D cultures *in vitro*. Data represent mean and SEM of seven mice per group. $^{**}p < 0.01$.

Similarly, after 72-h of ABTL0812 treatment, we observed a clear induction of nuclear fragmentation/condensation and caspase 3 activation of pathogenic glands but not in normal endometrium at 75 μM or higher concentrations of ABTL0812 (Fig. 3C, D and Fig. S3B). Finally, we analyzed TRIB3 gene expression in PTEN-deficient cells undergoing apoptosis after ABTL0812 treatment, and we found that TRIB3 levels were significantly increased upon ABTL0812 treatment in those pathogenic endometrium 3D cultures of TAM treated mice (Fig. 3E).

3.4. ABTL0812 treatment impairs the initial progression of endometrial tumorigenesis in a PTEN knock-out inducible mouse model

Next, we sought to investigate whether ABTL0812 was equally effective *in vivo* in our tamoxifen-inducible PTEN knock-out mouse model. We observed that 90% of the mice developed endometrial hyperplasia after three weeks of tamoxifen exposure (Fig. S4A and B). ABTL0812 treatment or placebo was started at this time point and was administrated 5 days per week for 3 weeks. ABTL0812 was well tolerated and

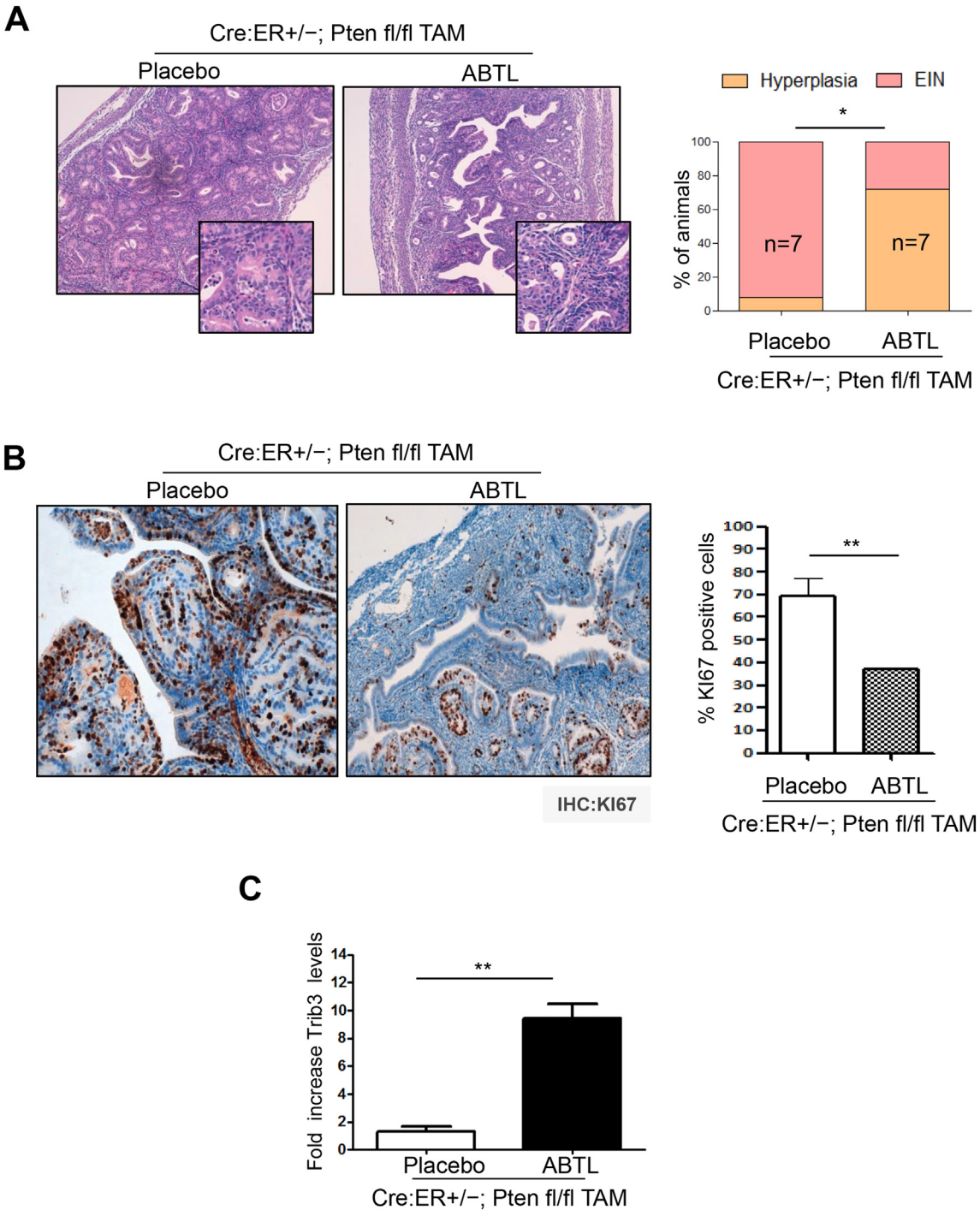

Fig. 4. ABTL0812 attenuates tumor progression in an endometrial PTEN-null model. A) Representative hematoxylin and eosin staining of uterine lesions from placebo and ABTL0812 (120 mg/Kg) - treated Cre:ER+/−; Pten fl/fl inducible mice models. Magnification x10. Quantification of number of animals with hyperplasia or *in situ* carcinomas (EIN) is shown as bar graph due to tamoxifen exposure. $^{*}p < 0.05$. B) Representative immunohistochemical images and quantification of Ki67 performed on uteri dissected from Cre:ER+/−; Pten fl/fl inducible mice models treated with placebo or ABTL0812 (120 mg/Kg). Data represent mean ± SEM of 7 animals per group. $^{**}p < 0.01$. C) RT-qPCR analysis of TRIB3 mRNA expression from Cre:ER+/−; Pten fl/fl TAM epithelial endometrial cell. Data represent mean ± SEM of 7 animals per group. $^{**}p < 0.05$.

no changes in body weight were seen in treated animals (Fig. S4C). Histopathological evaluation of the uteri removed from these animals revealed that ABTL0812-treated mice had endometrial lesions to a significantly lower extent than untreated mice. Placebo-treated mice showed EIN in most animals (92%), whereas most mice receiving ABTL0812 treatment showed just hyperplasia (72%) (Fig. 4A). At molecular level, uteri from mice treated with ABTL0812 showed a substantial reduction in expression of the Ki67 proliferation marker and increased levels of TRIB3 expression in comparison with placebo group (Fig. 4B-C). Importantly, these results proved that ABLT0812 is able to impair progression of hyperplasia, which is the most common precancerous lesion of the endometrium, to cancer.

3.5. ABTL0812 treatment reduces tumor growth in two clinically relevant endometrial carcinoma PDX models

In an attempt to have a more realistic overview of the ABTL0812 efficacy in the clinical scenario, we conducted a preclinical study to test ABTL0812 efficacy in two PDX models of EC: a grade 2 endometrioid (PDX 521) and a grade 3 serous (PDX 548) EC, both bearing PTEN mutations. Both PDX models retained the same tissue architecture and features of the original human tumor (Fig. S5). These models present the advantage of their high predictively drug-response value and their accuracy recapitulating main features of donor tumors [27]. In each model, we studied the use of ABTL0812 as a single agent and in combination with the current standardized first-line therapy in EC, which is carboplatin and paclitaxel (CBPT-PTX). We observed that ABTL0812 treatment inhibited tumor progression with a comparable efficacy to the standard first-line chemotherapy treatment (CBPT-PTX) on both PDX models (Fig. 5A and B). Remarkably, in the serous PDX we observed a significant synergistic effect in ABTL0812 + CBPT-PTX group showing an increased efficacy of the dual treatment in comparison to single treatments. This synergy was also observed *in vitro* using EC cell lines (Fig. S6). We also evaluated in the serous EC PDX model the use of ABTL0812 as a sustained treatment after the first-line of chemotherapy. As seen in Fig. 5B, ABTL0812 was sufficient to clearly retain tumor growth after CBPT-PTX treatment.

Once pharmacologic treatment was finished, we determined by immunohistochemistry that ABTL0812 significantly increased TRIB3 expression compared to untreated animals (Fig. 5C and D). These results indicate that TRIB3 might be a potential biomarker to monitor the therapeutically activity of ABTL0812 in endometrial tumors.

Expression of TRIB3 in blood from EC treated patients treated with ABTL0812.

Currently, a phase 1b/2a clinical trial is being performed in order to evaluate the safety and preliminary efficacy of ABTL0812 in patients with EC (NCT03366480). The study design includes the recruitment of advanced EC patients and the administration of ABLT0812 prior and concomitant to CBPT-PTX treatment. Since we observed that ABTL0812 treatment increases TRIB3 expression levels and it correlates

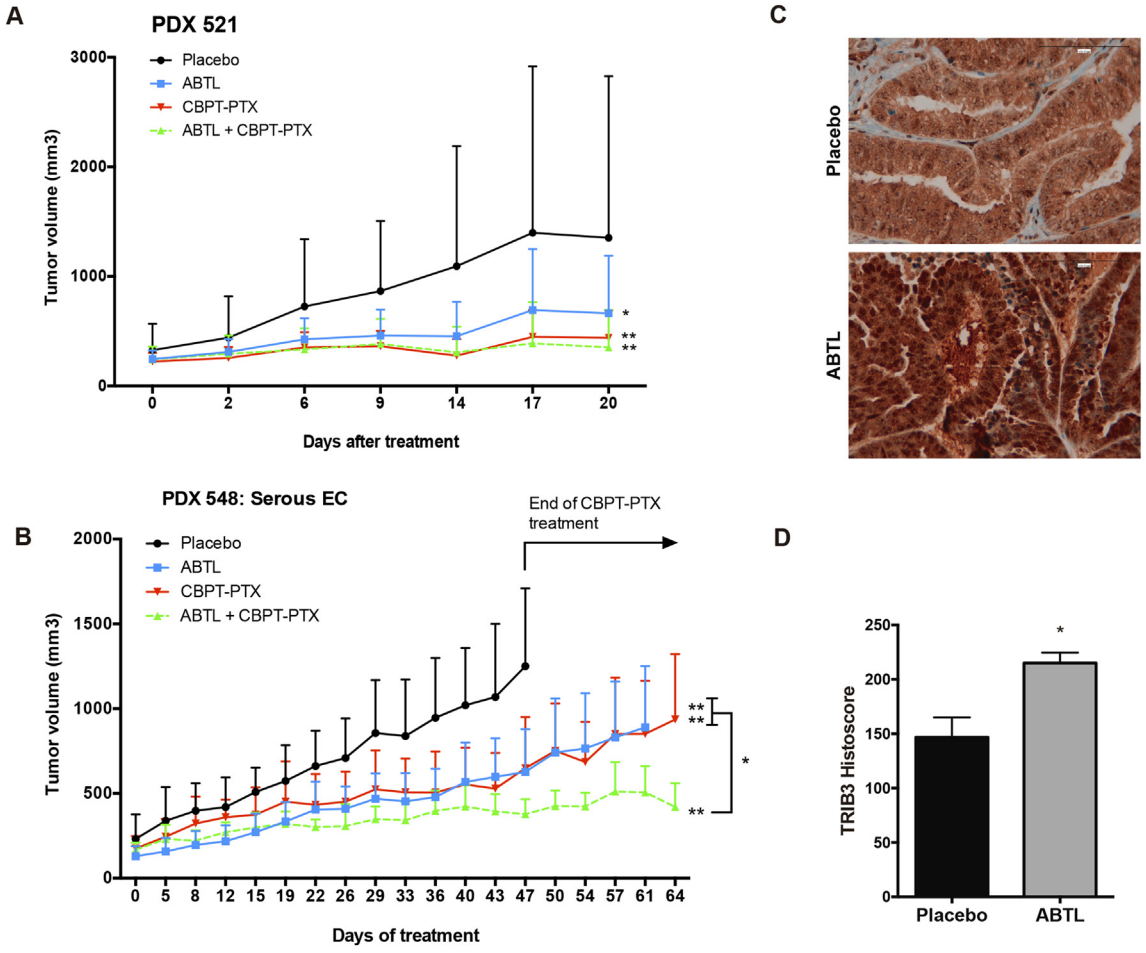

Fig. 5. ABTL0812 impairs tumor growth in two EC models of patient-derived xenografts. A) Mean tumor growth curves from PDX 521 models (Endometrioid IIIA G2 patient) treated for 3 weeks with ABTL0812 (120 mg/kg), Carboplatin/Paclitaxel (30 mg/kg and 50 mg/kg, respectively), ABTL0812 + Carboplatin/Paclitaxel and Placebo. Each data point represents tumor volume average and SD from each group of animals. B) Mean tumor growth curves from PDX 548 models (Serous carcinoma IIIC2 G3 patient) treated for 7–9 weeks with ABTL0812 (120 mg/kg), Carboplatin/Paclitaxel (30 mg/kg and 50 mg/kg respectively), ABTL0812 + Carboplatin/Paclitaxel and Placebo. Each data point represents tumor volume average and SD from each group of animals. C) Representative images of TRIB3 immunohistochemistry staining in sections of PDX 521 treated or not with ABTL0812 (120 mg/kg). D) TRIB3 immunohistochemistry quantification performed on tumor from PDX521 dissected from mice treated with placebo or ABTL0812 120 mg/kg.

with impaired tumor progression in different EC pre-clinical models, we aimed to determine whether if TRIB3 expression was also altered in the peripheral blood samples from EC patients upon ABTL0812 treatment. Blood samples from each patient were obtained before the first ABTL0812 intake, and after 8 h, 7 and 28 days of a daily oral administration with ABTL0812. At day 7, CBPT-PTX treatment started. The analysis of TRIB3 mRNA levels showed that TRIB3 is highly induced by ABTL0812 after 8 h and that this induction is sustained after 7 days of single therapy, and at day 28 when ABTL0812 had been already administered in combination with CBPT-PTX for 21 days (Fig. 6). Although the limited numbers of samples, these preliminary results potentially indicate that TRIB3 could be used as a pharmacodynamic biomarker to monitor ABTL0812 treatment.

4. Discussion

In the present study, we investigated the efficacy of ABTL0812, a novel first-in class antitumor drug, to improve the outcome of EC patients. Here we demonstrated that ABTL0812 treatment reduces viability of a wide panel of endometrioid and non-endometrioid EC cells. The therapeutic benefit was also confirmed in two clinically relevant PDX models of endometrioid and serous EC histologies. Importantly, ABTL0812 did not affect cell viability on non-tumorigenic endometrial epithelial cells and was able to avoid hyperplasic cells to progress to cancer. To achieve this therapeutic effect, we demonstrated that ABTL0812 acts through an induction of TRIB3 expression, impairing PI3K/AKT/mTOR pathway overactivation, and consequently, downregulating mTOR downstream kinases to induce autophagy-mediated cell death.

ABTL0812, unlike other PI3K/AKT/mTOR inhibitors, does not act as allosteric or competitive inhibitor of the PI3K/AKT/mTOR signaling pathway. Recently it has been shown the role of ABTL0812 as a therapeutic agent in lung and pancreatic cancer [14], involving binding and activation of PPAR-α and -γ transcriptional activities, and induction of autophagy through the upregulation of TRIB3. However, in contrast to EC, ABTL0812 induced cell death-autophagy without the activation of pro-apoptotic signaling in lung and pancreatic cancers.

Autophagy has been implicated in the pathogenesis of cancer, and it is usually referred as a 'double-edged sword' for its role in tumor suppression and tumor progression. Besides, autophagy has been proposed to protect from apoptosis, acting as an apoptosis alternative pathway to induce cell death, or act together with apoptosis as a combined mechanism for cell death [28]. However, in EC pathogenesis the contribution of autophagy and its therapeutic potential is still unexplored. Our results demonstrate that induction of autophagy is involved in the mechanism by which ABTL0812 treatment promotes the activation of the pro-apoptotic pathway, thus ending in cell-death. In this study, activation of autophagy occurred *via* TRIB3 overexpression, as it occurs in other cancers [14,29]. TRIB3 is a highly conserved protein belonging to Tribbles family of pseudokinases, responsible for a plethora of functions ranging from glucose regulation, migration of tumor cells, autophagy, and cell cycle control [30]. TRIB3 interacts with multiple targets including v-akt murine thymoma viral oncogene homologue (AKT) [31], transcriptional factors as ATF4 [32] or mitogen-activated protein kinases (MAPKs) [33] which play relevant roles in cancer. In correlation with our previous results, here we show that ABTL0812 up-regulates TRIB3 levels in EC cells thereby promoting inhibitory interaction of this protein with AKT. This interaction regulates the accessibility of AKT to mTOR2, an upstream kinase complex responsible for AKT phosphorylation on Serine 473 residue, consequently p-AKT^{ser473} is decreased as well as downstream mTOR-downstream-serine/threonine-kinase p70s6K.

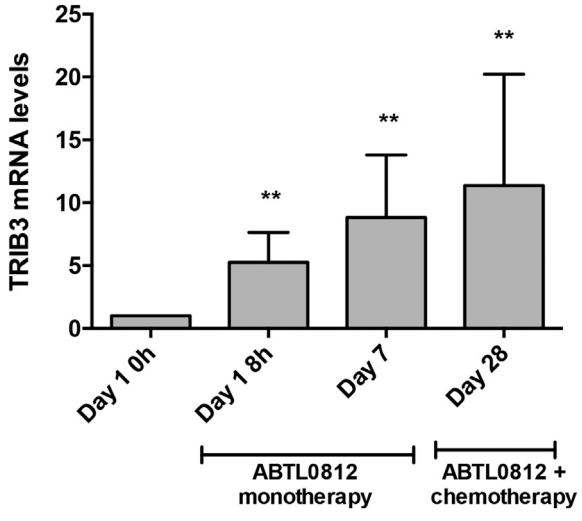

Fig. 6. ABTL0812 analysis in EC Phase 1b/2a clinical trial. TRIB3 mRNA levels evaluated in whole blood samples from 8 patients recruited in Phase 1b/2a clinical trial. Values represented in the graph correspond to the mean of 2^DDCt values and its associated SEMs. Statistical analysis was performed using DDCt values.

The role of TRIB3 in human cancers is largely unclear and dissecting its biological function has been shown to be challenging, presumably because of its context-dependent biology [34]. TRIB3 mRNA levels have been correlated with bad prognosis in certain types of human cancer [31,35]. Conversely, other studies demonstrate that TRIB3 can play onco-suppressive functions [36], which is in accordance with our results. Here we showed that ABTL0812 treatment increased TRIB3 mRNA and protein levels, correlated with tumor growth inhibition, autophagic-mediated cell death, and impairment of endometrial tumorigenesis progression in a PTEN-knockout inducible mouse model (Cre: ER+/— PTENfl/fl). However, normal endometrial epithelial cells did not modify its proliferation rates and autophagy state upon ABTL0812 treatment. We hypothesize that this unique specificity of ABTL0812 towards tumor cells might be related to the higher overactivation of the PI3K/AKT/mTOR signaling pathway in EC cell lines rather than in healthy endometrium.

Importantly, we proved the therapeutic effect of ABTL0812 *in vivo* by using two clinically relevant murine PDX models of advanced endometrioid and non-endometrioid tumors, both bearing PI3KCA mutations. We evaluated the use of ABTL0812 in monotherapy, compared to standard chemotherapy (carboplatin and paclitaxel), and the combination of both therapeutic regimens (chemotherapy and ABTL0812), and we found that ABTL0812 treatment inhibited tumor progression, with a comparable efficacy to the standard chemotherapeutic treatment for the serous EC. Noteworthy, the combination of ABTL0812 with chemotherapy exhibit synergistic activity without adding toxicity, showing great potential to arrest, almost completely, tumor growth in the serous EC PDX model. These results supported the clinical use of ABTL0812 as first-line treatment of EC, together with standard chemotherapy, which is currently being tested in a phase 1b/2a clinical trial. However, further research is needed in order to elucidate the mechanisms of synergy.

Previously, a Phase 1a/1b clinical trial was successfully completed proving a highly safety and tolerability profile of ABTL0812 administered orally [37]. Efficacy was not a primary objective of this study, however, a patient with a platinum-unresponsive IIIC-stage EC carrying mutations in AKT, PTEN, and PI3KCA showed the second longest disease stabilization (14 months) upon ABTL0812 treatment at 500 mg once a day. In the ongoing phase 1b/2a, safety and efficacy of ABTL0812 combined with chemotherapy is investigated, as well as the potential use of TRIB3 expression as a pharmacodynamic biomarker to monitor ABTL0812 treatment. To date, results from eight patients have shown an increase of TRIB3 expression in blood as soon as 8 h after ABTL0812 intake, and this induction was maintained after 7 of single therapy and 28 days of treatment when ABTL0812 had been administered in combination with chemotherapy, supporting TRIB3 as a pharmacodynamic biomarker.

In summary, we provide strong preclinical evidence of the therapeutic benefit of ABTL0812 as first-line treatment of EC, as monotherapy or in combination with standard chemotherapy. We proved that ABTL0812 promotes cell death through TRIB3 activation, which inhibits the PI3k/AKT/mTOR pathway and induces autophagy, specifically on tumor cells but not in non-pathogenic cells. The therapeutic benefit of ABTL0812 permits to also stop progression of precursor lesions to cancer. Our findings present a novel and clinically applicable therapeutic strategy for EC, including those with poorer prognosis.

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Author contribution

Study conception and design: IF, CPM, HP-M, JA, JML, EC, NE, CD, AG-M, XM-G.

Acquisition of data: IF, CPM, CM-L, CL-G, SS-S, PM-G, EM-R. MS, AR, AO, VR-F, NE.

Analysis and interpretation of data: IF, CPM, CM-L, CL-G, SC, SS-S, PM-G, EM-R. HP-M, JA, MY-V, MS, XD, AR, AO, VR-F, JML, CD, AG-M, XM-G, EC, NE.

Drafting of manuscript: EC, NE.

Critical revision: IF, CPM, HP-M, JA, JML, CD, AG-M, XM-G, EC, NE.

Conflicts of interest

ABTL0812 is a drug under development by Ability Pharmaceuticals. PM-G, SS-S, EM-R; HP-M, MY-V, CD and JA are Ability Pharmaceuticals employees; CD holds shares of the company; JML is member of its Scientific Advisory Board. The other authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.03.002.

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