FoxO3a Mediates the Inhibitory Effects of the Antiepileptic Drug Lamotrigine on Breast Cancer Growth

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Abstract

Breast cancer is a complex and heterogeneous disease, with distinct histologic features dictating the therapy. Although the clinical outcome of breast cancer patients has considerably improved, the occurrence of resistance to common endocrine and chemotherapy treatments remains the major cause of relapse and mortality. Thus, efforts in identifying new molecules to be employed in breast cancer therapy are needed. As a “faster” alternative to reach this aim, we evaluated whether lamotrigine, a broadly used anticonvulsant, could be “repurposed” as an antitumoral drug in breast cancer. Our data show that lamotrigine inhibits the proliferation, the anchorage-dependent, and independent cell growth in breast cancer cells (BCC), including hormone-resistant cell models. These effects were associated with cell-cycle arrest and modulation of related proteins (cyclin D1, cyclin E, p27Kip1, and p21Waf1/Cip1), all target genes of FoxO3a, an ubiquitous transcription factor negatively regulated by AKT. Lamotrigine also increases the expression of another FoxO3a target, PTEN, which, in turn, downregulates the PI3K/Akt signaling pathway, with consequent dephosphorylation, thus activation, of FoxO3a. Moreover, lamotrigine induces FoxO3a expression by increasing its transcription through FoxO3a recruitment on specific FHRE located on its own promoter, in an autoregulatory fashion. Finally, lamotrigine significantly reduced tumor growth in vivo, increasing FoxO3a expression.

Implications: The anticonvulsant drug lamotrigine shows strong antiproliferative activity on breast cancer, both in vitro and in vivo. Thus, drug repurposing could represent a valuable option for a molecularly targeted therapy in breast cancer patients. Mol Cancer Res; 1–12. ©2018 AACR.

Introduction

Breast cancer is the second most frequent malignancy and the most common cancer among women in the industrialized countries (1). Both the disease classification and the treatment are based on its histologic features. Therefore, the endocrine therapy (e.g., tamoxifen, fulvestrant, and aromatase inhibitors) is used for hormone-sensitive (ER+–) tumors (about 70%–80% of all breast cancers), the mAb trastuzumab is still the mainstay of treatment for HER2/neu overexpressing breast cancers (20%–30% of cases), while chemotherapy is used to cure triple-negative breast cancer (TNBC) patients (10%–20% of breast cancers; ref. 2). However, the occurrence of resistance to treatments and the consequent therapeutic inefficacy, demands a continuous effort in trying to discover new molecules to be employed as adjuvant therapy in breast cancer patients. Although research on new chemical entities is thriving, the slowness of drug approval procedures is discouraging, thus turning to off-label use of already approved medicines is a widespread practice among physicians in the attempt to care for several diseases.

In this perspective, in the past decades, several antiepileptic drugs (AED) have been associated with anticancer activity in several cancer cell types, including breast, both in vitro and in vivo (3–8). As very few and inadequate information is available in the current literature, here we aimed to investigate the potential antitumoral activity of lamotrigine, a synthetic phenyltriazine anticonvulsant with anticonvulsant properties, commercially available since mid 1990s for the treatment of epilepsy and bipolar disorder (9). The mechanism through which lamotrigine exerts its antiseizure action is ascribed to its ability of blocking the voltage-activated Na+ and Ca2+ channels (10). However, since lamotrigine effect on signal transduction has not been extensively studied and since the most common therapeutic targets for antitumoral drugs are receptors, protein tyrosine kinases and the phosphatidylinositol 3-kinase (PI3K)/Akt cell survival pathway, we explored that potential interference that lamotrigine could directly or indirectly...
exert on intracellular signaling molecules in breast cancer cells (BCC). The biological effects and molecular mechanism of lamotrigine were investigated in both ER⁺ (including tamoxifen-resistant cells models) and ER⁻ BCCs in vitro, while its influence on breast tumor growth was tested in vivo, using a xenograft model.

**Materials and Methods**

**Chemicals and reagents**

DMEM/Nutrient mixture F-12, Ham, DMEM, 100 bp DNA ladder, -glutamine, penicillin, streptomycin, BSA, PBS were purchased from Invitrogen. The RETROscript kit and DNase were provided from Ambion. Lamotrigine (Lamictal) was purchased from GlaxoSmithKline. Protease inhibitors, formaldehyde, NP-40, MITT, dimethyl sulfoxide were from Sigma (Milan, Italy). ECL System was from Bio-Rad.

**Cell cultures**

The human BCC lines MCF-7 and MDA-MB-231 were acquired from Interlab Cell Line Collection (ICLC, Genova, Italy), where they were authenticated. Cells were stored according to manufacturer’s instructions, and used within 6 months after frozen aliquots resuscitations. MCF-7 cells were grown in DMEM-F12 and MDA-MB-231 cells were cultured in DMEM. All media contained 5% FBS, 1% -Glutamine, and 1 mg/mL penicillin-streptomycin. Mycoplasma negativity was tested monthly (MycAlert, Lonza). Tamoxifen-resistant MCF-7 cells (MCF-7/TR) were developed as described previously (11), starting from MCF-7 wild type. The resistance to tamoxifen of MCF-7/TR cells has been described previously (12). Brieﬂy, cells were seeded in growing medium (GM), serum starved overnight to synchronize cells in G0 phase, or in reduced serum conditions, or in complete growing medium, giving comparable results.

**Cell proliferation assays**

Cell proliferation was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan (MTT) assay as described previously (12). Briefly, cells were seeded in growing medium (GM), serum starved overnight to synchronize cells in G0 phase and then treated or not with lamotrigine 30 µmol/L for 24, 48, and 72 hours. At each time point, 2 mg/mL MTT was added to the medium and the product of reaction was the dissolved in DMSO and quantified spectrophotometrically. The IC50 values were calculated using GraphPad Prism 4 (GraphPad Software, Inc.).

**Soft agar anchorage-independent growth assays**

Cells (10⁶/well) were resuspended in GM, containing 0.35% agarose, and plated on a 0.7% agarose bottom layer in 6-well plates. Two days after plating, GM containing vehicle or treatments (lamotrigine at 30 and 50 µmol/L) were added to the top layer and replaced every 3 days. After 14 days, 500 µL of MTT was added to each well and allowed to incubate at 37°C for 4 hours. Plates were then kept at 4°C overnight and colonies >50 µm diameter from triplicate assays were counted under an inverted microscope (Olympus BX51).

**Clonogenic assay**

Cells (1,000 cells/plate) were plated in GM in 12-multiwell plates and, the next day, treated with lamotrigine 30 or 50 µmol/L. Medium and lamotrigine treatment was renewed every 3 days. After 14 days, surviving colonies were fixed and stained with Crystal Violet stain and photographed at 4× magnification under an Olympus BX51 inverted microscope.

**Flow cytometry**

A total of 5 × 10⁵ cells were serum starved overnight and then treated or not with lamotrigine for 24 hours. Cells were harvested by trypsinization, resuspended in propidium iodide solution, and processed as described before (13). The DNA content was measured using a FACScan flow cytometer (BD Biosciences) and the data acquired using CellQuest and analyzed by ModFit LT softwares.

**Western blot analysis and antibodies**

Cells were treated as indicated in each experiment before lysis. Equal amounts of protein extracts were subjected to SDS-PAGE, as described previously (14). Western blot images are representative of at least three independent experiments. Antibodies: cyclin D1 (sc-718), cyclin E (sc-481), p27Kip1 (p27) (sc-53871), p21cip1/waf1 (p21) (sc-756), PTEN (sc-7974), AKT (sc-81434), GSK3β (sc-81462), GADD (sc-25778), and β-actin (sc-69879) were provided by Santa Cruz Biotechnology, Inc, while phosphorylated-FoxO3a (p-FoxO3a Ser253; cat# 13129), FoxO3a (cat# 2497), p-AKT (Ser473; cat# 4060), and p-GSK3β (Ser9; cat# 9323) were purchased from Cell Signaling Technology.

**RNA extraction, reverse transcription, and real-time PCR**

Subconfluent cells were serum starved overnight and then treated with lamotrigine for indicated times. Total RNA was isolated using NucleoZOL (Macherey-Nagel GmbH & Co. KG), according to the manufacturer’s instructions. Two µg of total RNA were reverse transcribed with RETROscript kit (Ambion, Life Technologies). One microliter of diluted (1:3) cDNA was analyzed in triplicate by qRT-PCR in iCycler iQ detection system (Bio-Rad), using SYBR green Universal PCR Master Mix (from Bio-Rad) and a FoxO3a-specific pair of primers [forward, 5’-CGGAAAACGCGTCCACTCTT-3’; rev, 5’-GGACCCCGATGACGCTTAT-3’ (FoxO3a)]. Samples were normalized versus GAPDH mRNA content, using the following primers: forward, 5’-CCCCACTCCCTCCACCTTGA-3’; reverse, 5’TGGTCTGTAGCCCCAAAATCGT–3’ (GAPDH). The results were calculated and expressed as previously reported (15).

**Chromatin immunoprecipitation**

Subconfluent cultures (70%) of MDA-MB-231 cells were serum starved overnight and then treated with lamotrigine for indicated times or left untreated. ChIP methodology was performed as described previously (15). The immuno-cleared chromatin was precipitated with anti-FoxO3a pAb (Cell Signaling Technology, #2497). Normal rabbit IgG (Cell Signaling Technology, #2729) was used instead of primary Ab as negative control. A sequence (including tamoxifen-resistant cells models) was used for amplification of the considered was amplified as control [forward, 5’-CGCTTCCCCAAGCGG-3’; reverse, 5’-CCGCCGCTTCATCAGCA-3’ (168 bp)]. Immunoprecipitated DNA was analyzed by qRT-PCR. A pair of primers (forward, 5’-ACCCACCGCCGATGACGCTTATC-3’; reverse, 5’-CCGCCGCTTCATCAGCA-3’; 175 bp) mapping the FHIRE-containing FoxO3a promoter region (16) was used. Results were expressed as fold differences of the ratio FoxO3a prom/Input over time 0.
data are reported as the mean ± SD of three different experiments, each performed in triplicates. ∗, P ≤ 0.05 versus control. In Western blot analysis, the band intensities were evaluated in terms of optical density and expressed as percentage versus control. Wilcoxon–Mann–Whitney test was used for the statistical comparisons for in vivo studies. P < 0.05 was considered statistically significant.

Results

Lamotrigine decreases BCC growth

The effect on cell viability of increasing doses of lamotrigine (1, 10, 20, 30, 50, and 100 μmol/L) was assessed on two human BCCs lines, the highly aggressive MDA-MB-231 and the less aggressive MCF-7. Our results show that lamotrigine treatment significantly inhibits cell proliferation in a dose-dependent manner in both cell lines (Fig. 1A). The half-maximal inhibitory concentration (IC_{50}) values for lamotrigine in the tested cell models are reported in Fig. 1B.

Similarly, lamotrigine treatment significantly inhibited, in a dose-dependent manner, the ability to form colonies in BCCs, in both anchorage-independent (soft agar assay, Fig. 1C) and anchorage-dependent conditions (clonogenic assay, Fig. 1D). These results suggest that lamotrigine is able to exert antiproliferative effects in breast tumor cells, regardless their ER status.

Effects of lamotrigine on cell-cycle profile of BCCs

To determine whether the cell growth inhibition induced by lamotrigine is a consequence of cell-cycle perturbation, flow cytometric cell-cycle analysis was performed in BCCs lines after 24 hours of treatment with 30 μmol/L lamotrigine. Lamotrigine caused a cell-cycle arrest in G0–G1 phase, with a concomitant reduction of the fraction of cells in G1–M-phase in both MCF-7 and MDA-MB-231 cells (Fig. 1E). To characterize the molecular mechanism associated with the antiproliferative effects induced by lamotrigine, the expression levels of proteins associated with the regulation of cell-cycle progression were also examined. Our results show that p27^{kip1} and p21^{Waf1/Cip1} were upregulated, whereas the levels of cyclin D1 and cyclin E were decreased in response to lamotrigine treatment in both BCCs (Fig. 1F).

Lamotrigine induces the expression of FoxO3a in BCCs through the inhibition of the PI3K/AKT signaling pathway

To unveil the molecular mechanism through which lamotrigine exerts its antiproliferative effect on BCCs, changes in the activation status of both the mitogen Ras/MAPK and the survival PI3K/AKT pathways were investigated.

In both MCF-7 and MDA-MB-231 cell lines, lamotrigine treatment for 24 hours markedly reduces AKT phosphorylation without affecting its protein levels, as compared with untreated cells. This result associated to an evident decrease in p-GSK3β levels (Fig. 2). No changes were observed neither in the activation nor in the expression of MAPK (Erk1/2; Supplementary Fig. S1A and S1B).

Being the PI3K/AKT pathway involved, lamotrigine effect was tested on two additional upstream and downstream members of the survival pathway, such as the main upstream negative regulator, PTEN, and the AKT downstream target, FoxO3a, a well-studied transcription factor known to control important cell-cycle regulators and proapoptotic genes (19), as well as PTEN itself (20, 21). Interestingly, lamotrigine treatment for 24 hours
Figure 1.
Lamotrigine (LTG) inhibits BCCs growth and cell-cycle progression. 

A, Cell viability (MTT assay). Cells were treated with vehicle (−) or with increasing concentrations (1, 10, 20, 30, 50, 100 μmol/L) of lamotrigine for 24, 48, and 72 hours, as indicated. The results are expressed as % respect to control cells (−/C0).

B, Values of lamotrigine half-maximal inhibitory concentration (IC50) for the indicated BCCs were calculated at 72 hours using GraphPad Prism 4 (GraphPad Software).

C, Soft agar growth assay. Cells were plated in 0.35% agarose and treated with vehicle (−/C0) or lamotrigine 30 and 50 μmol/L (see Materials and Methods for details).

D, Clonogenic assay. Cells were plated in duplicate in 6-well plates and treated as in C. After 2 weeks, colonies were stained with crystal violet and counted.

E and E1, Cells were treated with vehicle and lamotrigine at 30 μmol/L for 24 hours, and cell-cycle distribution was assessed by flow cytometry. The expression of cell-cycle-related proteins (cyclin D1, cyclin E, p27Kip1, and p21Waf1/Cip1) were evaluated by Western blot analysis. Cells were treated for 24 hours with vehicle (−) or lamotrigine 30 μmol/L. Equal amounts of total cellular extract (50 μg) were analyzed. GAPDH was used as loading control. The graphs (F1 and F2) show the densitometric analysis and protein normalization versus GAPDH.
was able to induce PTEN expression, which well correlates with the PI3K/AKT axis inhibition. Moreover, as expected, AKT inactivation in lamotrigine-treated samples was followed by FoxO3a dephosphorylation at Ser253 (one of the three AKT phosphorylation sites on FoxO3a), albeit a strong upregulation of FoxO3a protein expression was observed. Nevertheless, the concomitance of both these events (dephosphorylation and protein upregulation) is consistent with FoxO3a activation (Fig. 2).

Interestingly, FoxO3a protein increase observed in cells exposed to lamotrigine is accompanied by a gradual rise in FoxO3a mRNA expression, starting as soon as after 6 hours of treatment (Fig. 3A and B). Thus, a transcriptional regulation seems to be responsible of the lamotrigine-dependent FoxO3a induction. As a positive autoregulatory loop has been described for FoxO3a (16), ChIP experiments were performed on MDA-MB-231 cells, confirming that, in presence of lamotrigine, FoxO3a is recruited on a specific Forkhead response element (FHRE) containing region of its own promoter (ref. 16; Fig. 3C), in a time-dependent manner, reaching a peak at 1.5 hours of lamotrigine exposure (Fig. 3D).

PTEN and FOXO3a mediate the antiproliferative effect of lamotrigine on BCCs

Once verified that lamotrigine inactivates the PI3K/AKT axis and PTEN increased expression, we questioned whether PTEN increased expression following the exposure to the drug might be relevant for lamotrigine action. Indeed, PTEN silencing (siPTEN) abrogated the inhibitory effect exerted by the drug on the proliferation of MCF-7 and MDA-MB-231 BCCs (Fig. 4A and B). Noteworthy, lamotrigine did not show any relevant effect on the growth of PTEN-negative MDA-MB-468, nor in ZR-75-1 BCCs (Supplementary Fig. S2A–S2C), which bear a hemizygous deletion of PTEN and a missense mutation in the remaining allele (22), while lamotrigine shows an antiproliferative activity in another PTEN-positive BCCs, T47D (Supplementary Fig. S2A and S2D), corroborating the idea that PTEN is a pivotal mediator of lamotrigine activity.

Moreover, to confirm FoxO3a involvement in lamotrigine mechanism of action, it was silenced, in the same cells, by using a specific siRNA (siF3a). Similarly to siPTEN, FoxO3a silencing was also able to counteract lamotrigine-mediated growth retardation in both cell lines (Fig. 4C and D). These observations were paralleled by the restoration of cyclin D1 protein levels in lamotrigine-treated siF3a samples, compared with the relative control (siScramble), where lamotrigine treatment led to cyclin D1 downregulation. Concomitantly, siF3a inhibited the lamotrigine-mediated induction of p27Kip1 (Fig. 4E and F). These results suggest that lamotrigine modulates cell-cycle regulators through FoxO3a in BCCs.

Effects of lamotrigine on MCF-7/tamoxifen-resistant BCCs

The antineoplastic potential of lamotrigine was also investigated in tamoxifen-resistant MCF-7 cells (MCF-7/TR). To this aim, cells were subjected to the same experiments conducted in MCF-7 and MDA-MB-231 cells. Thus, lamotrigine effects on MCF-7/TR growth were assessed by MTT and anchorage-dependent and independent growth assays. Our results show that lamotrigine reduces cell vitality in a dose-dependent manner (Fig. 5A). The half-maximal inhibitory concentration (IC50) values for the active compound are shown in Fig. 5B. Interestingly, the concomitant exposure to tamoxifen and lamotrigine in parental MCF-7 showed the same synergistic effect observed in MCF-7/TR (Fig. 5C). In addition, the drug strongly interferes with the ability of MCF-7/TR cells to form colonies both in suspension (soft agar assay, Fig. 5D) and in adhesion (clonogenic assay, Fig. 5E). As observed in MCF-7 and MDA-MB-231 cells, also in MCF-7/TR these results were accompanied by a general inhibition of the PI3K/AKT axis with the consequent FoxO3a activation. In fact, a gradual increase in FoxO3a mRNA expression was observed after lamotrigine treatment up to 24 hours (Fig. 5F). Concomitantly,
both FoxO3a dephosphorylation and protein upregulation at the same endpoint well fit with its active state (ref. 23; Fig. 5G).

The efficacy of lamotrigine on tamoxifen-resistant breast cancer encourages its potential use as an adjuvant therapy for those patients who result refractory to the antiestrogen treatment.

Lamotrigine inhibits breast cancer tumor growth in vivo

Finally, the effects of lamotrigine were evaluated on the development and progression of breast carcinomas in nude mouse models. To this aim, female nude mice injected with MDA-MB-231, were treated with vehicle (–) or lamotrigine (10 and 20 mg/kg/day). All the in vivo procedures were well tolerated as no change in body weight or in food and water consumption, nor evidence of reduced motor function were observed. In addition, no significant difference in the mean weights and histologic features of the major organs (liver, lung, spleen, and kidney) after sacrifice was observed between vehicle-treated mice and those that received lamotrigine treatment. Our data show that on day 28, a significant reduction in tumor volume was observed in mice treated with lamotrigine (60% at a dose of 20 mg/mL and 40% at a dose of 10 mg/mL; Fig. 6A and B).

Hematoxylin and eosin staining of tumor tissue are shown in Fig. 6C. Moreover, the epithelial nature of the tumors was verified by immunostaining with antibody directed against human cytokeratin 18 (Fig. 6C). To determine whether lamotrigine effects on tumor growth were associated with changes in the mitotic index, the expression of Ki-67, as a marker of proliferation, was evaluated in xenograft tumor sections. As shown in Fig. 6D, a significant reduction of Ki-67 was observed in tumors explanted from mice treated with lamotrigine. Notably, a concomitant dose-dependent increase in FoxO3a, and p27Kip1 expression and the inhibition of AKT and FoxO3a phosphorylation as well as of cyclin D1 expression was also evidenced, confirming our in vitro findings (Fig. 6E and F).

Discussion

In recent years, major advances in breast cancer therapy have been obtained. Nevertheless, as resistance to treatment still occurs in a
relatively high percentage of patients, it remains the second most common cause of cancer-related death among women, thus new and more effective treatments are still required (1). Unfortunately, the development of new drugs is a long and costly process and only 5%–10% of preclinically efficacious molecules successfully translate to the clinical setting. Therefore, drug repositioning, that is, the use of already known drugs to treat other diseases, certainly gives a significant advantage over traditional drug development, having the molecule already passed the required toxicity and safety tests, thus greatly accelerating the whole approval process (24).

In this context, several AEDs have shown anticancer activity, influencing cell growth and apoptosis in several cancer cell types in vitro (4, 6). Here we investigated the potential use of the well-known anticonvulsant lamotrigine as an antitumoral agent in breast cancer therapy. Indeed, lamotrigine is able to inhibit the proliferation, the anchorage-dependent and independent cell growth of BCCs in vitro and tumor growth in vivo. Moreover, lamotrigine treatment interferes with cell-cycle progression, blocking cell population in G0–G1 phase, by reducing the expression of cyclin D1 and cyclin E, the main players of the G1–S phase transition, and by inducing the cyclin-dependent kinase (CDK) inhibitors p21Waf1/Cip1 and p27Kip1.

As p27Kip1, p21Waf1/Cip1, cyclin D1 and cyclin E are all downstream targets of FoxO transcription factors (19), we questioned if lamotrigine could exert its activity by affecting the PTEN/PI3K/AKT/FoxO axis. In fact, since activation of this pathway is implicated in tumor pathogenesis and in the development of resistance to anticancer therapies, targeting this signaling axis still represents one of the most promising options to explore in cancer treatment.

**Figure 4.**
PTEN and FOXO3a mediate lamotrigine (LTG) effects on BCCs. A and B, Indicated cells were transfected with scrambled siRNA (siScramble) or siPTEN as described in Materials and Methods and then treated or not with lamotrigine (30 μmol/L). Variation in cell proliferation were determined after 72 hours of drug treatment. *, P ≤ 0.05 versus control. Total proteins were extracted from a duplicate set of cells and subjected to Western blot analysis, to assess PTEN silencing efficacy. GAPDH was used as loading control. Representative images of four independent experiments are shown. C and D, A similar proliferation experiment was conducted by silencing FoxO3a (siF3a) in place of PTEN in presence or not of lamotrigine for 72 hours. *, P ≤ 0.05 versus control. E and F, A duplicate set of cells was treated as in C (MCF-7, E) and D (MDA-MB-231, F) and protein extracts were subjected to Western blot analysis to assess FoxO3a silencing efficacy and its effect on cyclin D1 and p27 expression. GAPDH was used as loading control. Representative images of three independent experiments are shown.
Figure 5.
Lamotrigine (LTG) growth-inhibitory activity on MCF-7/TR cells. A, Cell viability. MCF-7/TR cells were treated or not with increasing concentrations of lamotrigine for indicated time points. The results are expressed as % of growth inhibition respect to untreated cells (\( /C_0 \)). B, The IC\(_{50}\) values for MCF-7/TR cells are presented. C, MCF-7 cells were treated with lamotrigine (30 \( \mu \text{mol/L} \)), tamoxifen (1 \( \mu \text{mol/L} \)), or a combination of both for indicated time points. The graph shows the percentage of growth inhibition respect to untreated controls. D, Soft agar growth assay in MCF-7/TR cells treated at indicated lamotrigine concentrations. E, MCF-7/TR cells were treated as in Fig. 1D and the stained plate were photographed. The graph shows the % of reduction in clonogenic ability of lamotrigine-treated cells versus control. F, FoxO3a mRNA content evaluated by qRT-PCR in MCF-7/TR cells treated for 6, 12, and 24 hours with vehicle (\(-\)) or lamotrigine 30 \( \mu \text{mol/L} \). GAPDH mRNA levels were determined as a control. G, MCF-7/TR cells were treated (\(+\)) or not (\(-\)) with lamotrigine 30 \( \mu \text{mol/L} \) for 24 hours. Expression of indicated proteins (50 \( \mu \text{g/line} \)) was analyzed by Western blot analysis (G1, densitometric analysis: phosphorylated forms were normalized on relative total proteins which have been previously normalized versus GAPDH, used as loading control).
Lamotrigine (LTG) inhibits MDA-MB-231–derived xenograft tumors in vivo. A, MDA-MB-231 cells were injected subcutaneously in female nude mice (see Materials and Methods). Tumor growth was monitored by caliper, measuring the visible tumor sizes at indicated time points. *, P < 0.05 versus control. B, At the end of experiment, tumors were explanted and representative tumor images are shown. C, FFPE sections of tumor xenografts were stained with hematoxylin and eosin Y. The epithelial nature of the tumors was verified by immunostaining with anti-human cytokeratin 18 antibody. D, The expression of Ki-67 and FoxO3a were evaluated in tumor sections deriving from mice treated or not (−) with lamotrigine. NC, negative control. E and F, 50 μg of lysates from explanted tumors were subjected to Western blot analysis for the detection of the indicated proteins. β-Actin was used as loading control. Fifty micrograms of lysates from MDA-MB-231 cells were loaded as a positive control (PC).
(25). In particular, we focused our attention on FoxO3a, one of the members of the winged-helix forkhead transcription factors family, whose functions (regulation of genes promoting cell-cycle arrest, stress resistance, apoptosis, DNA damage repair and metabolism; refs. 26, 27) are negatively regulated by the PI3K/AKT signaling (28). FoxO3a has been recognized as a tumor suppressor gene in breast cancer (29) and restoration of its expression and activity is being exploited in cancer therapy (26, 30).

The results reported here confirm our hypothesis, as lamotrigine treatment markedly reduces the phosphorylation of AKT and its direct downstream targets FoxO3a and GSK-3β, while, in agreement with another study conducted on human neuroblastoma SH-SY5Y cell line (31), it did not have any effect on ERK/MAPK pathway. Moreover, our data show that lamotrigine exerts its antiproliferative action through PTEN and FoxO3a, since: (i) lamotrigine increases their protein expression (Fig. 2), which perfectly fits with an inhibited AKT signaling; (ii) either PTEN or FoxO3a knockdown abrogate the lamotrigine-dependent growth inhibition (Fig. 4). Lamotrigine was also able to induce FoxO3a mRNA transcripts, most likely as a consequence of FoxO3a recruitment on its own promoter, confirming the possibility of a positive autoregulatory loop already described by Lutzner and colleagues (16). It is worth noting that PTEN has been described as a FoxO3a target gene (20, 21), thus, in lamotrigine-treated samples, PTEN increase could very likely directly depend on FoxO3a activation.

The identification of lamotrigine main target is currently under investigation in our laboratory, starting from the assumption that lamotrigine exerts its antiseizure action by blocking the voltage-gated sodium channel (VGSC; ref. 32) and Ca2+ channels (N-, P/Q/R- and, weakly, T-types VGCCs; ref. 33).

VGSCs are drug targets for the treatment of epilepsy and cardiac arrhythmia, but they have recently become a research focus of interest as it emerged that VGSCs (in particular Na1,5) are also upregulated in various cancers, including breast, favoring an invasive/metastatic phenotype. Moreover, Na1,5 α-subunit expression associates with poor prognosis in clinical breast cancer specimens, suggesting that VGSCs may have utility as prognostic markers for breast cancer progression and may be a novel molecular target for breast cancer treatment. Consistently, AEDs have been shown to inhibit cancer cell proliferation, invasion, tumor growth and metastasis in preclinical models, including breast cancer (7, 34, 35), and their use is inversely associated with cancer risk (e.g., colorectal, lung, and gastric cancers and hematologic malignancies; ref. 36).

Similarly, T-type calcium channels are often deregulated and aberrantly expressed in cancer cells, including breast cancer (37), supporting their proliferation, survival, and resistance to treatment. Therefore, VGCCs blockers could represent a promising tool for anticancer therapy (38, 39). In fact, consistent with the observation that Ca2+ entry activates Akt/PKB in a PI3K-independent manner in neuroblastoma cells (40), T-type calcium channel inhibition was reported to interfere with mTOR/AKT pathway in human lung adenocarcinoma cells (41) and to disrupt Akt signaling, promoting apoptosis, in glioblastoma cells (42). Nevertheless, VGCCs blockers have also been reported to activate Akt, increasing GSK3 inhibitory phosphorylation (43), as well as to cause GSK3 dephosphorylation in several cell culture systems (44).

Therefore, considering that the role of VGSCs and VGCCs blockers strongly depends on the different cell model used (e.g., protective in neuronal cells (45, 46) and antiproliferative and/or antimetastatic in cancer cells (7, 42)), further studies are necessary to demonstrate our hypothesis on lamotrigine mechanism of action in breast cancer (schematically represented in Fig. 7).

Interestingly, lamotrigine activity does not depend on ER, as the same antitumoral behavior, accompanied by FoxO3a activation, was observed in ER− MCF-7, in ER− MDA-MB-231 (which are also a model of TNBC) and in cells with altered ER signaling, as the tamoxifen resistant BCCs. This broadens lamotrigine potential therapeutic application not only to ideally any subtype of tumors, but also to the subgroup of breast cancer patients developing hormone resistance (Fig. 5).

The clinical utility of lamotrigine in human breast cancer therapy was very well supported by our in vivo experiments (Fig. 6). Strikingly, an evident tumor growth reduction was evidenced in mice implanted with MDA-MB-231 BCCs and receiving lamotrigine treatment. Again, in tumor sections deriving from lamotrigine-treated mice, FoxO3a levels were significantly increased.

It is important to underline that the antiproliferative effect of lamotrigine in vitro and in vivo is not due to a dose-related toxicity. Indeed apoptotic cells were detected neither in cell-cycle analysis (Fig. 1E) nor through TEM observations (Supplementary Fig. S3A) and TUNEL assay (Supplementary Fig. S3B), nor hallmarks of apoptosis, necrosis, or necroptosis were detected in cells treated with increasing concentrations (10−30 μmol/L) of lamotrigine (Supplementary Fig. S4A) or in tumor extracts from lamotrigine-treated mice (Supplementary Fig. S4B), confirming that lamotrigine mainly acts as a cell-cycle inhibitor and not as a cytotoxic agent (see Supplementary Data). Moreover, lamotrigine safety for cancer patients without epilepsy has been recently confirmed by a.
phase III clinical trial aimed to evaluate the effect of lamotrigine on pain and other neuropathic symptoms deriving from the chemotherapy-induced peripheral neuropathy (CIPN; ref. 47). Although the results were negative regarding the relief from CIPN, the study reported mild and similar toxicities in lamotrigine-treated patients, compared with placebo receiving patients.

In summary, our data demonstrate that “repurposing” the anticonvulsant drug lamotrigine, for its ability to activate the attractive therapeutic target FoxO3a (30), might represent a promising alternative strategy for breast cancer treatment, also in those patients who result refractory to the hormonal therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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