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γ -Secretase inhibition promotes cell death, Noxa upregulation, and sensitization to BH3 mimetic ABT-737 in human breast cancer cells

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Abstract

Introduction: Inappropriate Notch signaling, downstream of γ -secretase activity, is understood to have tumor-promoting function and to be associated with poor outcome in cancer, of the breast in particular. The molecular basis of antitumoral effects of its inhibitors, however, remains poorly characterized. Moreover, the effects of their combination with the pro-apoptotic pharmacologic inhibitor of Bcl-2/Bcl-xL, ABT-737, have never been evaluated. In this study, we thus specifically addressed the biologic consequences of targeting γ -secretase and Bcl-2/Bcl-xL, alone or simultaneously, in breast cancer cell lines as well as in a novel human breast cancer *ex vivo* assay.

Methods: By using *in vitro* 2D or 3D cultures of breast cancer cells plus a novel preclinical short-term *ex vivo* assay that correctly maintains human mammary tissue integrity and preserves tumor microenvironment, we tested the effects of the pharmacologic γ -secretase inhibitor GSIXII used as a single agent or in combination with ABT-737.

Results: We show herein that the γ -secretase inhibitor, GSIXII, efficiently induces apoptosis in breast cancer cell lines by a process that relies on the induction of Noxa, a pro-apoptotic Bcl2-homology 3 domain (BH3)-only protein of the Bcl-2 family that functions as an inhibitor of antiapoptotic Mcl1. GSIXII also targets mammary cancer stem-like cells because it dramatically prevents *in vitro* mammosphere formation. Moreover, combining GSIXII treatment with ABT-737, a BH3-mimetic inhibitor of additional antiapoptotic proteins, such as Bcl-2 and Bcl-xL, leads to both a synergistic apoptotic response in breast cancer cells and to an inhibitory effect on mammosphere formation. These effects are also found when a Notch transcriptional inhibitor, SAHM1, is used. Finally, we evaluated individual human tumor responses to γ -secretase inhibition alone or in combination with ABT-737 in *ex vivo* assays. Analysis of a series of 30 consecutive tumors indicated that a majority of tumors are sensitive to apoptosis induction by GSIXII and that association of GSIXII with ABT-737 leads to an enhanced induction of apoptosis in tumor cells.

Conclusions: We thus provide evidence that γ -secretase, and downstream Notch signaling, are relevant targets in breast cancer. GSIXII, used as single agent or in combination with clinically relevant BH3-mimetics, is a promising innovative proapoptotic strategy to treat mammary tumors.

Introduction

Notch signaling impinges on a wide variety of cellular processes, including cell-fate specification, cell proliferation, differentiation, apoptosis, and maintenance of stem cells. Deregulation of Notch signaling leads to several

pathologic conditions, including cancer [1]. Notch was first identified as an oncogene in T-acute lymphoblastic leukemia with (7,9) chromosomal translocation [2] or activating mutation within *Notch1* gene [3]. The Notch pathway also participates in oncogenesis through aberrant activation related to deregulated expression of Notch receptors or ligands, or the loss of a negative regulator, as described for Numb. Such inappropriate activation of the Notch pathway has been reported in many solid tumors, including breast cancer, in which it was

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linked to poor clinical outcomes [4-6]. Of note, the Notch pathway may have a direct oncogenic effect by its aberrant activation in cancer but may also be involved in feedback-reactivation process after conventional anticancer therapy, thus participating in chemoresistance. Indeed, this pathway is turned on in breast cancer cells, on tamoxifen treatment of estrogen receptor (ER)-positive tumors [7,8], or after HER2 inhibition in HER2-amplified tumors [9]. This is due to the capacity of estradiol or the HER2 pathway intrinsically to inhibit Notch activity. Another important point is that the mammary microenvironment can trigger Notch paracrine signaling to mammary cells, making a potent niche for mammary stem cells [10,11].

After ligand binding to Notch transmembrane receptors, a series of proteolytic reactions leads to the release of Notch intracellular domain (NICD), allowing its translocation into the nucleus, where it interacts with DNA-bound protein factor CSL (or CBF1) and recruits MAML family member coactivators, such as MAML1. These events lead to the formation of a transcriptional activator complex that drives the transcription of targeted genes [12].

The final proteolytic cleavage step mediated by the γ -secretase complex is critical for Notch-signaling activation, and its inhibition can be exploited through emerging pharmacologic drugs identified as γ -secretase inhibitors (GSIs). These new agents attenuate signaling from all four receptors and are being investigated as candidates in cancer therapy. Recent studies provided evidence that GSI treatment suppressed growth of breast cancer cells, increasing the interest in validating this novel therapeutic approach [13-16].

A better understanding of molecular mechanisms involved in the antitumoral effect of Notch inhibition is needed to develop a comprehensive use of Notch inhibitors such as GSI. γ -Secretase activity and Notch signaling appear to be critical for cell survival [17,18], but evaluating how exactly their inhibition affects survival pathways in cancer cells remains to be performed. Along this line, it must be noted that the effects of γ -secretase inhibition have not been systematically assessed. In particular, their effects on intact human tumors in the presence of their microenvironment have not been evaluated. Aberrant survival signaling is a frequent feature of cancer cells, in part due to the acquisition of an increased apoptotic threshold leading to tumor chemoresistance [19]. This process often arises from the deregulation of Bcl-2 family members. This family is divided into three categories, (a) the antiapoptotic proteins (Bcl-2, Bcl-xL, and Mcl-1); (b) the proapoptotic BH3-only proteins, such as Noxa, Puma, and Bim; and (c) the proapoptotic multidomain proteins (Bax, Bak) that function downstream of the former. This family of proteins maintains a subtle survival/

cell-death balance by regulating mitochondrial integrity, caspase activation, and consequent cell demolition. Antiapoptotic proteins promote survival, in great part, by physically interacting with the BH3 domain of their proapoptotic counterparts via a well-characterized binding interface. Subtle yet significant differences exist in the BH3-binding interface of each Bcl-2 homologue, so that promiscuous but also selective interactions occur between these proteins and multidomain or BH3-only proteins. For instance, Bim or Puma interacts with all known Bcl-2 homologues, whereas Bad interacts preferentially with Bcl-2 and Bcl-xL, and Noxa, with Mcl-1 [20]. Thus, Bcl-2 homologues exert complementary effects on cell survival, and their simultaneous inhibition is expected to promote efficient cancer cell death.

The pivotal role of the Bcl-2 family in the apoptotic pathway has stimulated considerable interest in developing anticancer agents that specifically act to restore apoptotic cell death [21]. The BH3 mimetic, ABT-737, is a promising compound that potently binds to and neutralizes the prosurvival proteins Bcl-2, Bcl-xL, and Bcl-w, but not Mcl-1 or A1 [22]. Thus, expression of Mcl-1 confers resistance to ABT-737 when used as single agent and, conversely, approaches that lead to downregulation or inhibition of Mcl-1 are expected to enhance sensitivity to this compound.

Based on these premises, we investigated the impact of Notch inhibition on the apoptotic threshold in breast cancer cells, by focusing our analysis on the Bcl-2 family of proteins. We first pointed out that the γ -secretase inhibitor GSIXII, used as single agent, triggers apoptosis *in vitro* in breast cancer cells. It also exerts an inhibitory effect on breast cancer cells that have a stem-like phenotype, as does the Notch transcriptional inhibitor SAHM1. Importantly, GSIXII treatment also induced an apoptotic response in numerous intact breast tumors tested in an *ex vivo* assay developed in our laboratory. We further demonstrated that the GSIXII apoptotic effect depended mainly on the induction of Noxa, a BH3-only protein that inhibits Mcl-1. Consistently, GSIXII treatment combined with Bcl-2/Bcl-xL inhibition by ABT-737 potently enhanced the proapoptotic response of the breast cancer cells, including in *ex vivo* specimens. Thus, our results highlight the clinical relevance of targeting γ -secretase and downstream Notch signaling in breast cancer, especially in combination with the Bcl-2/Bcl-xL inhibitor ABT-737.

Materials and methods

Reagents and cell lines

MCF7, BT549, MDAMB231, ZR75.1, and T47D cell lines were from American Type Culture Collection, and Cal51, from DSMZ (Braunschweig, Germany). All cell lines were cultured by following the suppliers' recommendations.

SAHM1 and γ -secretase inhibitor XII (GSIXII, z-Ile-Leu-CHO) were purchased from Calbiochem (Darmstadt, Germany), Bortezomib and ABT-737 from Selleck Chemicals (Houston, TX, USA), and the pan-caspase inhibitor QVD-OPH, from R&D Systems (Abingdon, UK). Antibodies against cleaved Notch1 (N1ICD) were purchased from Cell Signaling (Saint Quentin en Yvelines, France); Puma, from Epitomics (Nanterre, France); and Bim and Noxa, from AbCam (Cambridge, UK). Antibodies to Bax and Actin were from Dako (Trappes, France) and Millipore, (Molsheim, France) respectively. Antibody against cleaved (that is, activated) caspase-3 used in IHC was from BD Bioscience (le Pont de Claix, France).

Apoptosis assays

Cell death was assessed with Apo2.7 (Beckman Coulter, Villepinte, France) staining and confirmed by an Annexin-V binding assay (Beckman Coulter, Villepinte, France), performed according to manufacturer's instructions. Flow-cytometry analysis was performed on a FACSCalibur by using the CellQuestPro software.

Mammosphere-formation assay

MCF7 or BT549 cells treated with the indicated treatment or siRNA were plated as single cells in ultra-low-attachment plates (Corning, Avon, France) at low density (500 viable cells/cm²). They were grown in serum-free mammary epithelial cell growth medium containing DMEM-F12 (Sigma, Saint-Quentin Fallavier, France) supplemented with B27 (Gibco, Saint Aubin France) and MEGM singlequots (Lonza, Levallois-Perret, France), as previously described [23]. Mammosphere-forming units (MFUs) were counted as the number of mammospheres $\geq 50 \mu\text{m}$. Mammosphere formation of the second and third generations was investigated in the presence of GSIXIII, or not, after trypsin treatment of the first- and second-generation mammospheres, respectively.

Immunoblot analysis

Patient samples for immunoblots were snap-frozen into liquid nitrogen and then prepared according to [24]. Cell-lines samples were prepared as previously described [25]. Fifty micrograms of protein was loaded for each lane and separated by 10% or 12.5% SDS-PAGE, then electrotransferred to PVDF membranes. Western blot analysis was performed by standard techniques with ECL detection (Pierce, IllKirch, France).

RNA interference

Cells were transfected by using Lipofectamine RNAiMax 2000 (Invitrogen), according to the manufacturer's instructions. Medium was changed 6 hours later, and compounds were added after 24 hours. The following siRNAs were used: control siRNA (sc44230) from Santa

Cruz, siRNA Bim (6461) from Cell Signaling, siRNA Puma from Dharmacon (Lafayette, USA), siRNA Noxa (AC2Z4U4) from Ambion (Saint Aubin, France), and siRNA Bax from IDT (Leuven, Belgium).

Quantitative PCR

Total RNA was isolated from cell lines with RNeasy Plus mini kit (Qiagen, France). The quality of the RNAs was assessed by analysis of the 28S:18S rRNA ratio by using the RNA 6000 Nano Assay kit and the Agilent 2100 bioanalyzer (Agilent Biotechnologies). Then 500 ng of total RNA was reverse transcribed by using the superscript III reverse transcriptase and random hexamers (Life Technologies, Saint Aubin, France). Quantitative PCR was done by using the Maxima SYBR Green/ROX qPCR Master Mix (Life Technologies) and the MX4000 instrument (Stratagene, Basel, Switzerland), according to the manufacturer's instructions. To control the specificity of the amplified product, a melting-curve analysis was done. No amplification of unspecific product was observed. Primer sequences were 5'-GCTGGAAGTCGAGTGTGCTA-3' (forward) and 5'-CCTGAGCAGAAGAGTTTGGA-3' (reverse) for *Noxa*. *RPLPO* AACCCAGCTCTGGAGAAACT (forward) and CCCCTGGAGATTTTAGTGGT (reverse), *HPRT1* 5'-ATGCTGAGGATTTGGAAAGG-3' (forward) and 5'-GATGTAATCCAGCAGGTCAGC-3' (reverse) and *RSP18* 5'-ATCCCTAAAAGTTCCAG-3' (forward) and 5'-CCCTCTTGGTGAGGTCAA-3' (reverse) were used for normalization. Relative quantification was carried out by using the $\Delta\Delta\text{Ct}$ method.

Promoter-reporter activity assay

The ability of NICD to bind to CBF1 and activate gene transcription was measured by the transfection of luciferase reporter plasmids that contain four copies of a binding site for CBF1 (CBF1-Luc) or mutated CBF1 (mCBF1-Luc) that were a kind gift from Dr. Diane Hayward (Johns Hopkins University, Baltimore, MD, USA) [26]. Cells were transfected by using Lipofectamine 2000 (Invitrogen). Medium was changed 6 hours later, and treatment was added after 24 hours. Cells were harvested 48 hours after transfection and analyzed by using the Stop&Glow kit (Promega, Lyon, France) and following the manufacturer's instructions. Results were expressed as ratios between the CBF1-Luc-transfected samples and the mCBF1-Luc-transfected one for each cell line in each condition, in three independent experiments.

Lentiviral infection

Recombinant lentivectors were produced by transient transfection of the transducing vector into 293T cells with two packaging vectors: pMD.G, a plasmid expressing the VSV-G envelope gene (Addgene plasmid 12259, Addgene, Cambridge, MA), and pCMVDeltaR8.91, a

plasmid expressing the HIV-1 gag/pol, tat, and rev genes (Addgene plasmid 8455) associated with a GFP control plasmid (Addgene plasmid 17618) or plasmid coding for N1ICD and GFP with two independent internal promoters (Addgene plasmid 17626), as described previously [27]. Cells were infected for 24 hours before treatment with GSIXII for 48 hours, and apoptosis was assessed on GFP⁺ cells by using Apo2.7 staining followed by flow-cytometry analysis.

Preclinical breast cancer *ex vivo* assay

Fresh human mammary samples were obtained from patients with invasive carcinoma after surgical resection at the Institut de Cancérologie de l'Ouest, René Gauducheau, Nantes, France. As required by the French Committee for the Protection of Human Subjects, informed consent was obtained from study patients to use their surgical specimens and clinicopathologic data for research purposes, and the local ethics committee approved protocols.

The tumors were cut into thin slices (250 μ m) by using a vibratome (Microm International, ThermoFischer Scientific, Illkirch, France) and incubated for 48 hours with or without 15 μ M GSIXII. Slices were then fixed in 10% buffered formalin and were paraffin embedded. Sections (3 μ m) were then cut for standard histologic analysis assessed by hematoxylin-eosin-saffron (HES) coloration. Immunohistochemistry (IHC) was performed to assess tumoral cell apoptosis with cleaved caspase-3 antibody. In brief, after deparaffinization in xylene and rehydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Samples were steamed for antigen retrieval with citrate buffer (pH 6.0). Slides were incubated for active caspase-3 (clone C92-605, dilution 1:1,200) on an automated immunostainer (Autostainer Plus, Dako) by using a standard labeled streptavidin-biotin method (Dako, LSAB +, Dako REAL Detection Systems kit) followed by 3,3'-diaminobenzidine chromogen detection. Immunostained slides were counterstained with hematoxylin (Dako, Trappes, France). Negative controls (omission of the primary antibody) were included in each run. Active caspase-3 immunostained cells were assessed according the percentage of labeled cells in 200 carcinomatous cells counted. Nonneoplastic cells were excluded from counting.

Statistical analysis

Statistical analysis was performed by using a one-tailed paired Student *t* test and one-way ANOVA test on GraphPad Prism. Errors bars represent standard errors of the mean (SEM). The symbols correspond to a *P* value inferior to *0.05, **0.01, or ***0.001, and ns for not statistically significant.

Results

Notch inhibition induced growth arrest and cell death in breast cancer cells

To investigate the proapoptotic effects of the γ -secretase inhibitor GSIXII and to define the range of active concentrations, we treated the breast cancer cell line MDAMB231 with increasing concentrations (4 to 20 μ M) for 48 hours before evaluation of apoptosis by measuring the expression of Apo2.7 antigen (whose expression is restricted to dying cells) by using flow-cytometry analysis. In comparison to DMSO (mock) treatment, GSIXII treatment induced specific apoptosis, and concentrations from 8 μ M to 15 μ M triggered increasing cell death (Figure 1A). The concentration of 15 μ M, corresponding to a plateau (inducing 40% of apoptotic cells), was used. We further tested a panel of six human breast cancer cells lines either expressing estrogen receptor (ER⁺) (ZR75.1, T47D and MCF7) or not (ER⁻, without amplification of the *HER2* oncogene) (BT549, Cal51, MDAMB231), for their cell-death response to this treatment (Figure 1B). All of them showed significant sensitivity to GSIXII. Interestingly, ER⁻/HER2⁻ cell lines exhibited higher sensitivity to GSIXII (40% versus 20% in ER⁺ cell lines), as previously observed by Lee *et al.* [15]. Apoptotic response to GSIXII treatment was further confirmed by Annexin-V binding assay, as shown for BT549, MDAMB231, and MCF7 cell lines in Additional file 1.

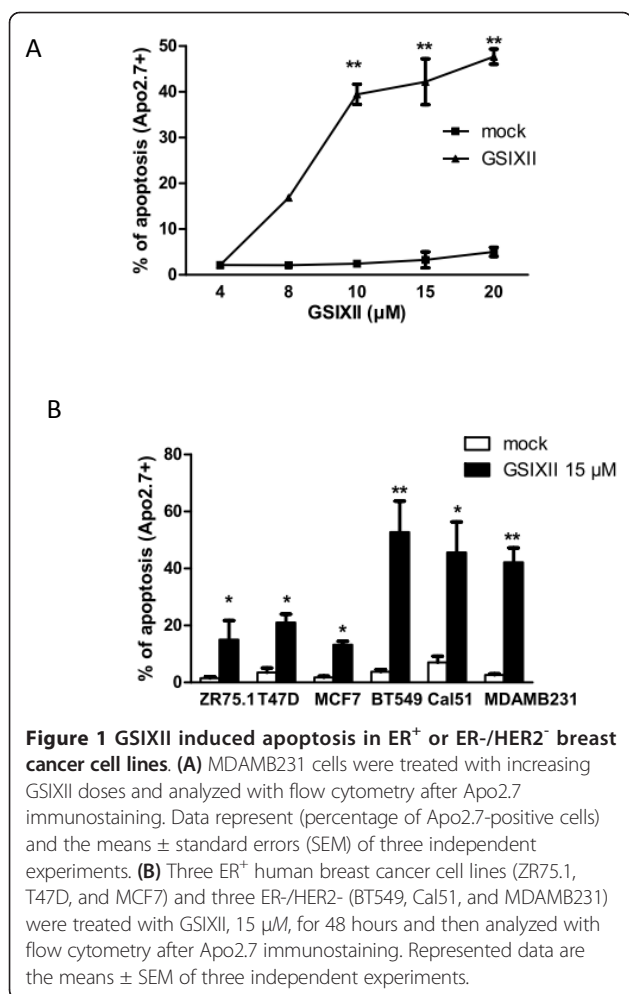
Numerous observations confirmed that GSIXII potently triggered an apoptotic response in breast cancer cells through inhibition of Notch activity in the breast cancer cells used.

First, we evaluated, with immunoblot analysis, the expression of the active form of Notch1, N1ICD, in GSIXII-treated cells compared with control cells, and found that GSIXII treatment downregulated N1ICD expression (Figure 2A).

Second, we measured Notch transcriptional activity, with a Notch promoter luciferase assay containing CBF1 or mutated CBF1 boxes, and this assay pointed out the efficient inhibition of Notch-driven luciferase transcription on GSIXII treatment (Figure 2B).

Third, overexpression of the human N1ICD obtained by lentiviral infection efficiently protected breast cancer cells from GSIXII-induced apoptosis (Figure 2C). Altogether, these results indicate that GSIXII potently interfered with Notch activity, and that this effect contributed in its impact on cell survival.

γ -Secretase inhibitors may also inhibit proteasome activity, and this effect might contribute to their biologic activity. We thus compared the effects of GSIXII and the well-known proteasome inhibitor bortezomib on both proteasome activity and cell survival. These assays



showed that GSIXII had a significant effect on proteasome activity (see Additional file 2A). However, bortezomib treatment that recapitulated this effect did not promote cell death, in contrast to treatment with GSIXII (see Additional file 2B). The lack of correlation between inhibition of proteasome activity and apoptotic activity in these assays indicates that apoptosis induction by GSIXII cannot solely rely on its ability to inhibit proteasome activity, even though we cannot formally rule out that this effect contributes to cell-death induction.

GSIXII treatment triggered Noxa-dependent apoptosis in breast cancer cells

The proapoptotic effects of GSIXII were strongly prevented by co-treatment with the chemical pancaspase-inhibitor QVD-OPH in all breast cancer cell lines (as shown for three of them in Figure 3A). As Bax is a major actor in the onset of apoptosis by the mitochondrial pathway, the impact of its knockdown by RNA interference on GSIXII induction of cell death was evaluated. Results shown in Figure 3B indicate that siRNA

targeting Bax significantly preserved breast cancer cells from the deleterious effects of GSIXII. Thus, GSIXII-induced apoptosis appears to occur mainly through the canonic mitochondria-dependent pathway requiring Bax and caspase activation.

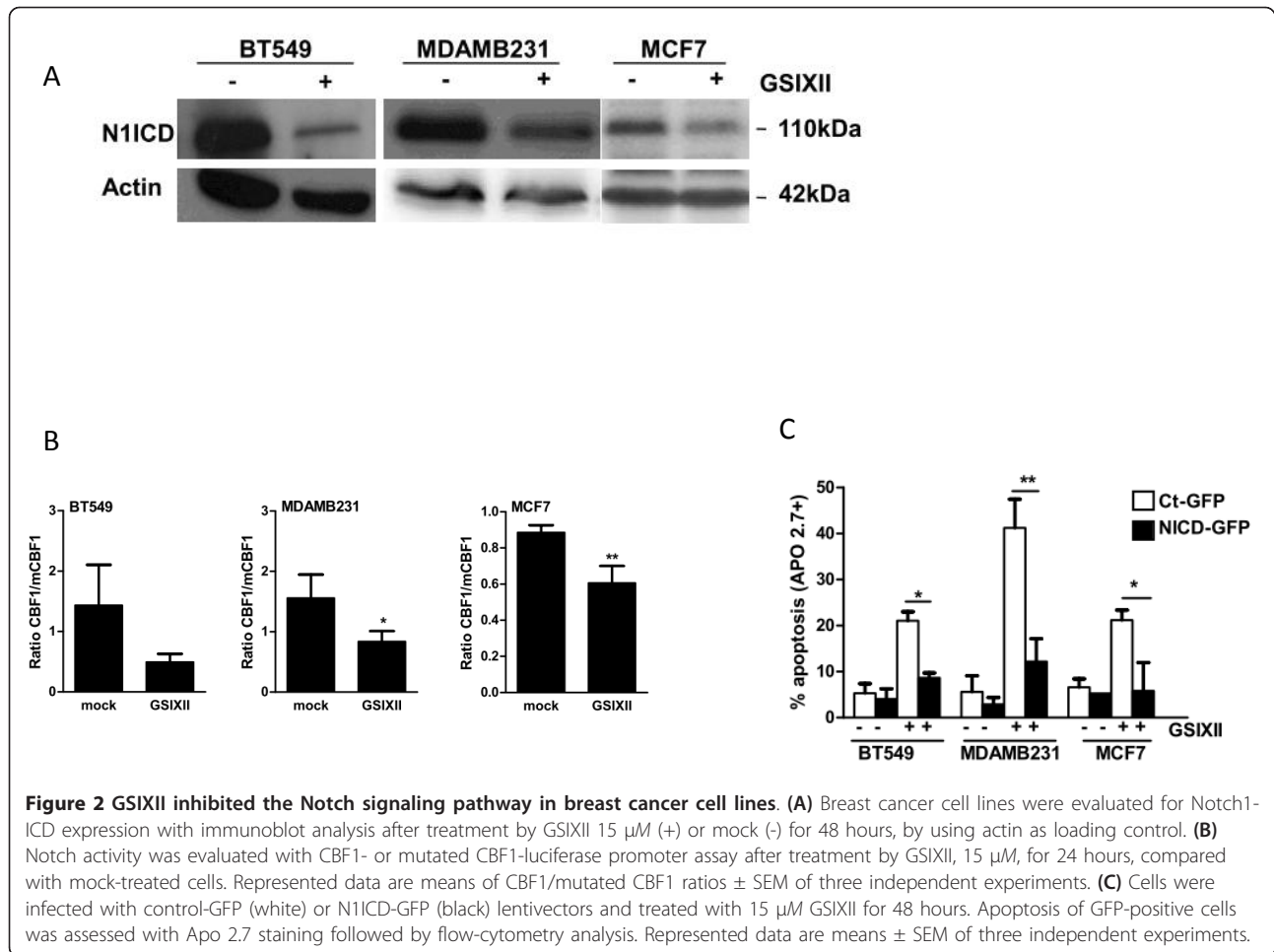
To investigate further the molecular pathways involved in GSIXII induction of cell death, we performed siRNA-based experiments against Noxa, Bim, or Puma before treating cells with GSIXII. Of major importance, the sole depletion of Noxa by RNA interference led to decreased cell sensitivity to GSIXII in all cell lines tested (Figure 4A and Additional file 3A). In contrast, neither Puma nor Bim depletion had a significant impact on the cell-death response to GSIXII. Of note, protection against cell death by Noxa knockdown was not complete, but this might rely on residual partial Noxa expression after Noxa siRNA treatment (see Additional file 3B). Thus GSIXII induces cell death preferentially by a Noxa-dependent cell-death pathway.

We then assessed the expression of the BH3-only proapoptotic proteins, Bim, Puma, and Noxa, with immunoblot analysis on treatment with GSIXII. In all breast cancer cell lines, a strong induction of Noxa protein expression was evidenced in response to GSIXII treatment (Figure 4B). In contrast, Puma or Bim expression was not enhanced (as shown in MDAMB231 in Additional file 4). The better to understand the mechanisms involved in Noxa protein accumulation on GSIXII treatment, RTqPCR analysis was performed to quantify Noxa mRNA. Data indicated that GSIXII induced Noxa mRNA, arguing for regulation of Noxa expression at a transcriptional level (Figure 4C).

GSIXII treatment strongly impaired *in vitro* mammosphere formation

Transformed mammary epithelial cells, including established breast cancer cell lines, exhibit an inherent phenotypic plasticity and harbor a subpopulation of cancer-initiating cells with features resembling those of stem cells. The latter cells, which are characterized by numerous criteria, including their ability to form spherical colonies in nonadherent fetal bovine serum-free culture conditions (mammospheres), were frequently described as being resistant to cell-death induction by numerous stimuli, and they may therefore rely on survival signals distinct from the bulk population. Moreover, the Notch pathway might be involved in cell stemness. We thus evaluated whether GSIXII treatment had an impact on mammosphere formation by breast cancer cell lines and whether this relied on cell-death induction.

A dramatic decrease in mammosphere formation was observed after GSIXII treatment of MCF7 or BT549 cell lines compared with mock-treated cells (Figure 5A). This effect was recapitulated by the SAHM1 cell-permeable peptide (a dominant-negative fragment of MAML1 that



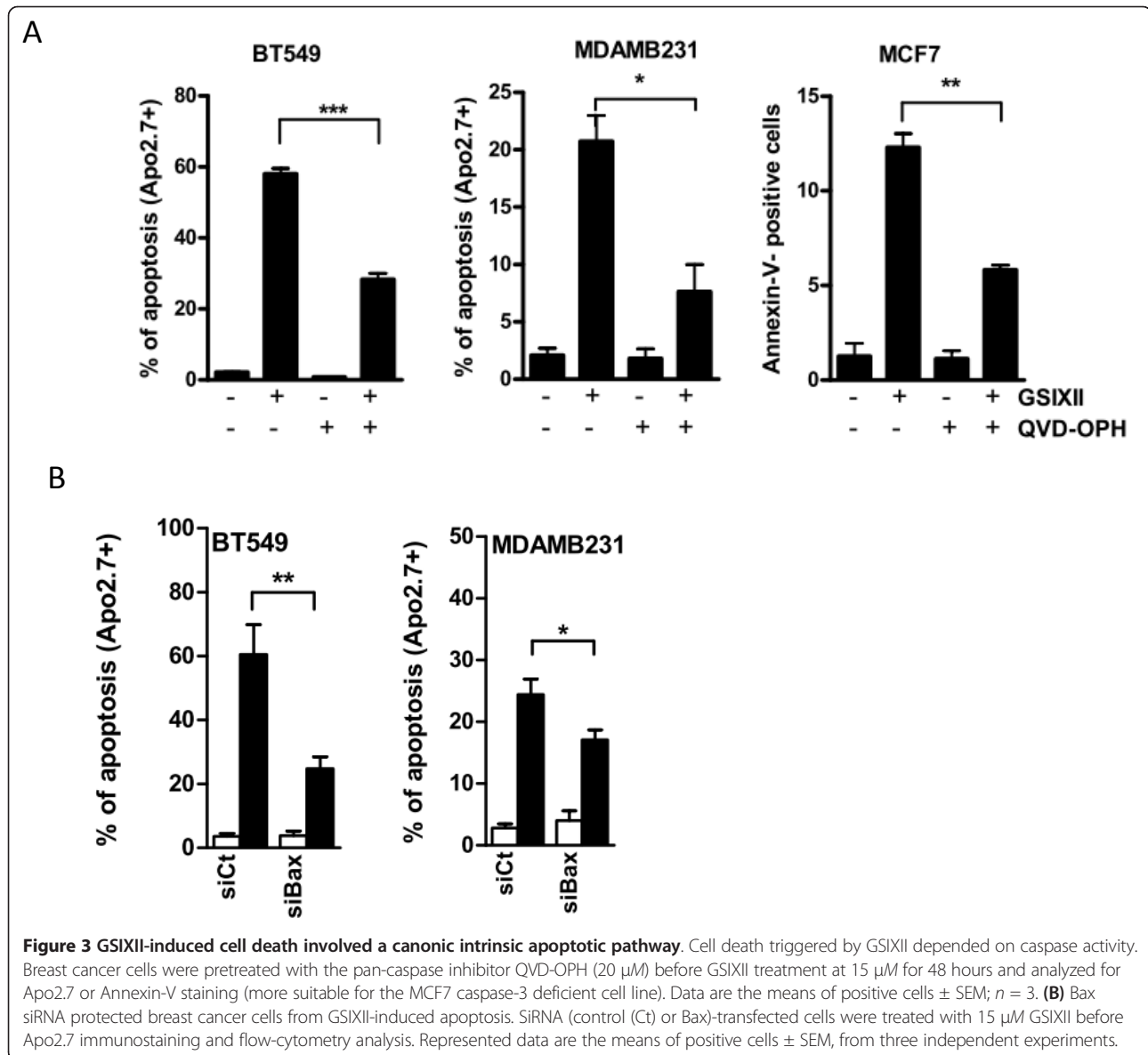
specifically prevents assembly of the active transcriptional complex and blocks Notch transcription activity [28]), used at 20 μ M (Figure 6C). In addition, GSIXII not only inhibited first-generation mammosphere formation but also decreased the mammosphere formation of second and third generations (Figure 5B), which are further enriched in self-renewing cells. This argues that the treatment affects not only cells that can give progeny, but also cells that can self-renew. Of importance, Noxa depletion by RNA interference combined with GSIXII treatment partially but significantly rescued mammosphere formation (Figure 5C). Thus, GSIXII potently prevents mammosphere formation, and this effect relies, at least in part, on Noxa-dependent cell-death mechanisms. This argues for the capacity of GSIXII to target mammary stem-like cells.

GSIXII and the BH3 mimetic ABT-737 strongly synergized to induce apoptosis in breast cancer cells

As GSIXII induced the expression of proapoptotic Noxa, which inhibits the survival activity of Mcl-1, we inferred that its combination with the BH3 mimetic ABT-737,

which targets Bcl-2 and Bcl-xL but not Mcl-1, might improve apoptosis induction in breast cancer cells. We observed that combined treatment of breast cancer cell lines with a suboptimal concentration (that is, 8 μ M GSIXII and 1 μ M ABT-737) strongly synergized to induce cell death (Figure 6A). In these conditions, GSIXII induced cell-death rates lower than 20%, and ABT-737 induced death rates lower than 10%, whereas the combination of both drugs triggered cell-death rates ranging from 50% to 70%. Interestingly, this synergy was also observed when using the other γ -secretase inhibitor DAPT in combination with ABT-737 (see Additional file 5).

To confirm that Noxa induced on treatment with GSIXII functions as an inhibitor of Mcl-1, we further evaluated its interaction with Mcl-1 on GSIXII treatment, with co-immunoprecipitation assays. We observed an increase of the interaction in the GSIXII-treated cells compared with mock-treated cells (see Additional file 6), demonstrating that Noxa could sequester Mcl-1 in treated cells. In addition, the presence of Noxa was greatly decreased in the Mcl-1-immunodepleted supernatants of GSIXII-treated cell lysates compared with the corresponding not-depleted



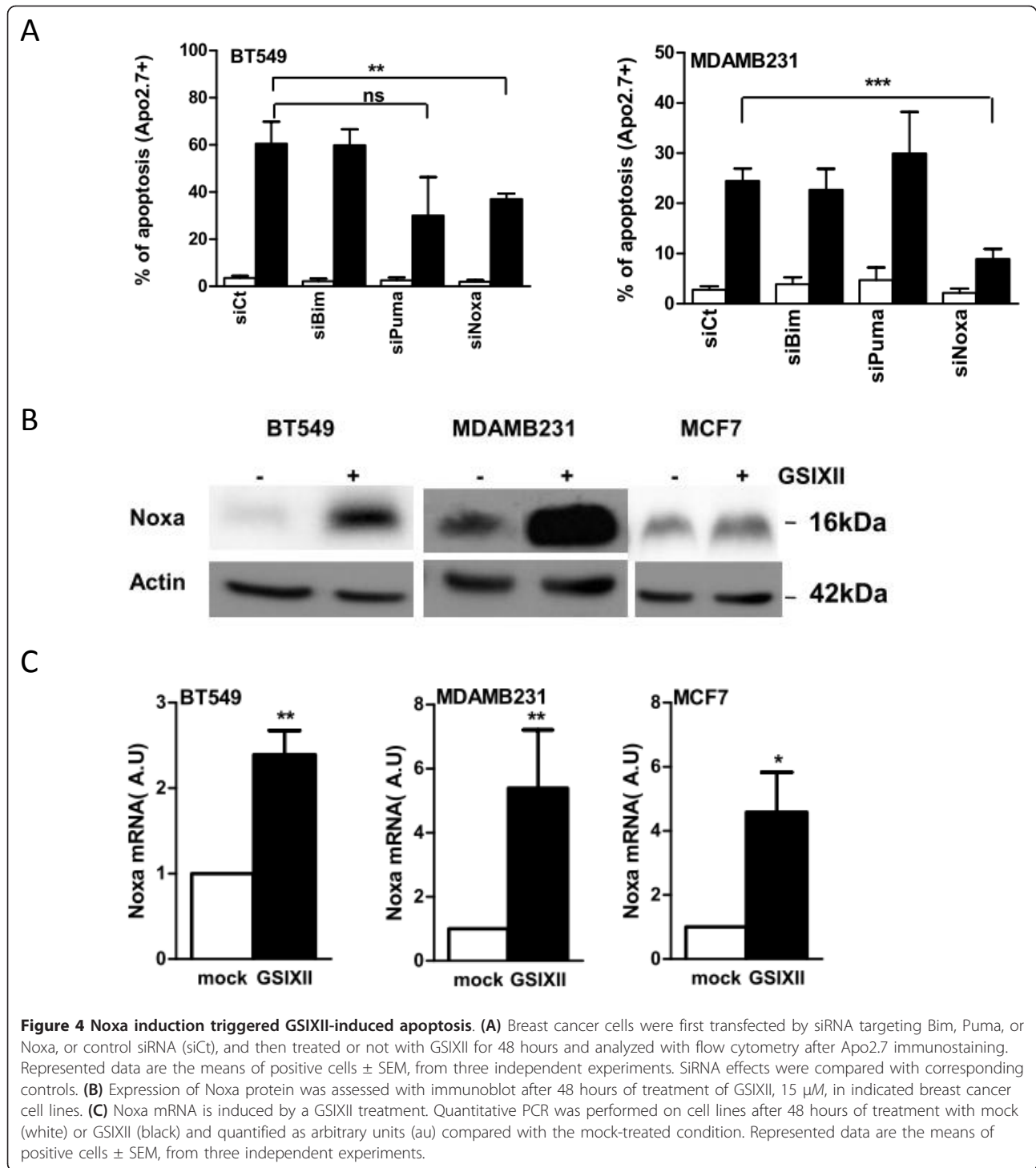
ones, indicating that GSIXII-induced Noxa was in the majority complexed to Mcl-1 (data not shown). Interestingly, the GSIXII and ABT-737 combination led to inhibition of mammosphere formation in both MCF7 and BT549 cell lines (Figure 6B). The SAHM1 and ABT-737 combination also decreased mammosphere formation (Figure 6C). These results argue that potent apoptotic synergy is induced by γ -secretase inhibitors and ABT-737 in stem-like breast cancer cells, as well as in more-differentiated cells.

Preclinical evaluation of GSI treatment on human mammary tumors

The tumor microenvironment is particularly important for Notch activation. We thus developed a model of 3D

culture of human primary breast tumors in which the architectural integrity of the tumor, including its microenvironment, is preserved. In brief, fresh tumors were rapidly cut into thin slices and incubated in full medium alone or with drugs (GSIXII+/- ABT-737) for 48 hours. Tumor slices were then paraffin embedded and analyzed with IHC for active caspase-3 expression, as a marker of apoptotic response. We studied a series of 30 consecutive primary tumors from patients with untreated breast cancer for their sensitivity to the Notch inhibitor GSIXII with this short-term *ex vivo* culture of human breast cancer tissues.

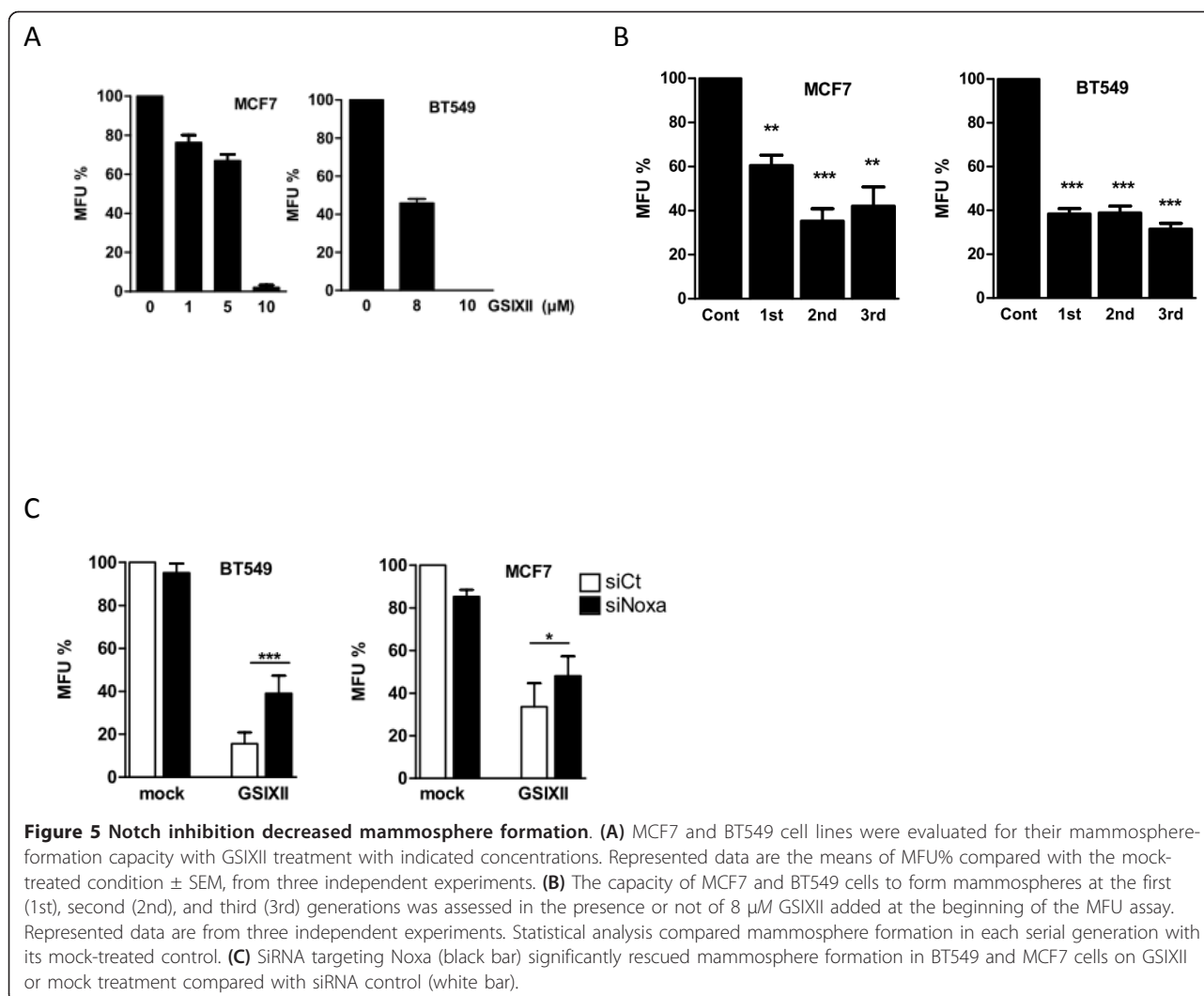
To evaluate the specific response to GSIXII of each tumor sample, we systematically kept one slice untreated (to evaluate spontaneous rates of cell death)



and treated another slice from the same tumor with 15 μ M GSIXII for 48 hours. Apoptosis was evaluated by counting the percentage of tumor cells that stained positive with an anti-active caspase-3 antibody, as evaluated by IHC analysis of the specimen fixed after incubation in either condition (Figure 7A); 23 ER-positive and

seven ER-negative tumors were included in this preclinical study.

Investigation of untreated slices showed low cell-death rates in each tumor, with the mean percentage of active caspase-3-positive cells in these specimens reaching 5.7%. In comparison, the mean percentage of active



caspase-3-positive cells in GSIXII-treated specimens was 44%. To classify individual tumors according to their apoptotic response to GSIXII, we arbitrarily defined a positive threshold above 17% of active caspase-3-positive tumor cells (corresponding to the highest score in untreated samples). Of 30 specimens, 24 showed a response to GSIXII above this threshold and can thus be considered GSIXII-sensitive tumors (Figure 7B). In contrast, six of 30 GSIXII-treated specimens showed cell-death rates that were undistinguishable from those found in control untreated specimens, which defined them as GSIXII-resistant tumors. Among sensitive specimens, we could identify two groups: an intermediate group of nine tumors that displayed 17% to 40% positive cells and a highly GSIXII-sensitive group of 15 tumors showing more than 40% of apoptotic cells under the conditions used. Importantly, a robust correlation was noted between the percentage of active caspase-3 tumor cells and tumor cell integrity, as evaluated with the

standard hematoxylin-eosin-saffron staining performed on the same sample (see Additional file 7). This strongly suggests that the effects of GSIXII treatment on the tumor samples in this *ex vivo* test predominantly rely on an apoptotic response, which can be marked and quantified by caspase-3 activation. In addition, and consistent with this, Noxa induction could be detected in breast cancer tissues after GSIXII *ex vivo* treatment, as shown in two sensitive tumors (for which we obtained sufficient material to perform immunoblot analysis of untreated and GSIXII-treated specimens) compared with the corresponding untreated tissues (Figure 7C). To evaluate whether ABT-737 treatment might enhance the apoptotic response of breast tumor samples to GSIXII induction of cell death, we also regularly treated, from the same series of tumor samples, one additional slice with 1 μM ABT-737 and another one with a combination of GSIXII and ABT-737 before evaluation of the apoptotic response, as described earlier. Six

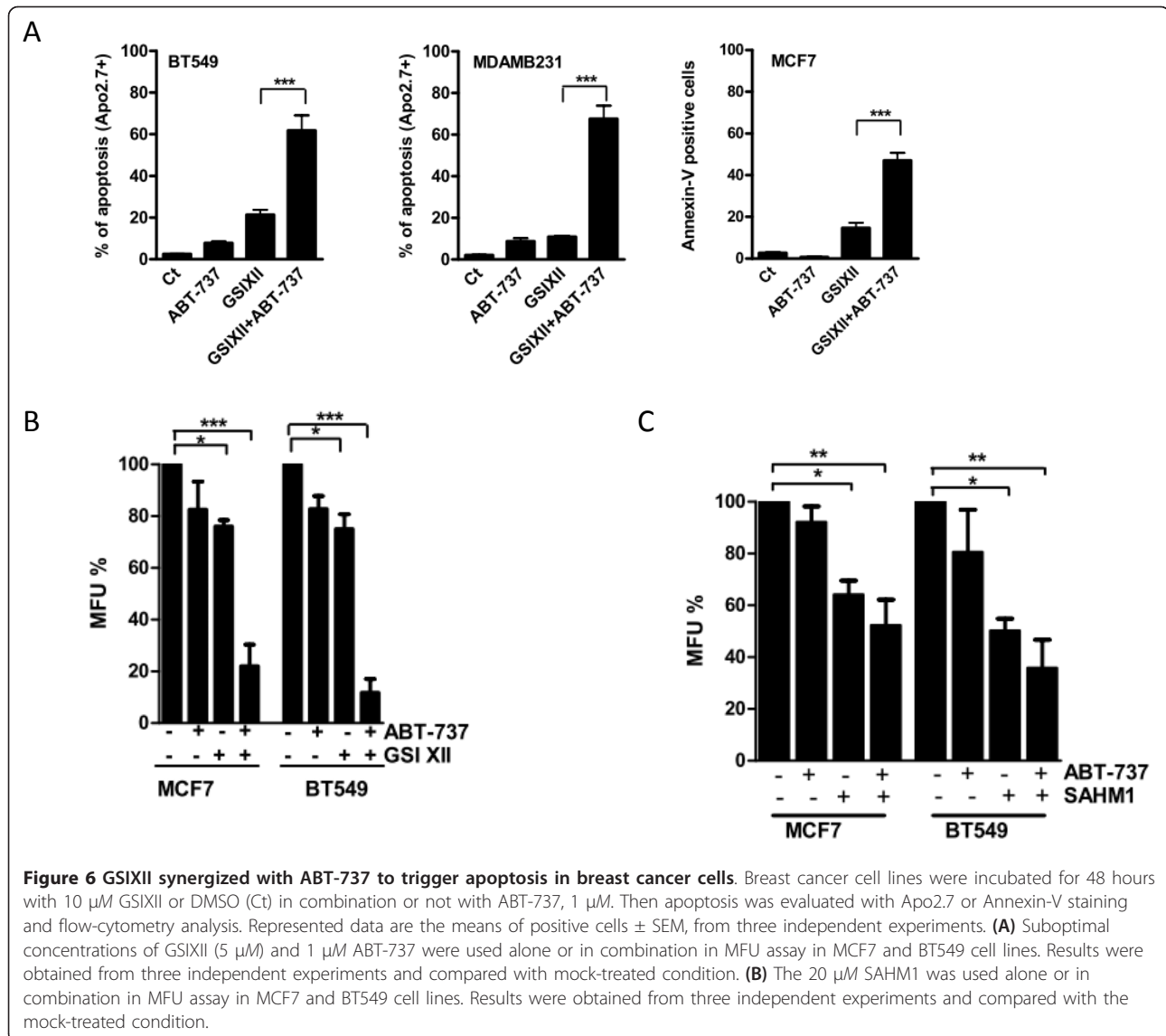


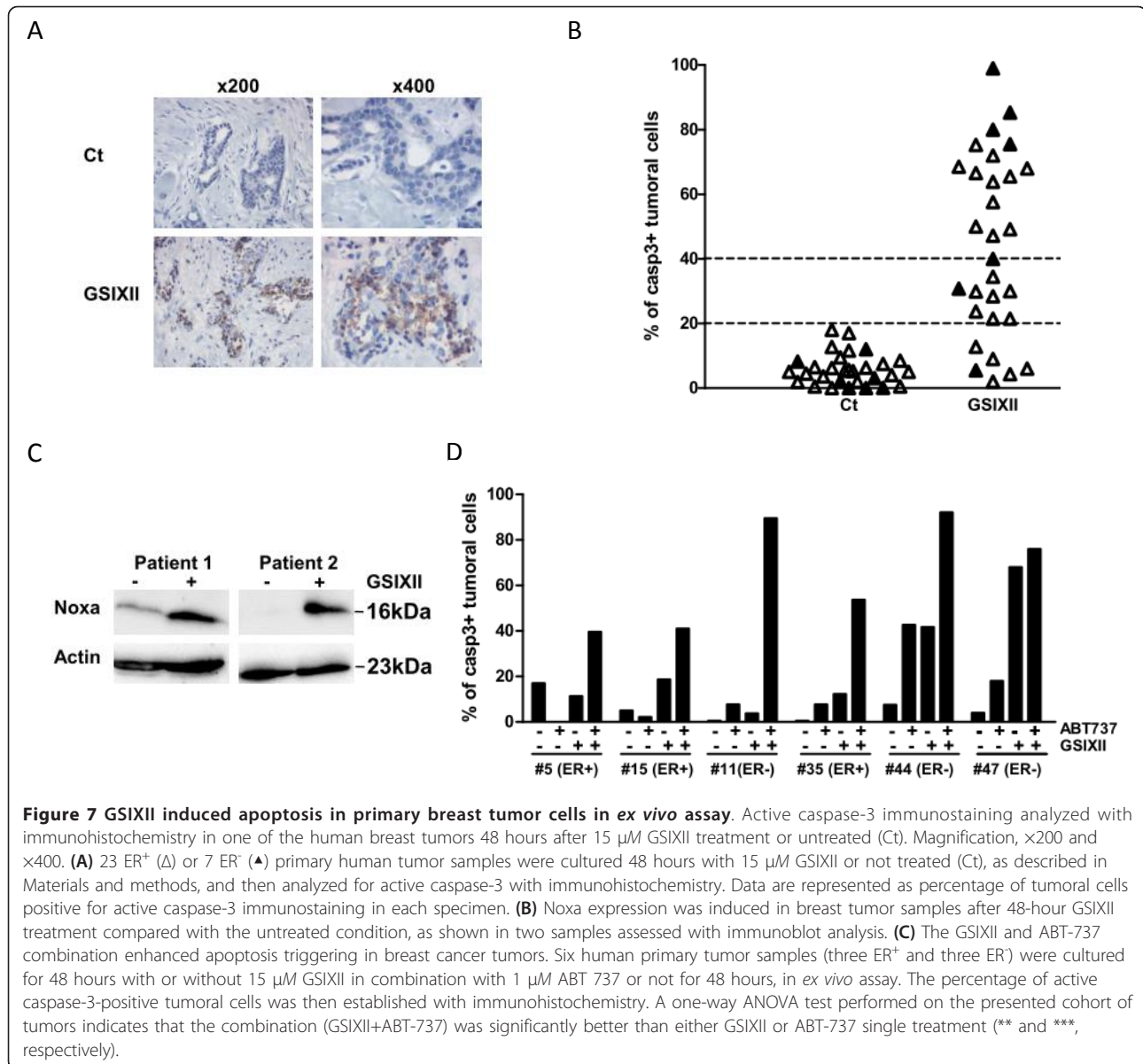
Figure 6 GSIXII synergized with ABT-737 to trigger apoptosis in breast cancer cells. Breast cancer cell lines were incubated for 48 hours with 10 μ M GSIXII or DMSO (Ct) in combination or not with ABT-737, 1 μ M. Then apoptosis was evaluated with Apo2.7 or Annexin-V staining and flow-cytometry analysis. Represented data are the means of positive cells \pm SEM, from three independent experiments. (A) Suboptimal concentrations of GSIXII (5 μ M) and 1 μ M ABT-737 were used alone or in combination in MFU assay in MCF7 and BT549 cell lines. Results were obtained from three independent experiments and compared with mock-treated condition. (B) The 20 μ M SAHM1 was used alone or in combination in MFU assay in MCF7 and BT549 cell lines. Results were obtained from three independent experiments and compared with the mock-treated condition.

specimens (three ER⁺ and three ER⁻) proved to be informative in these assays, in that their apoptotic response to GSIXII and ABT-737, used as single agents, gave sufficiently low apoptotic responses, thus allowing synergy detection. Three of these specimens were GSIXII resistant, one intermediate and two GSIXII-sensitive tumors. Moreover, regarding the ABT-737 response, four specimens were resistant, one was intermediate, and one, mildly sensitive. In all cases, the combination of ABT-737 treatment with that of GSIXII led to significantly enhanced cell death compared with that induced by each compound alone (Figure 7D). We conclude that at least some additivity occurs in the effects of the two compounds in both GSIXII-sensitive samples 44 and 47 and significant synergy in the four remaining tumors, for which the response to the combined treatment is

higher than the sum of those obtained for each of the treatment-alone tumors.

Discussion

Aberrant activation of the Notch pathway has been involved in solid-tumor pathogenesis, triggering protection against apoptosis or increased cell proliferation, yet the molecular basis for these effects remains unclear. To investigate these, and because the γ -secretase complex is a critical step in Notch-pathway activation, we evaluated the cell-death effects of inhibition of γ -secretase activity by GSIXII in breast cancer. Our results clearly indicate that GSIXII elicited potent apoptosis in breast cancer cells and that this effect occurred through the strong induction of the proapoptotic BH3-only protein Noxa. Of note, we showed that GSIXII treatment truly



inactivated the Notch pathway, because it decreased both the expression of the Notch1 active form (N1ICD) and the global Notch transcriptional activity. Importantly, N1ICD overexpression rescued breast cancer cells from GSI-induced apoptosis. These latter results strongly argue that the potent cell-death effect of GSIXII relies on the inhibition of Notch processing into its active form. We cannot formally rule out, however, that additional effects, such as that on proteasome activity previously reported for the structurally related GSI-I [29], contribute to cell-death induction, but preliminary data with DAPT, that was described as a specific γ -secretase inhibitor devoid of inhibitory effect on proteasome activity by Han and colleagues [29], could also

sensitize breast cancer cells to cell death. Importantly, the cytotoxic effects of GSIXII could be detected not only in the bulk of breast cancer cell lines but also in their stem cell-like compartment. This effect most likely results from Notch inhibition, as it was recapitulated by the Notch transcription factor inhibitor SAHM1. Intriguingly, this compound was more effective, in our hands, on this subpopulation enriched in self-renewing cells than on the bulk of cell lines. This may be due to a weak ability of the peptide to enter cells and/or to a higher dependency on Notch signaling of the stem-like cells compartment compared with the bulk population. Such a specific effect of Notch inhibition on mammosphere cultures has been observed by Farnie and

colleagues [30,31]. Importantly, freshly explanted human breast cancer cells maintained in their microenvironment are also sensitive to induction of apoptosis by GSIXII. In addition, simultaneous treatment with GSIXII and the Bcl-2/Bcl-xL inhibitor ABT-737 led to synergistic effects in all three paradigms.

Altogether, our results strongly argue for the use of the γ -secretase inhibitors in breast cancer therapy, especially in combination with Bcl-2/Bcl-xL inhibition, which may help to reduce the dose of GSI used.

In an attempt to define molecular mechanisms involved in apoptosis triggered by GSIXII treatment, we first demonstrated that it was related to Bax and the caspase-dependent pathway. We then identified the BH3-only Bcl-2 family member Noxa as a pivotal actor. Indeed, its expression was strongly induced on GSIXII treatment, in breast cancer cell lines as well as in human primary breast tumors. Moreover, its knockdown by RNA interference potently blocked apoptosis in breast cancer cell lines, as previously observed in melanoma cell lines with GSI-I treatment [32]. We also detected Noxa mRNA accumulation on GSIXII treatment, arguing for an increase of its gene transcription rather than stabilization of the protein.

Previous studies have indicated Bcl-2 family members as major regulators of apoptosis triggering by Notch inhibition. Some reported the decrease of antiapoptotic Bcl-2 members, such as Bcl-xL, on GSI treatment in cancer cells [14,16]. Others described inefficient p53 response after treatment by potent p53 activators, such as genotoxic drugs acquired on Notch activation [17]. Of note, activated Notch1 could suppress Noxa expression even in mutated p53 cells, possibly through the regulation of E2F-1 [7]. Currently, and in contrast to the antiapoptotic gene *survivin* that is a direct target of Notch [15], no direct transcriptional regulation of Bcl-2 family genes by Notch has been reported. However, numerous regulators of Noxa expression have been described, among them p53, c-myc, and E2F-1 [reviewed in [33]], and further experiments are needed to investigate whether these, or additional factors, are involved in Noxa induction on Notch inhibition.

Importantly, Noxa specifically inhibits the survival activity of Mcl-1 and can also target it for proteosomal degradation [34]. This presumably occurs in GSIXII-treated cells, because we found that induced Noxa potently binds to Mcl-1. This event is a prerequisite for cell death induced by various stimuli (UV, cytokine deprivation, or treatment with anticancer agents) [reviewed in [33]]. Thus our work establishes that GSIXII, which triggers Noxa expression, functions as an indirect inhibitor of one key survival protein, Mcl-1. Because evasion of apoptosis has been recognized as one of the hallmarks of cancer, pharmacologic inhibition

of antiapoptotic proteins is a potential strategy to restore apoptosis function in cancer cells. Several molecules, including ABT-737, have been designed to mimic the binding of BH3-only proteins to the hydrophobic groove of antiapoptotic proteins, blocking their activity. ABT-737 nevertheless binds preferentially to Bcl-2 and Bcl-xL but not to Mcl-1. Thus ABT-737 is ineffective in killing tumor cells expressing high levels of Mcl-1 compared with those of Bcl-2/Bcl-xL [35].

Breast cancer cells often express high levels of Bcl-2, Bcl-xL, and/or Mcl-1 [36]. Therefore, a rationale exists to use BH3 mimetics to circumvent apoptosis resistance in these cancers. We recently reported, in particular, that Mcl-1 participates in survival maintenance of breast cancer cells, at the very least in that of the HER2-amplified subtype [23]. Thus, combining Bcl-2/Bcl-xL and Mcl-1 inhibition by ABT-737 and GSIXII, respectively, should restore apoptosis sensitivity efficiently and affect survival maintenance, in breast cancer cells. Our results are consistent with this and clearly indicate that ABT-737 and GSIXII co-treatment led to synergistic apoptosis in breast tumors, suggesting the potential use of this combination to overcome cellular resistance. Interestingly, previous reports indicated that GSI treatment sensitized cancer cells to other chemotherapeutic drugs, such as oxaliplatin or 5-FU in colon cancer cells [16]. In the same line, the combination of BH3 mimetics with potent inducers of Noxa, such as vinblastin [37] or cisplatin [38], induced cancer cell sensitization to apoptosis.

Mammary stem cells, defined by indefinite self-replication that ensures tissue self-renewing by asymmetric cell division and generation of progenitor cells, have been isolated from both normal breast tissues and breast tumors [10]. This cell population exhibits an inherent capacity to form clonal mammospheres in suspension in *in vitro* assays, and in breast cancer stem cells, to initiate tumors in *in vivo* assays. Importantly, these cells show resistance to toxic agents [39]. Indeed, conventional chemotherapy often kills a majority of differentiated cancer cells but spares cancer stem cells, thus probably participating in cancer recurrence. We assessed this cellular compartment by using mammosphere-formation assay and showed that inhibition of Notch signaling, by using either GSIXII or SAHM1, successfully decreased mammosphere formation. This highlights the importance of the Notch pathway in mammary stem cell maintenance, as previously reported in breast cancer stem-like cells [40] or in normal mammary stem cells [10]. In addition, we found evidence that Noxa is involved in the effects of GSIXII, at least in part, because its knockdown significantly rescued mammosphere formation in GSIXII-treated cells. However, we cannot exclude the involvement of other mechanisms in

the process, because this rescue was partial. Of particular interest, the simultaneous treatment with GSIXII and ABT-737 strongly impaired mammosphere formation. These results revealed that the Bcl-2 family of proteins might play an important role in maintaining the survival of breast cancer stem-like cells. Interestingly, this observation is supported by a recent report that shows that the co-silencing of Bcl-2, Bcl-xL, and Mcl-1 in breast cancer cell lines potently reduced mammosphere formation [41]. Practically, targeting breast cancer stem-like cells with BH3-mimetics should improve therapeutic outcomes.

Recent data suggest that Notch signaling is also important in the tumor microenvironment, as observed in myeloma [42] or head and neck squamous carcinoma cells [43]. The 3D short-term *ex vivo* model we developed, similar to the one described by van der Kuip and colleagues [44], allowed correct maintenance of intact breast tumoral tissues where cells remained viable and still proliferated because of sufficient diffusion of oxygen or nutrients. This assay preserves specific interactions between tumor cells and surrounding nontumoral tissue components and provides a powerful, rapid, and reproducible tool for studying the differential responses of individual tumors (and their various components) to specific drugs. Our results clearly demonstrate that GSI treatment is efficient in breast cancer cells embedded in their microenvironment.

We evaluated a series of 30 human primary breast tumors in the *ex vivo* assay and found evidence that 24 tumors exhibited high levels of caspase-3 activity on GSI treatment. Crucially, this method can be used to predict tumor sensitivity to drugs in a patient-specific manner and to help to identify patients who could benefit from the specific therapy. Moreover, combining GSIXII treatment with ABT-737 treatment led to a synergistic proapoptotic effect in six tumors tested. Among them, three were resistant to GSIXII, and four were resistant to ABT-737, each used as single agent. Thus, these results strongly argue for potent proapoptotic cooperation between GSIXII and ABT-737 in breast cancer cells maintained in their microenvironment.

Conclusions

Altogether, our data provide strong evidence that γ -secretase inhibition triggers potent apoptosis in breast cancer cells. Moreover, the induction of Noxa expression played a major role in this process. Combining GSIXII treatment with ABT-737 strongly enhanced the apoptotic response in breast cancer cells, especially in tumors for which both molecules used as single agents led to a moderate proapoptotic effect. Thus, our data suggest that γ -secretase inhibition might offer a potent novel approach to treating breast cancers. Experimental

treatments with Notch inhibitors in animal models were very promising [45]. However, they resulted in serious gastrointestinal side effects or immunosuppression [46]. A therapeutic window may exist if GSI could be given for short periods or in smaller doses. On the basis of our data, we propose that combining γ -secretase inhibition with Bcl-2/Bcl-xL targeting, might allow us to use concentrations of GSI under the side-effect limit in breast cancer therapy.

Additional material

Additional file 1: GSIXII treatment induced Annexin-V-positive staining in breast cancer cells. Cells were incubated with 15 μ M GSIXII or with DMSO for 48 hours, and then assessed for Annexin-V expression with flow cytometry.

Additional file 2: Proteasome activity inhibition and apoptosis induction on GSIXII or bortezomib treatment did not correlate in breast cancer cell lines. Proteolytic activity of 20S proteasome was quantified in breast cancer cell lines treated with GSIXII (15 μ M) or bortezomib (10 nM) with the fluorimetric substrate assay by using the substrate Suc-Leu-Leu-Val-Leu-AMC, according to the manufacturer's recommendation (Tebu Bio, Le Perray-en-Yvelines, France) (A). Apo2.7-positive cells were evaluated on 15 μ M GSIXII treatment or 10 nM bortezomib for 48 hours, as previously described (B). Data are represented as percentage mean of inhibition compared with control (mock-treated) cells \pm SEM; $n = 3$.

Additional file 3: BH3-only proteins expression in MDAMB231 cells after RNA interference. Extinction of proteins expression was evaluated with immunoblot analysis after siRNA transfection in MDAMB231 for Bim and Puma (A) and Noxa on GSIXII treatment or not for 48 hours (B).

Additional file 4: GSIXII treatment did not induce other Puma and Bim BH3-only proteins. Expression of Noxa, Puma, and Bim proteins was assessed with immunoblot after 48 hours of treatment of GSIXII in MDAMB231 cells. *Nonspecific band.

Additional file 5: DAPT synergized with ABT-737 to trigger apoptosis in breast cancer cells. MDAMB231 cells were incubated for 48 hours with 10 μ M DAPT (Sigma, Saint-Quentin Fallavier, France) in combination or not with ABT-737, 1 μ M. Then apoptosis was evaluated with Apo2.7 immunostaining and flow-cytometry analysis. Represented data are the means of positive cells \pm SEM, from three independent experiments.

Additional file 6: Noxa co-immunoprecipitated mainly with Mcl-1 after GSIXII treatment. Cells were treated for 48 hours with GSIXII and QVD-OPH (to avoid cell death and obtain sufficient protein material in treated cells) before lysis in CHAPS buffer. Whole lysates were incubated overnight with the capture antibody (Mcl-1 S19 clone; Santa Cruz (Santa Cruz, USA), and then immunocomplexes were captured by using protein G-magnetic beads according to manufacturer's instructions (Millipore, Molsheim, France), in GSIXII-treated or untreated indicated cells. The immunoprecipitates were analyzed for the presence of Mcl-1 and Noxa proteins with immunoblotting.

Additional file 7: Correlation between HES and active caspase-3 IHC on GSIXII-treated tumors. Each specimen was scored for active caspase-3 IHC and HES staining, allowing cell-morphology analysis. Active caspase-3 was scored as percentage of positive tumor cells. HES score was established in three groups, depending on the percentage of cells with altered morphology: group 1 (< 25%), group 2 (25% to 50%), and group 3 (> 50%).

Abbreviations

BH3 domain: Bcl-2-homology 3 domain; ER: estrogen receptor; GSI: γ -secretase inhibitor; HER2: epidermal growth factor receptor 2; HES:

hematoxylin-eosin-saffron; IHC: immunohistochemical analysis; MFU: mammosphere-forming unit; NICD: Notch intracellular domain; PVDF: polyvinylidene fluoride; SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis; 2D or 3D: 2- or 3-dimensional.

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Authors' contributions

CS performed the majority of *in vitro* experiments and improved the *ex vivo* assay. DL conducted tumor histology analysis. SB participated in immunoblot experiments. MC helped to obtain human breast tumors. SBN performed mammosphere assays. PJ and SBN conceived the study and participated in the design of the project and the preparation of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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