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Activation of the Farnesoid X-receptor in breast cancer cell lines results in cytotoxicity but not increased migration potential

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Abstract

Breast cancer is the commonest form of cancer in women, but successful treatment is confounded by the heterogeneous nature of breast tumours: Effective treatments exist for hormone-sensitive tumours, but triple-negative breast cancer results in poor survival. An area of increasing interest is metabolic reprogramming, whereby drug-induced alterations in the metabolic landscape of a tumour slow tumour growth and/or increase sensitivity to existing therapeutics. Nuclear receptors are transcription factors central to the expression of metabolic and transport proteins, and thus represent potential targets for metabolic reprogramming. We show that activation of the nuclear receptor FXR, either by its endogenous ligand CDCA or the synthetic GW4064, leads to cell death in four breast cancer cell lines with distinct phenotypes: MCF-10A (normal), MCF-7 (receptor positive), MDA-MB-231 and MDA-MB-468 (triple negative). Furthermore, we show that the mechanism of cell death is predominantly through the intrinsic apoptotic pathway. Finally, we demonstrate that FXR agonists do not stimulate migration in breast cancer cell lines, an important potential adverse effect. Together, our data support the continued examination of FXR agonists as a novel class of therapeutics for the treatment of breast cancer.

Keywords: Apoptosis, Autophagy, Nuclear Receptor, Triple negative breast cancer, Bile acids,

Abbreviations: CDCA = chenodexoycholic acid; ER = estrogen receptor; FXR = Farnesoid X-receptor; Her2 = Human epidermal growth factor receptor 2; PR = progesterone receptor; TNBC = Triple negative breast cancer

1. Introduction

Breast cancer represents one of the largest killers of women in the Western world, with a lifetime risk of one in eight [1]. In the past decades a number of successful therapeutics targeting breast cancer have been developed, used in both single and combination therapies. However, improvements in the five year survival rate have not been as high as hoped. This is to a large degree due to the heterogeneous nature of breast tumours, with multiple molecular landscapes being classified as a single disease. These diverse tumour phenotypes result in varied responses to therapeutic intervention, often leading to sub-optimal patient response [2]. To aid optimisation of treatment regimens, breast tumours are commonly classified into three clinically significant groups: hormone sensitive, Her2 positive and triple negative breast cancers (TNBC) [3]. Both hormone sensitive and Her2-positive tumours have a range of good therapeutic options, but TNBC tumours are characterized by a lack of molecular targets and tendency to develop drug resistance. Not surprisingly, TNBC represents the leading cause of death in breast cancer [4]. It is thus imperative to develop novel therapeutic options that would exploit tumour vulnerabilities, mitigate drug resistance and lead to improved patient response, and especially in the case of TNBC tumours.

Two areas of therapeutic intervention have received increasing attention in the past few years: Synthetic lethality and metabolic vulnerability. These related concepts exploit the greater understanding of network biology to predict synergistic combination therapies [5]. In synthetic lethality, the concept of 'rescue pathways' is exploited: In essence, targeting of a single species in a biological pathway is often negated by re-routing of the biological network to exploit a secondary pathway. In synthetic lethality, drug combinations are used

to target both the primary and secondary pathways, producing a synergistic cytotoxicity [6]. In metabolic vulnerability, this theory of network targeting is further expanded, looking for novel agents that will work with existing therapeutics. Tumour cells have a high metabolic load, due to their requirement to make all the precursors required for constant cell proliferation [7]. As such, the development of drugs that target metabolism may reduce cell proliferation, and synergise with existing drugs to produce more effective combination therapies [8].

Members of the nuclear receptor family of ligand-activated transcription factors generally regulate expression of genes that encode proteins involved in metabolic and transport processes [9]. As such, they may represent important targets to instigate metabolic reprogramming of tumour cells, leading to a reduction in supply of the components required for cell growth [10]. Nuclear receptor expression is widespread throughout the body [11], with a number being (over)expressed in breast tumours [12]. Indeed, two of the three major classifiers for breast cancer, the estrogen receptor (ER; NR3A1) and the progesterone receptor (PR; NR3C3), are nuclear receptors, and disruption of their regulatory action is a successful treatment in receptor positive tumours [13, 14]. The farnesoid X-receptor (FXR; NR1H4) is an adopted nuclear receptor with oxysterols such as primary and secondary bile acids as its endogenous ligands [15, 16]. Bile acids are the metabolic products of cholesterol, and can be toxic to the body at high concentrations. FXR acts to prevent accumulation of bile acids by inducing expression of a second nuclear receptor small heterodimer partner (SHP, NR0B2), which inhibits expression of CYP7A1, the rate-limiting enzyme in bile acid synthesis [17, 18]. The main site of bile acid production and secretion are the liver and intestine, respectively, and this is mirrored by high levels of FXR expression in these organs [19]. However, as approximately 70% of bile acids are re-absorbed by the

body, they can reach micromolar concentrations in the plasma, which may underlie the expression of FXR in a number of other tissues [20]. FXR has been shown to be expressed in both normal breast tissue and breast tumours, with its activation *in vitro* stimulates apoptosis and inhibits aromatase [21]. These features suggest that FXR agonists may represent a novel class of breast cancer therapeutics due to their ability to alter the FXR-regulated metabolic network.

In the present work, we first fully delineate the molecular mechanisms by which FXR agonists activate apoptosis in breast cancer cell lines, demonstrating this to be through the intrinsic pathway. Next, we examine the ability of FXR agonists to stimulate migration in these cell lines, concluding that they do not possess this ability. Together, these data support the further examination of FXR agonists as cancer chemotherapeutics.

2. Materials and Methods

2.1. Materials

The breast tumour cell line MDA-MB-468 (ATCC-HTB-132), and the normal breast cell line MCF10A (ATCC-CRL-10317) were purchased from the ATCC (Teddington, UK), while the breast tumour cell lines MCF-7 (ECACC 86012803) and MDA-MB-231 (ECACC 92020424) were purchased from the ECACC (Porton Down, UK). CDCA and GW4064 were purchased from Sigma Aldrich (Poole, UK) and Tocris Biosciences (Abingdon, UK), respectively.

2.2. Cell Culture

MCF7, MDA-MB-468 and MDA-MB-231 cells were grown in Dulbecco's modified eagle medium (DMEM) with phenol red and L-glutamine, supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 0.1mg/ml streptomycin sulphate, and 0.25µg/ml amphotericin B. MCF10A cells were cultured in Dulbecco's modified eagle medium/F12 (1:1

v/v DMEM/F12) with phenol red, supplemented with 5% horse serum, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 100 μg/ml streptomycin, and 100 units/ml penicillin.

2.3. Viability Assay

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay, as previously described [22]. Briefly, breast cancer cell lines were plated in 96-well tissue culture flasks at a density of 5 x 10^4 cells per well, and allowed to attach overnight. For experiments using serum-starved conditions, cells were grown for a further 24 hours in serum-free medium. Next, cells were exposed to test chemicals for the required time; during the final 60 min of incubation, 0.5 mg/mL MTT was added. At the end of the incubation period, the resultant formazan salt was dissolved in DMSO and its absorbance measured at 540 nm. Results are expressed as a percentage of vehicle control; each data point represents the mean of a minimum of three independent experiments of 6 wells per experiment, with error bars representing the standard error of the mean (SEM).

2.4. Caspase Analysis

Caspase activity was measured using the Caspase-Glo assay system (Promega, UK) as previously reported [23]. Briefly, breast cancer cell lines were seeded in 96-well plates at a density of 1 x 10^4 cells per well, allowed to attach overnight, serum-starved for 24 hours, and then exposed to test chemicals (± specific caspase inhibitor) as required. Next, Caspase-Glo reagent was added, mixed, incubated at room temperature for one hour, and then luminescence measured. Results are expressed as a percentage of vehicle control; each data point represents the mean of a minimum of three independent experiments of 6 wells per experiment, with error bars representing the standard error of the mean (SEM).

2.5. Protein Analysis

Western blot analysis was undertaken as previously described [22]. Briefly, total protein was extracted using RIPA buffer, and protein quantified by the method of Smith [24] . Thirty micrograms of total protein was separated on precast 6-18% polyacrylamide gels, and then transferred to PVDF membrane. Protein were detected overnight at 4˚C using the following primary antibodies and dilutions: FXR 1:500 (sc13063, Santa Cruz Biotechnology); MMP2 1:200 (sc-10736, Santa Cruz Biotechnology); MMP9 1:200 (sc-10737, Santa Cruz Biotechnology); PARP 1:1000 (#9542, Cell Signalling); cytochrome c 1:500 (sc-7159, Santa Cruz Biotechnology); Bax 1:250 (sc-493, Santa Cruz Biotechnology); Bcl2 1:250 (sc-7382, Santa Cruz Biotechnology); SHP 1:250 (sc-15283, Santa Cruz Biotechnology); LC3 1:1000 (#2775, Cell Signalling); and, B-actin 1:2,000 (A2228, Sigma-Aldrich). Next, membranes were incubated with an IRDye 800 CW goat, anti-rabbit secondary antibody (1:10,000) for one hour at room temperature, and imaged using an Odyssey CLx infrared imaging system (LI-COR Biosciences).

2.6. Autophagy Analysis

In addition to examination of autophagy through Western blotting of LC3 cleavage (see above), the accumulation of was p62/SQSTM1 in autophagosomes was quantified using the Premo Autophagy Sensor GFP-p62 assay (Molecular Probes, OR, USA) [25]. Breast cancer cell lines were seeded in 24-well plates at a density of 2 x 10^5 cells per well, allowed to attach overnight, exposed to pGFP-62 induction mix in serum-free medium for 24 hours, and then exposed to test chemicals as required. p62/SQSTM1 fluorescence was quantified and expressed as a percentage of vehicle control; each data point represents the mean of a minimum of three independent experiments, with error bars representing the standard error of the mean (SEM). 3-methyladenine and rapamycin (Sigma-Aldrich) were used as inhibitors and activators of autophagy, respectively [26-28].

2.7. Cell Migration Analysis

For the transwell assay, 24-well transwell inserts with an 8 μm pore size (Corning Inc, USA) were used. Breast cancer cell lines were seeded into the upper chamber at a density of $1x10^5$ cells per well in serum-free medium. Plates were incubated 24 -72 hours, and then cells in the lower chamber counted; to ensure counting of all migrated cells, the lower surface of the transwell was exposed to 1x trypsin:EDTA for 30 minutes.

For the wound-healing assay, 35 mm wound healing- inserts (Ibidi, Martinsried, Germany) were used. Breast cancer cell lines were seeded into the chamber at a density of $1x10^5$ cells per well in serum-free medium and allowed to attach overnight. Inserts were removed, and gap closure measured at time 0 and 12 hours after exposure to test chemical using tScratch program [29].

2.8. Inhibition of FXR activity by FXR dominant-negative over-expression

To demonstrate that the observed effects were FXR-dependent, experiments were undertaken using over-expression of a dominant negative FXR mutant [30]. MCF-7 and MDA-MB-231 cells were transfected with the FXR-DN expressing plasmid using Fugene 6 (Promega, UK) according to the manufacturer's instruction, and incubated overnight to allow protein expression. Cells were then exposed to GW4064 and analysed as previously described.

2.9. Statistical Analysis

Statistical analysis was undertaken using GraphPad Prism v6.01 (GraphPad Software Inc., La Jolla, USA). Datasets were compared through either a one-way ANOVA with Tukey's multiple comparison test or a two-way ANOVA with Sidak's multiple comparison test, as appropriate. The level of statistical significance was set *a priori* at $p < 0.05$.

3. Results and Discussion

3.1. Activation of FXR induces cell death in breast cancer cell lines

The nuclear receptor FXR is classically associated with bile acid homeostasis in the body and its target genes impact on the metabolic and transporter-mediated clearance of both bile acids and their precursors [15]. However, FXR activation has also been reported to elicit a number of other phenotypes, including cell death. To confirm this phenotype in breast cancer cell lines, we examined the effect of the endogenous ligand CDCA and the more potent and selective artificial ligand GW4064 [31]. Four cell lines were chosen to represent different breast cancer phenotypes: normal (i.e. MCF-10A), receptor positive tumour (i.e. MCF-7) and triple negative tumours (i.e. MDA-MB-231 and MDA-MB-468). We confirmed that FXR is expressed in these cell lines, and for MCF-7 and MDA-MB-231 cells that the FXR-dependent signalling cascade is intact, with activation of SHP expression in response to FXR activation (supplemental figure S1).

Both CDCA and GW4064 caused a concentration-dependent toxicity in all cell lines (Figure 1), with the effect generally being more marked in serum-free conditions. This is consistent with the observation that both CDCA and GW4064 are lipophilic, with LogP values of 4.15 and 8.3, respectively [20]. In serum-containing medium, the free concentration of both compounds are expected to be reduced due to protein binding [32]. IC50 values were determined for all conditions and are presented in Table 1. We note that, as expected, IC50 values for GW4064 are approximately three orders of magnitude lower than for CDCA, consistent with the higher affinity of this ligand for FXR [GW4064 Kd=0.01µM versus CDCA Kd=50µM [15, 31]]. Data consistent with these values were also obtained with MTT analysis following 72 hours of exposure of cell lines to each compound (data not shown).

Despite the diverse phenotypes of the four cell lines studied, all responded in the same manner to CDCA and GW4064, both qualitatively and quantitatively, with no statistically significant differences in response for either agonist between cell lines. This would suggest that FXR-dependent cytotoxicity is a general cell response, and not dependent of any emergent phenotypic property of the different genotypes of each cell line.

3.2. FXR-mediated cytotoxicity is mediated through the intrinsic apoptotic cascade

Having determined that FXR agonists stimulate cytotoxicity in all four breast cancer cell lines, we next examined the molecular mechanisms underlying this response in the receptor positive MCF-7 and triple negative MDA-MB-231 cell lines.

Activation of FXR has been previously reported to cause apoptotic cell death in breast cancer MCF-7 and MDA-MB-468 cell lines [21]. As depicted in Figure 2, exposure of MCF-7 and MDA-MB-231 cells to GW4064 or CDCA for 24 hours resulted in a statistically significant activation of the executioner caspases 3/7, and cleavage of its substrate poly(ADP-ribose)polymerase-1 (PARP) [33]. This confirms the data of Swales et al, and extends it to cover another triple negative cell line. In addition, we note Swales et al used high concentrations of GW4064 (30μM; 3,000 x Kd); here we use a lower concentration that will cause 99.9% receptor occupancy while reducing the risk of off-target effects [31]. FXR agonist-mediated activation of caspases 3/7, and subsequent PARP cleavage, is more potent in the MDA-MB-231 cell line compared to the MCF-7 cell line. This is consistent with reports that the MCF-7 cell line lacks expression of caspase 3 [34, 35], meaning only caspase 7 activity is present.

Having confirmed that FXR activation by GW4064 or CDCA causes apoptosis in breast cancer cell lines, we next examined the molecular mechanism(s) underlying this effect. The apoptotic cascade can occur through the intrinsic and extrinsic pathways, with each being

composed of distinct molecular events that converge at the activation of executioner caspases such as caspase 3 and 7 [36]. Caspase 8 activation is classically associated with the extrinsic apoptotic pathway, while caspase 9 activation is indicative of apoptosis via the intrinsic pathway. Exposure of MCF-7 and MDA-MB-231 cells to either 3μM GW4064 or 30μM CDCA for 9 hours caused a statistically significant increase in caspase 9 activity, which could be prevented by co-incubation with the caspase-9 inhibitor Z-LEHD-FMK (Figure 3a). Fold increases in caspase 9 activity were the same order of magnitude as that observed with the positive control, 1μM staurosporine. In contrast, neither compound was able to activate caspase 8 activity in either MCF-7 or MDA-MB-231 cell lines (Figure 3b); presence of a functional extrinsic pathway was demonstrated through the use of the positive control chemical Fas ligand [37]. Together, these data are consistent with activation of FXR resulting in cell death through activation of the intrinsic pathway of apoptosis.

To confirm the ability of GW4064 and CDCA to induce intrinsic pathway-mediated apoptosis, we examined several other molecular markers for this pathway. A key stage in the intrinsic pathway, and one that distinguishes it from the extrinsic pathway, is mitochondrial dysfunction. Members of the BCL2 family are central regulators of this process, with the relative expression of pro- (e.g. Bcl2) and anti- (e.g. BAX) apoptotic family members determining cell fate [36]. Figure 4a demonstrates that FXR activation results in a significant increase in BAX:Bcl2 ratio, with GW4064 increasing the ratio by 2.9±0.3-fold and 4.8±1.0-fold, and CDCA by 2.8±0.4-fold and 2.5±0.2-fold in MCF-7 and MDA-MB-231 cells, respectively. Alteration in this ratio is associated with changes in mitochondrial membrane integrity, leading to cytochrome c release into the cytoplasm, which complexes with Apaf-1 to form the apoptosome [38]. We observe a time-dependent release of cytochrome c following exposure of MCF-7 and MDA-MB-231 cells to both GW4064 and CDCA (Figure 4b).

As noted above, the MCF-7 cell line may have limited apoptotic capacity due to a lack of expression of caspase 3 [34, 35]. Under such conditions, alternative pathways may be initiated. Autophagy is a cell rescue pathway involving degradation and recycling of damaged cell components [39], and the relative roles of autophagy and apoptosis in determining cell fate appears to be highly context dependent, with factors including cellular background, type of perturbation, and degree of damage. Conjugation of cytosolic LC3-I to phosphatidylethanolamine, forming autophagosomal LC3-II is an important marker of autophagy [40], and in Figure 5a we present the impact of GW4064 and CDCA on the LC3-I:LC3-II ratio in both cell lines. In MCF-7 cells, GW4064 is observed to cause a 4.4±0.3-fold increase in this ratio, consistent with activation of the autophagy pathway, but CDCA does not elicit a similar response. In MDA-MB-231 cells, neither compound was able to elicit a significant change in LC3-I:LC3-II. A second experimental approach was used to confirm the activation of autophagy, namely accumulation of p62/SQSTM1, a ubiquitin-binding protein that targets ubiquitinylated proteins for degradation via autophagy [41]. Both 3µM GW4064 and 30µM CDCA elicited a statistically significant increase in p62-GFP fluorescence in MCF-7 cells, but not in MDA-MB-231 cells. This increase was reduced by the addition of 5mM 3-methyladenine, an inhibitor of autophagy. Assay specificity was demonstrated by the positive control compound 2.5µM rapamycin [26].

These data are consistent with MCF-7 cells using the autophagic response as a complement to apoptosis, which is necessary due to the compromised capacity to undergo apoptosis caused by lack of expression of the executioner caspase 3. In support of this, when autophagy is inhibited by 3-methyladenine, no significant increase in caspase 3/7 activation is seen in MCF-7 cells, suggesting that the cells are already utilising the caspase-dependent apoptotic cell death pathway at close to its capacity (Figure 5c).

3.3. Induction of apoptosis and autophagy in breast cancer cell lines is FXR-dependent

As previously noted, while GW4064 is a highly selective ligand against FXR, the actions of CDCA are more promiscuous. It is thus important to demonstrate that the observed effects are mediated via FXR and not an alternate mechanism. To examine this, we have used a dominant negative FXR mutant (FXR-DN) to squelch FXR-mediated signalling [21, 30, 42]. As can be seen from figure 6a, transfection of the FXR-DN plasmid into MCF-7 and MDA-MB-231 cells effectively blocks FXR-dependent signalling, as evidenced by the ablation of SHP induction, a classical FXR target gene, in response to GW4064.

Blockade of FXR signalling by FXR-DN also reduces GW4064 and CDCA-dependent cell death (Figure 6b), consistent with previous reports that this is FXR dependent [21]. The reduction in cell death is due to decreased apoptosis in both cell lines, as evidence by reduced activation of the execution caspases 3/7 (Figure 6c). As previously demonstrated, MCF-7 cells, which are deficient in caspase-3, are also stimulated to undergo autophagy by GW4064, and we demonstrate that this is also FXR-dependent, being reduced in the presence of the FXR-DN mutant (figure 6d).

Together, these data strongly support that the observed effects are mediated through the action of FXR, and not via another, non-specific, mechanism.

3.4. Activation of FXR in breast cancer cell lines does not cause an increase in cell migration

The data presented herein supports the cytotoxic potential of FXR activation in breast cancer cell lines. In addition, we further elucidate the molecular mechanisms underlying this, which are through the programmed apoptotic and autophagic pathways. As such, these data would support the potential of FXR agonists as cancer chemotherapeutic agents.

However, a number of reports have suggested that activation of FXR can alter the migration/invasion phenotype of tumour cells, although the data in this area is controversial. For example, Fukase *et al.* have reported a positive action on migration in the Hep3B hepatoma cell line [43], while Strauch *et al.* demonstrated a similar effect in intestinal epithelial cells [44]. Conversely, Zhang et al reported that FXR suppression promoted proliferation and migration of HepG2 and Hep3B hepatoma cell lines [45], while Yoyo et al. demonstrated a repressive effect on vascular smooth muscle cell migration [46]. Any potential for enhanced cell migration/invasion is a major liability in the development of a therapeutic agent, and we therefore examined the potential of GW4064 and CDCA to effect migration in breast cancer cells.

In the transwell migration assay we were able to detect a significant number of migrated cells following 72 hours incubation for both MCF-7 and MDA-MB-231 cell lines, important as MCF-7 are considered poorly invasive [47]. Exposure of cells in the upper chamber to either 3 μM GW4064 or 30 μM CDCA throughout the incubation period did not result in any increase in migration rates (Figure 7a). In addition, we note that extension of the incubation period to include a 1-hour pre-incubation with FXR agonist also did not impact upon migration rates (data not shown). A second experimental approach was also used to examine migration, the scratch assay. Data from this assay was consistent with the transwell assay; while significant migration could be observed over the 24 hour incubation period, there was no significant increase when MCF-7 or MDA-MB-231 cells were exposed to FXR agonists (Figure 7b).

This data appears in conflict with that of Silva et al, who demonstrated that the secondary bile acid deoxycholate could induce migration of MDA-MB-231, but not MCF-7 cells [48]. However, the methodology used by Silva et al was significantly different from the present

work, and only the secondary bile acid deoxycholate was examined. This bile acid is a product of bacterial metabolism of CDCA, and at the concentration used likely to cause both FXR-dependent and FXR-independent effects [49].

Given the important negative liability associated with any pro-migratory effect, and the conflicting data in this area, we also examined MCF10A and MDA-MB-468 cells for their migratory response upon FXR activation. As presented in supplementary figure S2, no evidence was found to support a pro-migratory phenotype in either of these cell lines. As such, these data support the notion that FXR activation in breast cancer cell lines does not enhance migratory potential. This suggests that any interaction between FXR and cell migration/invasion is complex, and almost certainly context-dependent.

Conflict of interest

KES is an employee of the Institute of Cancer Research. NA, RM, LM and NJP declare that they have no conflicts of interest

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References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer, 136 (2015) E359-E386.

[2] B. Weigelt, A. Mackay, R. A'Hern, R. Natrajan, D.S.P. Tan, M. Dowsett, A. Ashworth, J.S. Reis-Filho, Breast cancer molecular profiling with single sample predictors: a retrospective analysis, Lancet Oncology, 11 (2010) 339-349.

[3] J.S. Reis-Filho, L. Pusztai, Breast Cancer 2 Gene expression profiling in breast cancer: classification, prognostication, and prediction, Lancet, 378 (2011) 1812-1823.

[4] J. Crown, J. O'Shaughnessy, G. Gullo, Emerging targeted therapies in triple-negative breast cancer, Ann. Oncol., 23 (2012) 56-65.

[5] A. Kolodkin, F.C. Boogerd, N. Plant, F.J. Bruggeman, V. Goncharuk, J. Lunshof, R. Moreno-Sanchez, N. Yilmaz, B.M. Bakker, J.L. Snoep, R. Balling, H.V. Westerhoff, Emergence of the silicon human and network targeting drugs, Eur J Pharm Sci, 46 (2012) 190-197.

[6] W.G. Kaelin, The concept of synthetic lethality in the context of anticancer therapy, Nat. Rev. Cancer, 5 (2005) 689-698.

[7] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell, 100 (2000) 57-70.

[8] B. Al-Lazikani, U. Banerji, P. Workman, Combinatorial drug therapy for cancer in the post-genomic era, Nature Biotechnology, 30 (2012) 679-691.

[9] N. Plant, S. Aouabdi, Nuclear receptors: the controlling force in drug metabolism of the liver?, Xenobiotica, 39 (2009) 597-605.

[10] H. Gronemeyer, J.A. Gustafsson, V. Laudet, Principles for modulation of the nuclear receptor superfamily, Nat. Rev. Drug Discov., 3 (2004) 950-964.

[11] A.L. Bookout, Y. Jeong, M. Downes, R.T. Yu, R.M. Evans, D.J. Mangelsdorf, Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network, Cell, 126 (2006) 789-799.

[12] M. Uhlen, L. Fagerberg, B.M. Hallstroem, C. Lindskog, P. Oksvold, A. Mardinoglu, A. Sivertsson, C. Kampf, E. Sjoestedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani,

C.A.-K. Szigyarto, J. Odeberg, D. Djureinovic, J.O. Takanen, S. Hober, T. Alm, P.-H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J.M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen, F. Ponten, Tissue-based map of the human proteome, Science, 347 (2015) 394-+.

[13] N.E. Hynes, H.A. Lane, ERBB receptors and cancer: The complexity of targeted inhibitors, Nat. Rev. Cancer, 5 (2005) 341-354.

[14] X.J. Cui, R. Schiff, G. Arpino, C.K. Osborne, A.V. Lee, Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy, J. Clin. Oncol., 23 (2005) 7721-7735.

[15] M. Makishima, A.Y. Okamoto, J.J. Repa, H. Tu, R.M. Learned, A. Luk, M.V. Hull, K.D. Lustig, D.J. Mangelsdorf, B. Shan, Identification of a nuclear receptor for bile acids, Science, 284 (1999) 1362-1365.

[16] D.J. Parks, S.G. Blanchard, R.K. Bledsoe, G. Chandra, T.G. Consler, S.A. Kliewer, J.B. Stimmel, T.M. Willson, A.M. Zavacki, D.D. Moore, J.M. Lehmann, Bile acids: Natural ligands for an orphan nuclear receptor, Science, 284 (1999) 1365-1368.

[17] B. Goodwin, S.A. Jones, R.R. Price, M.A. Watson, D.D. McKee, L.B. Moore, C. Galardi, J.G. Wilson, M.C. Lewis, M.E. Roth, P.R. Maloney, T.M. Willson, S.A. Kliewer, A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis, Molecular Cell, 6 (2000) 517-526.

[18] G. Rizzo, B. Renga, A. Mencarelli, R. Pellicciari, S. Fiorucci, Role of FXR in regulating bile acid homeostasis and relevance for human diseases., Current Drug Targets for Immune, Endocrinology and Metabolic Disorders, 5 (2005) 289-303.

[19] G.R. Mishra, M. Suresh, K. Kumaran, N. Kannabiran, S. Suresh, P. Bala, K. Shivakumar, N. Anuradha, R. Reddy, T.M. Raghavan, S. Menon, G. Hanumanthu, M. Gupta, S. Upendran, S.

Gupta, M. Mahesh, B. Jacob, P. Mathew, P. Chatterjee, K.S. Arun, S. Sharma, K.N. Chandrika, N. Deshpande, K. Palvankar, R. Raghavnath, R. Krishnakanth, H. Karathia, B. Rekha, R. Nayak, G. Vishnupriya, H.G.M. Kumar, M. Nagini, G.S.S. Kumar, R. Jose, P. Deepthi, S.S. Mohan, T.K.B. Gandhi, H.C. Harsha, K.S. Deshpande, M. Sarker, T.S.K. Prasad, A. Pandey, Human protein reference database - 2006 update, Nucleic Acids Res., 34 (2006) D411-D414.

[20] D.S. Wishart, T. Jewison, A.C. Guo, M. Wilson, C. Knox, Y. Liu, Y. Djoumbou, R. Mandal, F. Aziat, E. Dong, S. Bouatra, I. Sinelnikov, D. Arndt, J. Xia, P. Liu, F. Yallou, T. Bjorndahl, R. Perez-Pineiro, R. Eisner, F. Allen, V. Neveu, R. Greiner, A. Scalbert, HMDB 3.0-The Human Metabolome Database in 2013, Nucleic Acids Res., 41 (2013) D801-D807.

[21] K.E. Swales, M. Korbonits, R. Carpenter, D.T. Walsh, T.D. Warner, B.-B. D., The farnesoid X receptor is expressed in breast cancer and regulates apoptosis and aromatase expression., Cancer Res., 66 (2006) 10120-10126.

[22] R.H. Gee, J.N. Spinks, J.M. Malia, J.D. Johnston, N.J. Plant, K.E. Plant, Inhibition of prenyltransferase activity by statins in both liver and muscle cell lines is not causative of cytotoxicity, Toxicology, 329 (2015) 40-48.

[23] K.E. Plant, E. Anderson, N. Simecek, R. Brown, S. Forster, J. Spinks, N. Toms, G.G. Gibson, J. Lyon, N. Plant, The neuroprotective action of the mood stabilizing drugs lithium chloride and sodium valproate is mediated through the up-regulation of the homeodomain protein Six1, Toxicol. Appl. Pharmacol., 235 (2009) 124-134.

[24] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Analytical Biochemistry, 150 (1985) 76-85.

[25] N.J. Dolman, K.M. Chambers, B. Mandavilli, R.H. Batchelor, M.S. Janes, Tools and techniques to measure mitophagy using fluorescence microscopy, Autophagy, 9 (2013) 1653-1662.

[26] Y.-p. Yang, L.-f. Hu, H.-f. Zheng, C.-j. Mao, W.-d. Hu, K.-p. Xiong, F. Wang, C.-f. Liu, Application and interpretation of current autophagy inhibitors and activators, Acta Pharmacol. Sin., 34 (2013) 625-635.

[27] Y. Sheng, B. Sun, W.-T. Guo, Y.-H. Zhang, X. Liu, Y. Xing, D.-L. Dong, 3-Methyladenine induces cell death and its interaction with chemotherapeutic drugs is independent of autophagy, Biochem. Biophys. Res. Commun., 432 (2013) 5-9.

[28] K.A. Tekirdag, G. Korkmaz, D.G. Ozturk, R. Agami, D. Gozuacik, MIR181A regulates starvation- and rapamycin-induced autophagy through targeting of ATG5, Autophagy, 9 (2013) 374-385.

[29] T. Gebaeck, M.M.P. Schulz, P. Koumoutsakos, M. Detmar, TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays, Biotechniques, 46 (2009) 265-+.

[30] T.A. Kocarek, S.D. Shenoy, N.A. Mercer-Haines, M. Runge-Morris, Use of dominant negative nuclear receptors to study xenobiotic-inducible gene expression in primary cultured hepatocytes, J. Pharmacol. Toxicol. Methods, 47 (2002) 177-187.

[31] P.R. Maloney, D.J. Parks, C.D. Haffner, A.M. Fivush, G. Chandra, K.D. Plunket, K.L. Creech, L.B. Moore, J.G. Wilson, M.C. Lewis, S.A. Jones, T.M. Willson, Identification of a chemical tool for the orphan nuclear receptor FXR, Journal of Medicinal Chemistry, 43 (2000) 2971-2974.

[32] T. Ghafourian, Z. Amin, QSAR models for the prediction of plasma protein binding, BioImpacts : BI, 3 (2013) 21-27.

[33] S.H. Kaufmann, S. Desnoyers, Y. Ottaviano, N.E. Davidson, G.G. Poirier, Specific proteolytic cleavage of poly(adp-ribose) polymerase - an early marker of chemotherapy-induced apoptosis, Cancer Res., 53 (1993) 3976-3985.

[34] M.J. Abedin, D. Wang, M.A. McDonnell, U. Lehmann, A. Kelekar, Autophagy delays apoptotic death in breast cancer cells following DNA damage, Cell Death and Differentiation, 14 (2007) 500-510.

[35] R.U. Jaenicke, MCF-7 breast carcinoma cells do not express caspase-3, Breast Cancer Research and Treatment, 117 (2009) 219-221.

[36] S. Elmore, Apoptosis: A review of programmed cell death, Toxicologic Pathology, 35 (2007) 495-516.

[37] S. Nagata, P. Golstein, The FAS death factor, Science, 267 (1995) 1449-1456.

[38] H. Zou, Y.C. Li, H.S. Liu, X.D. Wang, An APAF-1 center dot cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9, J. Biol. Chem., 274 (1999) 11549-11556.

[39] D.J. Klionsky, S.D. Emr, Cell biology - Autophagy as a regulated pathway of cellular degradation, Science, 290 (2000) 1717-1721.

[40] Y. Kabeya, N. Mizushima, T. Uero, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, Embo Journal, 19 (2000) 5720-5728.

[41] S. Barth, D. Glick, K.F. Macleod, Autophagy: assays and artifacts, Journal of Pathology, 221 (2010) 117-124.

[42] F.T. He, J. Li, Y. Mu, R. Kuruba, Z. Ma, A. Wilson, S. Alber, Y. Jiang, T. Stevens, S. Watkins, B. Pitt, W. Xie, S. Li, Downregulation of endothelin-1 by farnesoid X receptor in vascular endothelial cells, Circulation Research, 98 (2006) 192-199.

[43] K. Fukase, H. Ohtsuka, T. Onogawa, H. Oshio, T. Ii, M. Mutoh, Y. Katayose, T. Rikiyama, M. Oikawa, F. Motoi, S. Egawa, T. Abe, M. Unno, Bile acids repress E-cadherin through the induction of Snail and increase cancer invasiveness in human hepatobiliary carcinoma, Cancer Science, 99 (2008) 1785-1792.

[44] E.D. Strauch, J. Yamaguchi, B.L. Bass, J.Y. Wang, Bile salts regulate intestinal epithelial cell migration by nuclear factor-kappa B-induced expression of transforming growth factor-beta, Journal of the American College of Surgeons, 197 (2003) 974-984.

[45] Y. Zhang, W. Gong, S. Dai, G. Huang, X. Shen, M. Gao, Z. Xu, Y. Zeng, F. He, Downregulation of Human Farnesoid X Receptor by miR-421 Promotes Proliferation and Migration of Hepatocellular Carcinoma Cells, Molecular Cancer Research, 10 (2012) 516-522.

[46] Y.T.Y. Li, K.E. Swales, G.J. Thomas, T.D. Warner, D. Bishop-Bailey, Farnesoid X receptor ligands inhibit vascular smooth muscle cell inflammation and migration, Arteriosclerosis Thrombosis and Vascular Biology, 27 (2007) 2606-2611.

[47] D.A. Zajchowski, M.F. Bartholdi, Y. Gong, L. Webster, H.L. Liu, A. Munishkin, C. Beauheim, S. Harvey, S.P. Ethier, P.H. Johnson, Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells, Cancer Res., 61 (2001) 5168-5178.

[48] J. Silva, S. Dasgupta, G.H. Wang, K. Krishnamurthy, E. Ritter, E. Bieberich, Lipids isolated from bone induce the migration of human breast cancer cells, Journal of Lipid Research, 47 (2006) 724-733.

[49] P. Lefebvre, B. Cariou, F. Lien, F. Kuipers, B. Staels, Role of Bile Acids and Bile Acid Receptors in Metabolic Regulation, Physiol. Rev., 89 (2009) 147-191.

Table 1: IC50 values for CDCA- and GW4064-elicited cell death in breast cancer cell lines

	CDCA		GW4064	
	Complete medium	Serum-free medium	Complete medium	Serum-free medium
MCF-7	n.d.	244.7±9.5 µM	10.6±2.6 µM	12.6±2.8 µM
MDA-MB-231	n.d.	273.3±32.2 µM	9.5±3.9 µM	3.7±3.3 µM
MDA-MB-468	n.d.	188.5±124.4 µM	n.d.	4.8±1.4 µM
MCF-10A	n.d.	142.0±87.4 µM	2.6±2.6 µM	3.8±1.1 µM

n.d. = not determined as slope did not converge

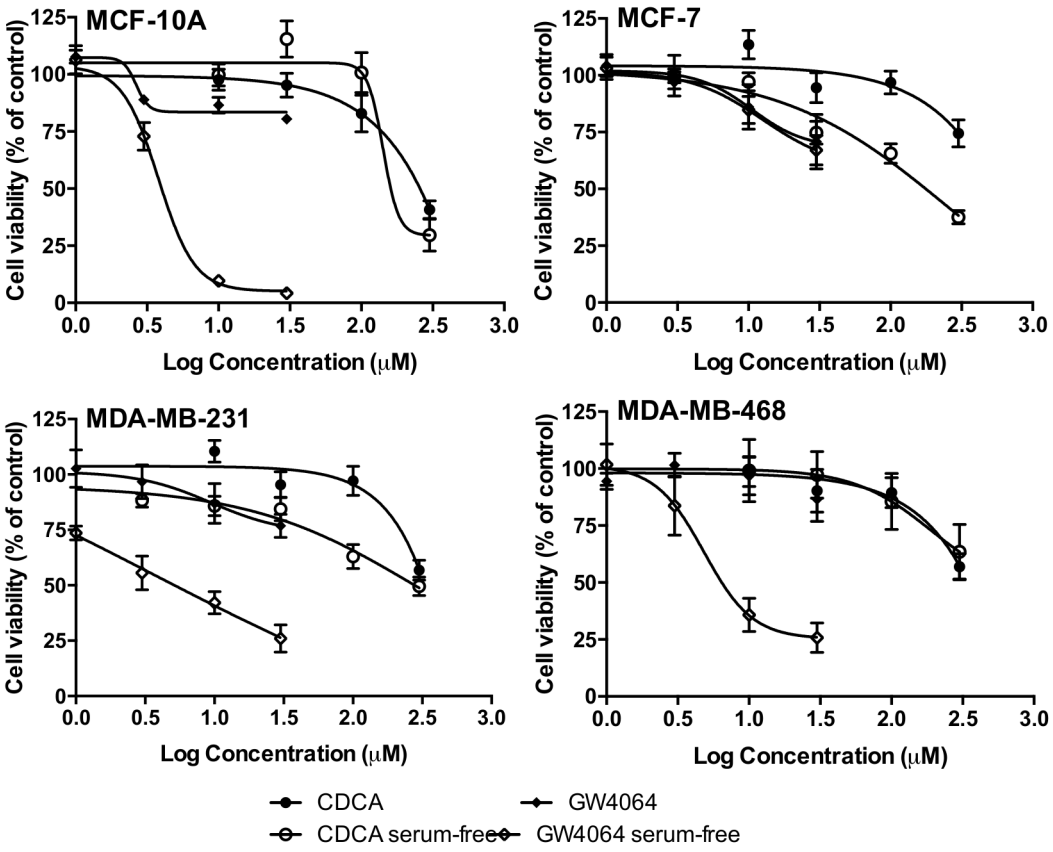


Figure 1: The FXR agonists GW4064 and CDCA cause concentration-dependent toxicity in breast cancer cell lines. MCF-10A (A), MCF-7 (B), MDA-MB-231 (C) and MDA-MB-468 (D) cells were exposed to varying concentrations of GW4064 or CDCA in complete or serum-free medium. Following 48 or 72 hours of exposure, cell viability was assessed by MTT. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

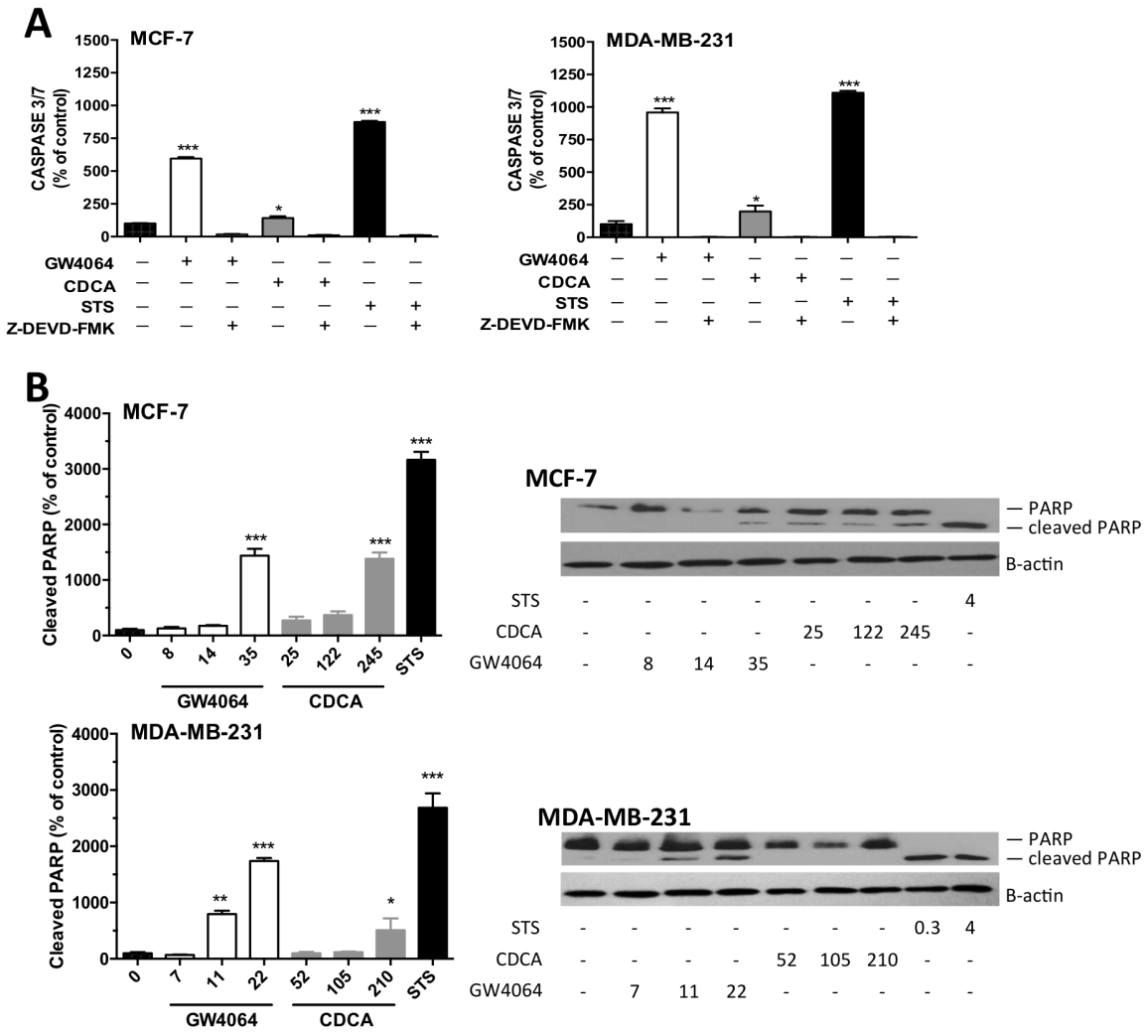


Figure 2: The FXR agonists GW4064 and CDCA cause concentration-dependent activation of caspase 3 and caspase 7 in the MCF-7 and MDA-MB-231 cell lines. (A) MCF-7 and MDA-MB-231 cells were exposed to 3µM GW4064, 30µM CDCA, or 1µM staurosporine in serum-free medium, in the presence or absence of specific caspase inhibitors for 24 hours, and then Caspase 3 and caspase 7 activity measured. (B) MCF-7 and MDA-MB-231 cells were exposed to indicated concentrations of GW4064, CDCA or staurosporine in serum-free medium for 24 hours and then poly(ADP-ribose)polymerase-1 (PARP) cleavage assessed by Western blot. Each data point represents the mean of three independent experiments,

while error bars represent the standard error of the mean (SEM). * = p<0.05, ** = p<0.01, *** = p<0.001

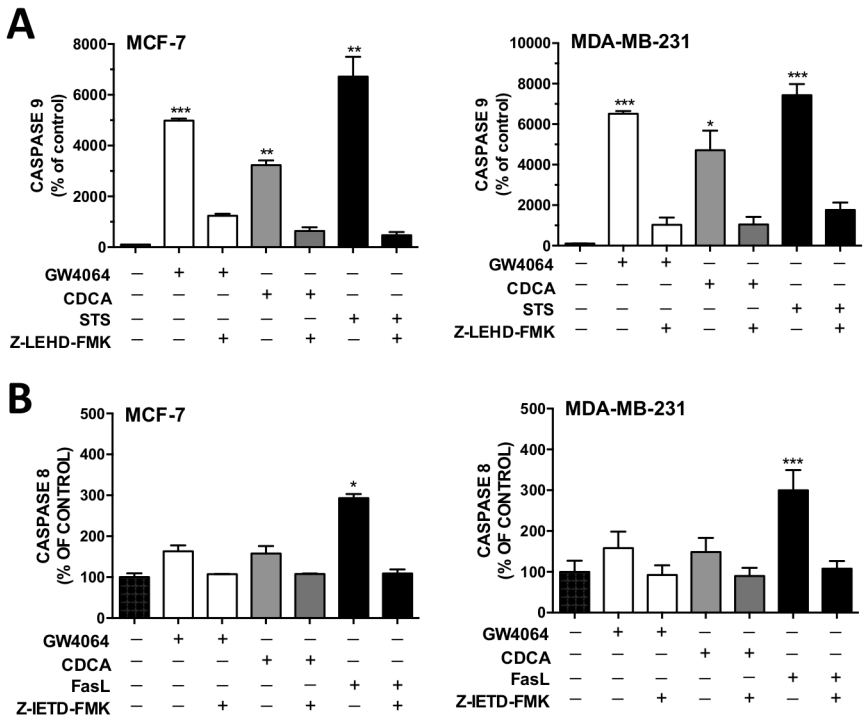


Figure 3: The FXR agonists GW4064 and CDCA activate caspase 9, but not caspase 8, activity in the MCF-7 and MDA-MB-231 cell lines. MCF-7 and MDA-MB-231 cells were exposed to 3µM GW4064, 30 µM CDCA or 1µM staurosporine in serum-free medium for 24 hours, and then Caspase 8 and caspase 9 activity measured. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM). * = p<0.05, ** = p<0.01, *** = p<0.001

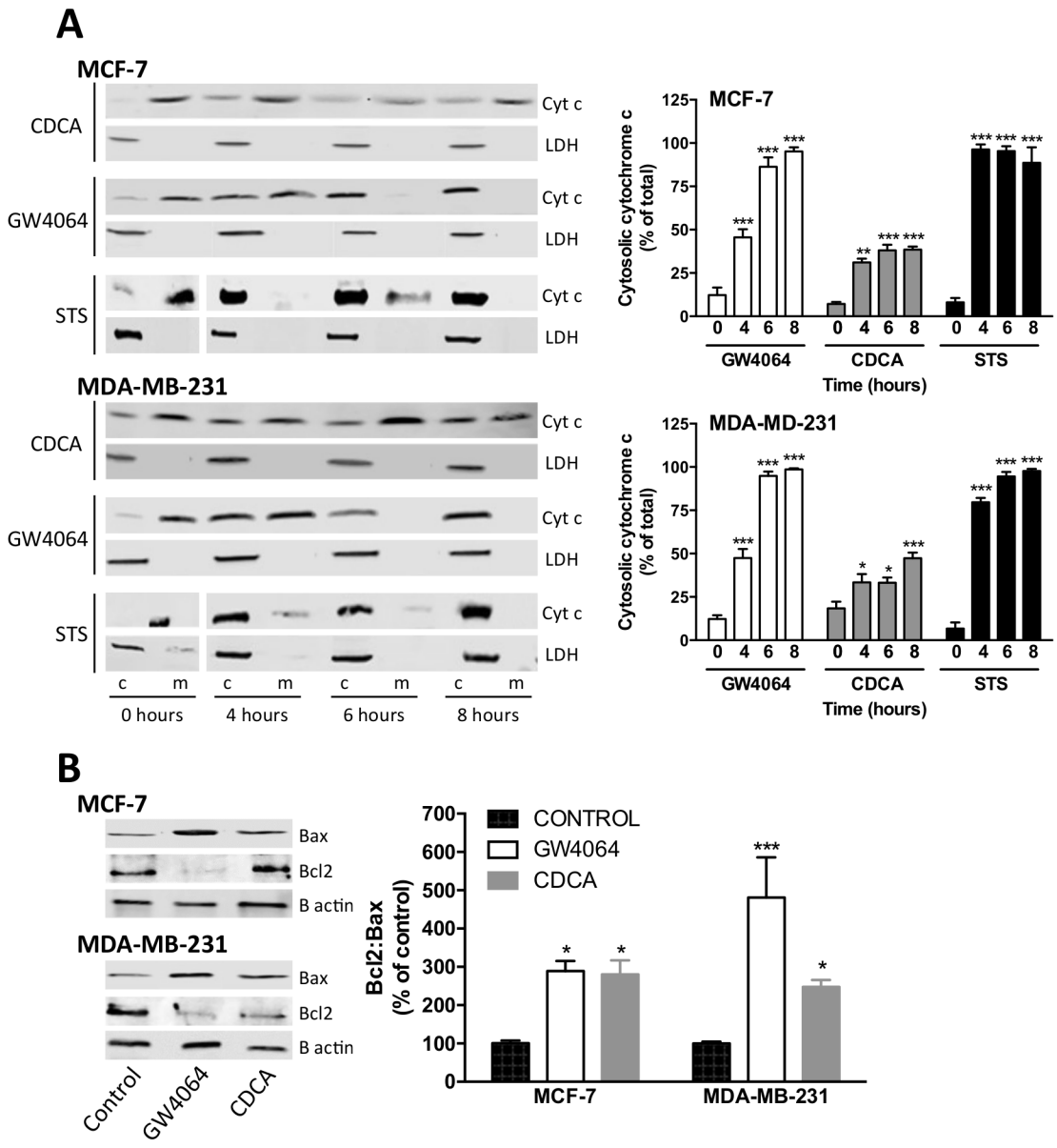


Figure 4: The FXR agonists GW4064 and CDCA stimulate apoptosis through the intrinsic pathway in MCF-7 and MDA-MB-231 cell lines. MCF-7 and MDA-MB-231 cells were exposed to 3µM GW4064, 30 µM CDCA or 1µM staurosporine in serum-free medium. (A) Subcellular localisation of cytochrome c was examined following 0, 4, 6 and 8 hours of exposure, with LDH as a marker for the cytosolic fraction. (B) Bax and Bcl2 protein levels were measured in total protein following 24 hours of exposure. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM). * = p<0.05, ** = p<0.01, *** = p<0.001

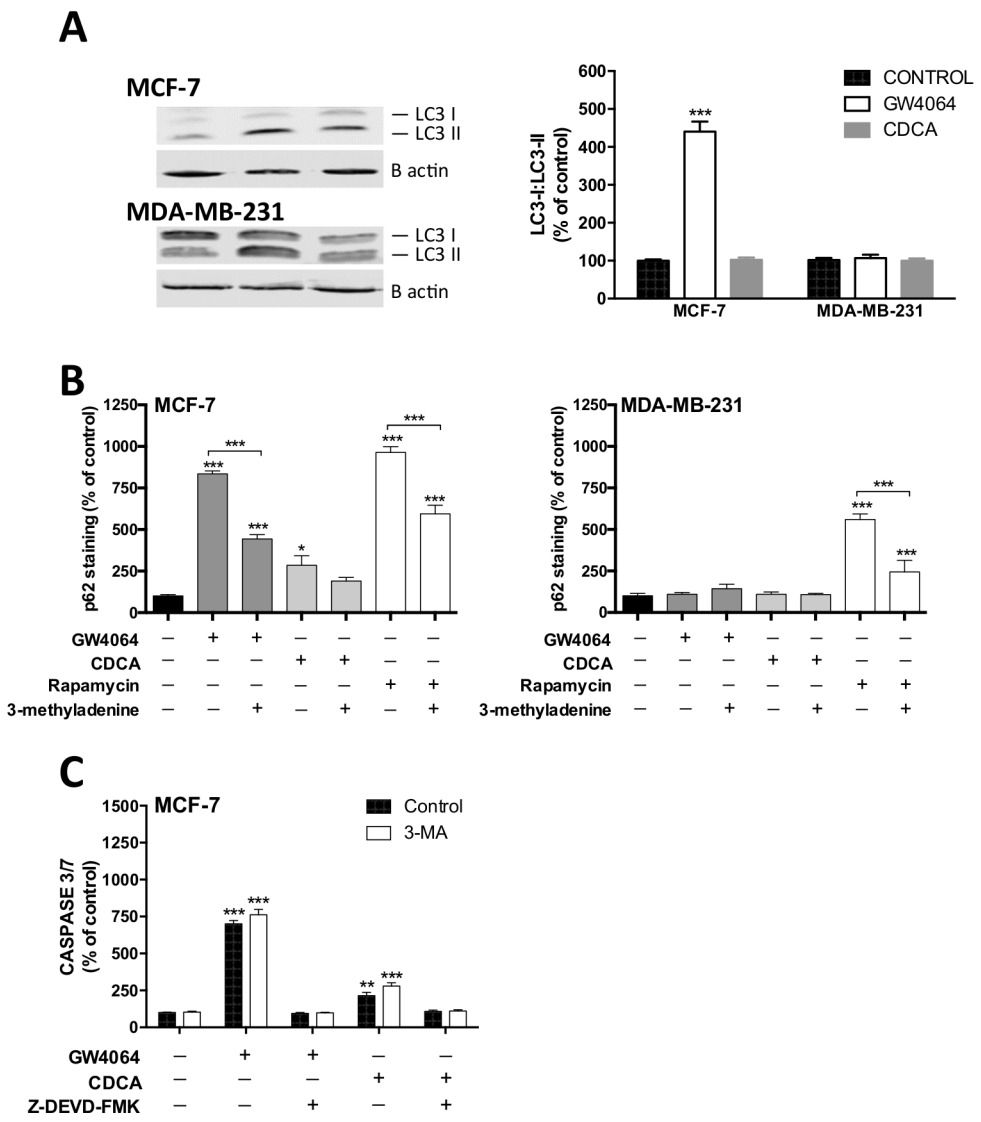
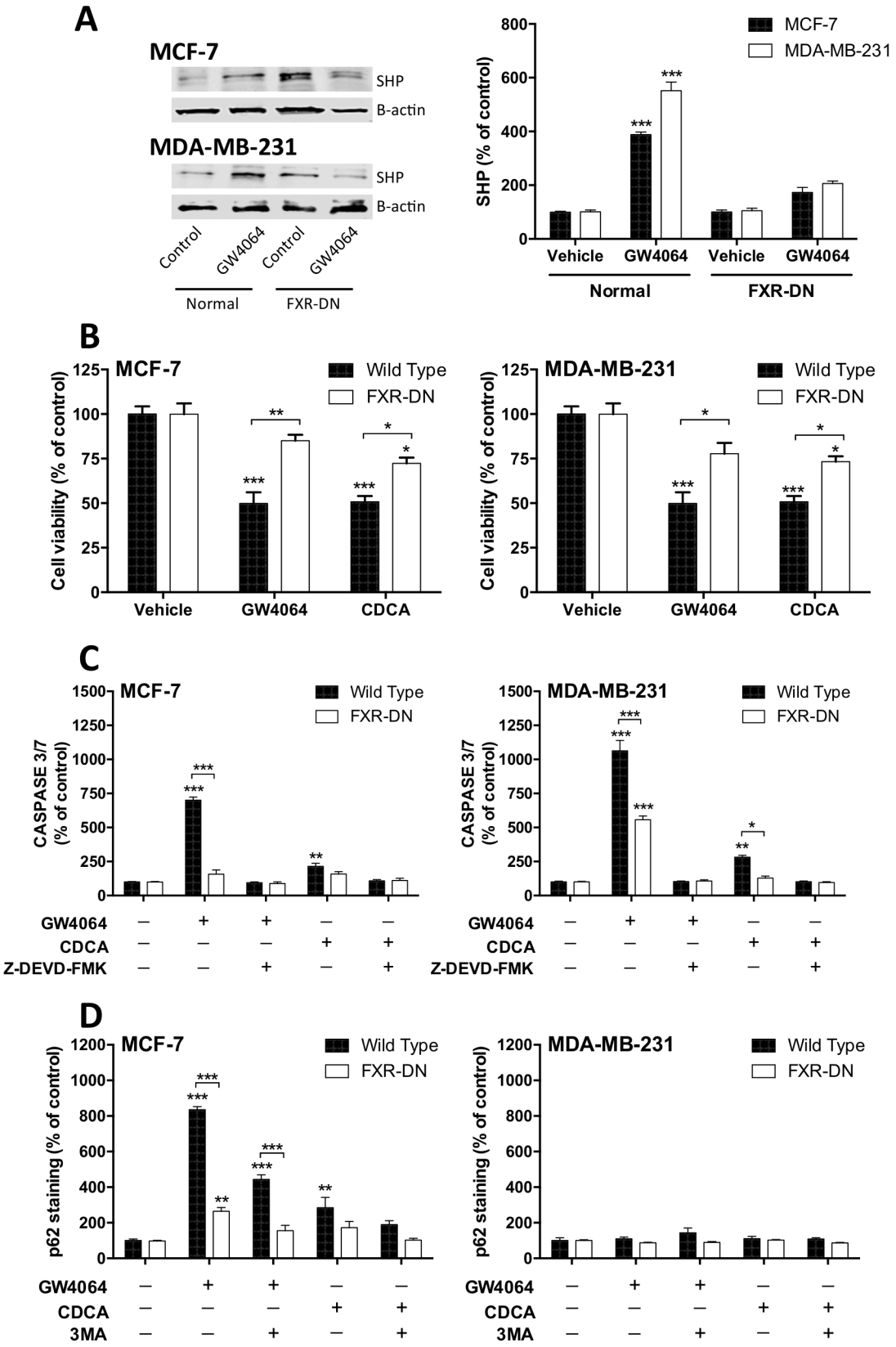


Figure 5: The FXR agonist GW4064 stimulates autophagy in MCF-7 cells. MCF-7 and MDA-MB-231 cells were exposed to 3µM GW4064 or 30 µM CDCA in serum-free medium for 24 hours and autophagy assessed by (A) LC3 cleavage and (B) p62/SQSTM1 accumulation. 5mM 3-methyladenine and 2.5µM rapamycin were included where indicated as an inhibitor and activator of autophagy, respectively. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM). * = $p<0.05$, *** = $p<0.001$


A
MCF-7
SHP
B-actin
MDA-MB-231
SHP
B-actin
Control
GW4064
Control
GW4064
Normal
FXR-DN
MCF-7
MDA-MB-231
SHP (% of control)
800
600
400
200
0
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***
Vehicle
GW4064
Vehicle
GW4064
Normal
FXR-DN
B
MCF-7
Wild Type
FXR-DN
Cell viability (% of control)
125
100
75
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25
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Vehicle
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Wild Type
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Cell viability (% of control)
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CASPASE 3/7 (% of control)
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CDCA – – – + +
3MA – – + – +

Figure 6: Induction of apoptosis and autophagy in breast cancer cell lines by GW6064 and CDCA is FXR-dependent. MCF-7 and MDA-MB-231 cells were exposed to GW4064 or CDCA in serum-free medium for 24 hours in the presence or absence of a dominant negative FXR protein (FXR-DN). (A) Cells were exposed to 3µM GW4064 or 30 µM CDCA and expression of the FXR target gene SHP monitored by Western blotting. (B) Cells were exposed to IC50 concentration of GW4064 or CDCA and cell viability assessed by MTT assay. (C) Cells were exposed to 3µM GW4064 or 30 µM CDCA and apoptosis assessed by caspase 3/7 activity assay. (D) Cells were exposed to 3µM GW4064 or 30 µM CDCA and autophagy assessed by p62/SQSTM1 accumulation. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

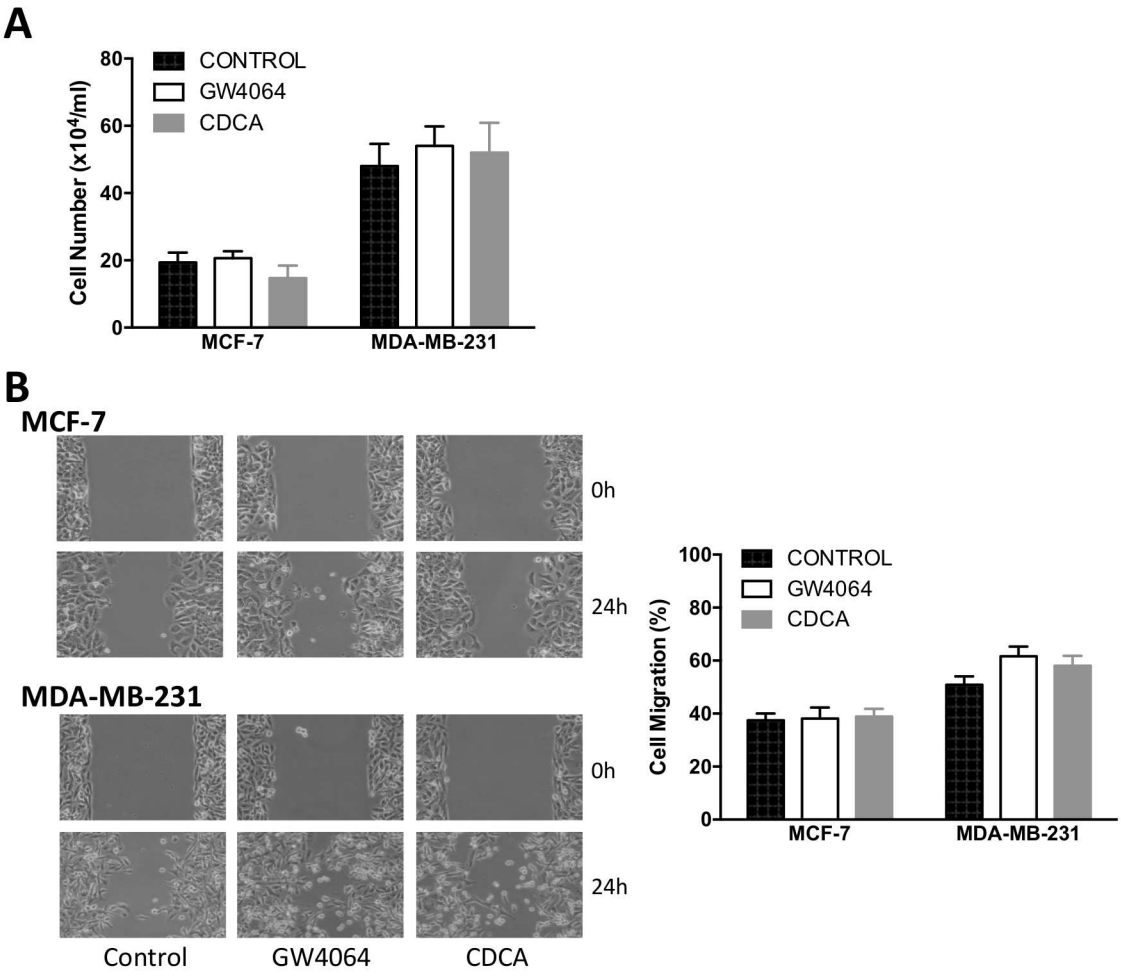

Figure 7: The FXR agonists GW4064 and CDCA do not stimulate migration of MCF-7 or MDA-MB-231 cell lines. (A) MCF-7 and MDA-MB-231 cells were seeded in transwell plates and exposed to 3µM GW4064 or 30 µM CDCA in serum-free medium for 72 hours. The number of cells migrating into the bottom chamber was quantified. (B) MCF-7 and MDA-MB-231 cells were seeded in a wound-healing assay format and exposed to 3µM GW4064 or 30 µM CDCA in serum-free medium for 24 hours. Cell migration was quantified by closure of the scratch area using the tScatch program. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM).

Supplementary Figures

Activation of the Farnesoid X-receptor in breast cancer cell lines results in cytotoxicity but not increased migration potential

Noura Alasmael, Rati Mohan, Lisiane B. Meira, Karen E. Swales and Nick J. Plant

Supplementary Figure 1: Breast cancer cell lines express a functional FXR gene regulatory network

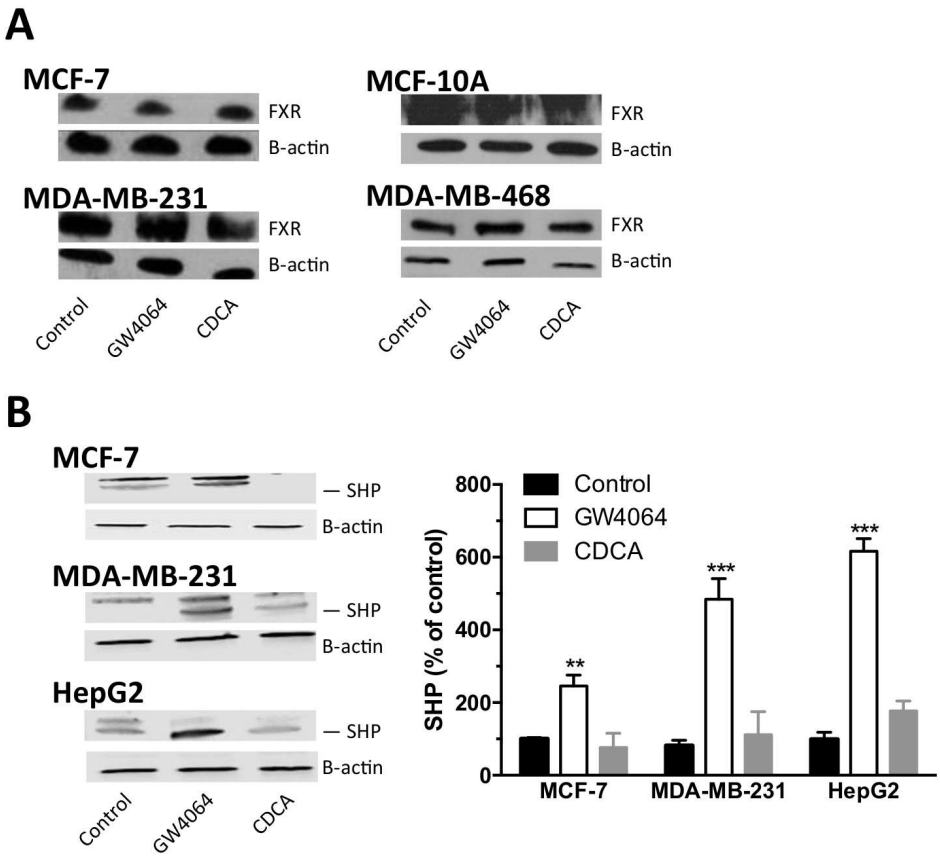

Figure S1: Breast cancer cell lines express a functional FXR gene regulatory network. (A) MCF-10A, MCF-7, MDA-MB-231 and MDA-MB-468 cells were exposed to 3µM GW4064, 30µM CDCA or vehicle control in serum-free medium for 24 hours, and then Farnesoid X-receptor (FXR) expression assessed by Western blot. (B) MCF-7, MDA-MB-231 or HepG2 (positive control) cells were exposed to 3µM GW4064, 30µM CDCA or vehicle control in serum-free medium for 24 hours, and then small heterodimer partner (SHP) expression assessed by Western blot. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM). ** = $p<0.01$, *** = $p<0.001$

Supplementary Figure S2: The FXR agonists GW4064 and CDCA do not stimulate migration of MCF-10A or MDA-MB-468 cell lines

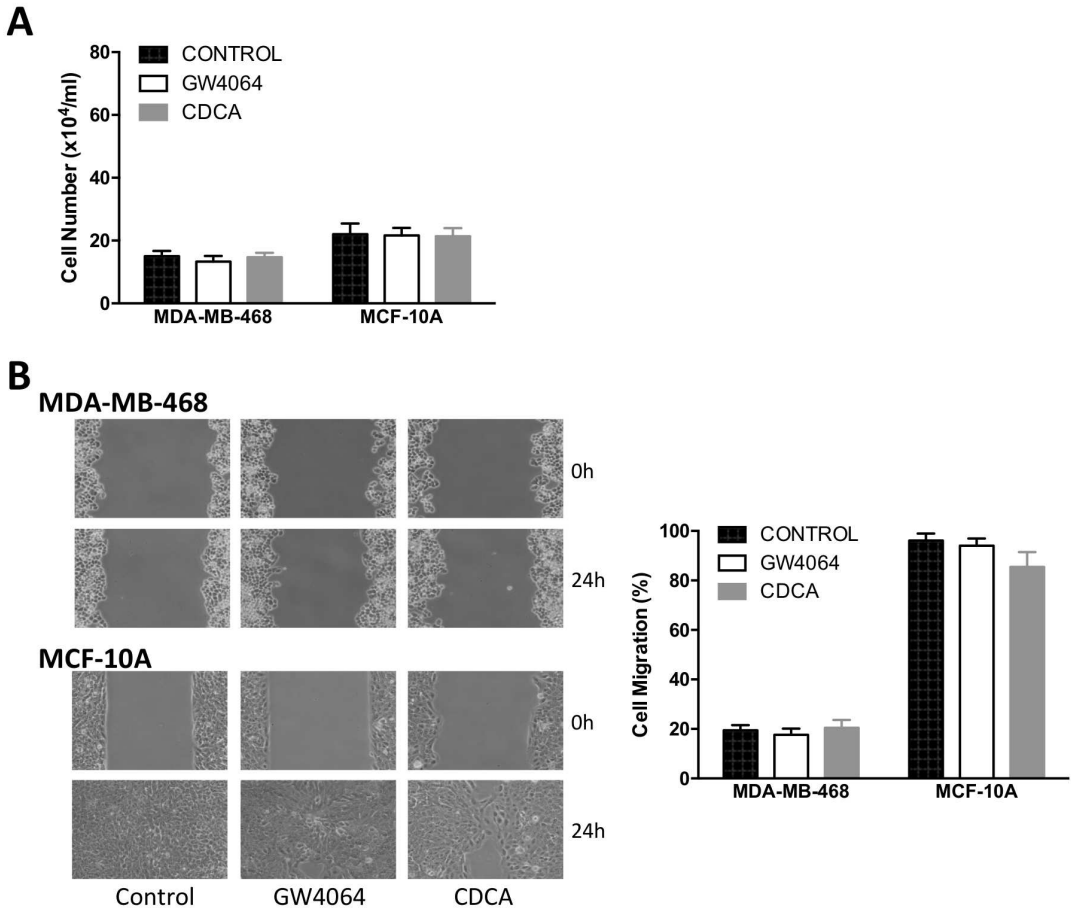

Figure S2: The FXR agonists GW4064 and CDCA do not stimulate migration of MCF-10A or MDA-MB-468 cell lines. (A) MCF-10A and MDA-MB-468 cells were seeded in transwell plates and exposed to 3µM GW4064 or 30 µM CDCA in serum-free medium for 72 hours. The number of cells migrating into the bottom chamber was quantified. (B) MCF-7 and MDA-MB-231 cells were seeded in a wound-healing assay format and exposed to 3µM GW4064 or 30 µM CDCA in serum-free medium for 24 hours. Cell migration was quantified by closure of the scratch area using the tScatch program. Each data point represents the mean of three independent experiments, while error bars represent the standard error of the mean (SEM).