Breast Cancer Research

Tamoxifen enhances the cytotoxic effects of nelfinavir in breast cancer cells

Breast Cancer Research 2010, **12**:R45 doi:10.1186/bcr2602

Ansgar Bruning (ansgar.bruening@med.uni-muenchen.de)
Klaus Friese (klaus.friese@med.uni-muenchen.de)
Alexander Burges (alexander.burges@med.uni-munenchen.de)
Ioannis Mylonas (ioannis.mylonas@med.uni-muenchen.de)

ISSN 1465-5411

Article type Research article

Submission date 1 March 2010

Acceptance date 1 July 2010

Publication date 1 July 2010

Article URL http://breast-cancer-research.com/content/12/4/R45

Tamoxifen enhances the cytotoxic effects of nelfinavir in breast cancer cells

Ansgar Brüning[1,2,]†, Klaus Friese[1,2], Alexander Burges[2*], Ioannis Mylonas[1*]

1) Department of Obstetrics and Gynaecology, Campus Innenstadt, Ludwig-Maximilians-University Munich, 11 Maistrasse, Munich 80337, Germany

2) Department of Obstetrics and Gynaecology, Campus Grosshadern, Ludwig-Maximilians-University Munich, 15 Marchioninistrasse, Munich 81377, Germany

[*]Contributed equally

†Address for correspondence:

Ansgar Brüning

ansgar231@web.de

Abstract

Introduction: The HIV protease inhibitor nelfinavir is currently under investigation as a new anti-cancer drug. Several studies have shown that nelfinavir induces cell cycle arrest, endoplasmic reticulum stress, autophagy, and apoptosis in cancer cells. Here, the effect of nelfinavir on human breast cancer cells was examined, and potential combination treatment options investigated.

Methods: The effects of nelfinavir and tamoxifen on the human breast cancer cell lines MCF7, T47D, MDA-MB-453, and MDA-MB-435 were tested by analyzing their effects on cell viability (via 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay), apoptosis (annexin binding, poly [ADP-ribose] polymerase (PARP) cleavage), autophagy (autophagy marker light chain 3B (LC3B) expression), endoplasmic reticulum stress (binding protein (BiP) and activating transcription factor 3 (ATF3) expression), and the occurrence of oxidative stress (intracellular glutathione level).

Results: Nelfinavir induced apoptosis in all of the four breast cancer cell lines tested, although the extent of autophagy and endoplasmic reticulum stress varied among the cell lines. The concentration of nelfinavir needed for an efficient induction of apoptosis in breast cancer cells could be reduced from 15 µg/ml to 6 µg/ml when combined with tamoxifen. At a concentration of 6 µg/ml, tamoxifen substantially enhanced the endoplasmic reticulum stress reaction in those cell lines that responded to nelfinavir with BiP upregulation (MCF7, T47D), and enhanced autophagy in cell lines that responded to nelfinavir treatment with LC3B upregulation (MDA-MB-453). Although tamoxifen has been described to be able to induce oxidative stress at concentrations similar to those applied in this study (6 µg/ml), we observed that nelfinavir but not tamoxifen reduced the intracellular glutathione levels of breast cancer

cells within hours of application by up to 32 %, suggesting the induction of oxidative stress was an early event and an additional cause of the apoptosis induced by nelfinavir.

Conclusions: The results demonstrate that nelfinavir may be an effective drug against breast cancer and could be combined with tamoxifen to enhance its efficacy against breast cancer cells. Moreover, the cytotoxic effect of a tamoxifen and nelfinavir combination was independent of the oestrogen receptor status of the analysed breast cancer cells, suggesting a potential benefit of a combination of these two drugs even in patients with no hormone-responsive tumours. We therefore recommend that clinical studies on nelfinavir with breast cancer patients should include this drug combination to analyse the therapeutic efficacy as well as the safety and tolerability of this potential treatment option.

Introduction

Breast cancer is the most frequent cancer in the female population [1]. Although tremendous progress in the treatment of breast cancer has been achieved during the past decades, it is still the principal cause of cancer death for females worldwide [1, 2].

Tamoxifen is a selective oestrogen receptor antagonist and since its introduction in cancer therapy, it has become the standard treatment option for hormone-responsive breast cancer patients [2-5]. However, not all breast cancer patients benefit from an endocrine therapy with tamoxifen [3]. Interestingly, several hormone receptor-independent effects of tamoxifen have been described, leading to apoptosis when higher concentrations of tamoxifen are applied [6, 7]. Therefore, a combination therapy of tamoxifen with other drugs that cause synergistic anti-tumour effects might be an interesting option in the therapy of breast carcinomas.

Nelfinavir (Viracept®) is an HIV protease inhibitor that has long been an essential component of the antiviral combination therapy HAART (highly active antiretroviral therapy). Several recent *in vitro* studies have indicated that nelfinavir has potential anti-tumoral effects [8, 9] and clinical studies on nelfinavir are ongoing to confirm its efficacy against human cancers *in vivo* [10-13]. Nelfinavir exerts pleiotropic effects on cancer cells, including induction of apoptosis, necrosis, and autophagy [9, 14, 15]. It is believed that nelfinavir either cross-reacts with a protease of the cytoplasmic proteasomal protein degradation machinery or with endoplasmic reticulum-resident proteases [15, 16]. In both cases, this protease inhibition can lead to the accumulation of misfolded proteins that cause the unfolded protein response (UPR) or endoplasmic reticulum stress response [17-19]. These pathways are primarily associated with a transient cell cycle arrest and upregulation of molecular chaperones such as BiP and other members of the heat shock family, in order to

repair and prevent further cell damage [18]. However, a prolonged or irreparable stress reaction eventually switches from a repair and survival mechanism to cell death executed by apoptosis [20, 21]. This non-classical cell death mechanism has recently become of interest because of its ability to act even on otherwise chemoresistant human cancer cells [15, 22].

Since the orally available drugs tamoxifen and nelfinavir have anti-tumoral properties, a combination of these medications might be an intriguing option in the therapy of breast cancer patients. However, no data regarding their potential synergistic effects are available yet. Therefore, we tested the effect of nelfinavir and tamoxifen with regard to its influence on apoptosis, endoplasmic reticulum stress, autophagy, and oxidative stress in breast cancer cells with different oestrogen receptor status.

Materials and methods

Cells and cell culture

The breast cancer cell lines T47D (ATCC HTB 133; oestrogen receptor positive), MCF7 (ATCC HTB 22; oestrogen receptor positive), MDA-MB-453 (ATCC HTB 131; oestrogen receptor negative), and MDA-MB-435S (ATCC HTB 129; oestrogen receptor negative), all kindly provided by G. Saretzki, Newcastle, UK, were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and antibiotics at 37°C in a humidified atmosphere with 5% CO_2. All cell culture reagents were from PAA, Pasching, Austria.

Drugs and drug treatment

Nelfinavir (Viracept®) was generously provided by Pfizer, Groton, CT, USA. Nelfinavir was dissolved in ethanol and kept at −20°C as a 100 mg/mL stock solution. Tamoxifen (Sigma, Germany) was dissolved in DMSO at a concentration of 100 mg/mL. In control experiments, equal amounts of DMSO or ethanol were added.

Cell proliferation analysis

A total of 2×10^4 cells per well were seeded in quadruplicate in 24-well cell culture plates and incubated with nelfinavir for up to 4 days. The number of viable, trypan blue-excluding cells was determined by a haemocytometer.

MTT assay

For MTT assay analysis, 20 µL of an MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma, Germany) stock solution (5 mg/mL PBS) was added to viable cells in 200 µL cell culture medium for 1 h under cell culture conditions. The water-insoluble precipitate was dissolved in 100 µL DMSO and analysed by an ELISA reader at 595 nm.

Annexin binding assay

FITC-labeled annexin V (Biocat, Heidelberg, Germany) was applied to viable cells as recommended by the supplier in combination with propidium iodide and analyzed by FACScan with an FL-1 setting (propidium iodide) at 575 nm and an FL-2 setting (FITC) at 530 nm. FACScan analysis was performed using a Becton Dickinson FACScan analyzer (Becton Dickinson, Heidelberg, Germany).

Western blot analysis

Cell extracts of cancer cells cultured in cell culture plates were prepared with RIPA-buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% doxycholine, 0.1% SDS) and 20 µg of protein (BioRad Bradford Assay, BioRad, München, Germany), and were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes in a BioRad Mini Protean II Cell (BioRad, Munich, Germany) at 1 mA/cm^2 membrane in 10% methanol, 192 mM glycine, 25 mM Tris, pH 8.2. Membranes were blocked with 4% non-fat milk powder in PBS-0.05% Tween for 4 h. Primary antibodies were applied in blocking buffer and incubated at room temperature overnight. Antibodies against PARP, phospho-ERK1/2 (pp44/pp42), AKT, phospho-AKT, mcl-1, IkappaB, and LC3B were all purchased from Cell Signaling Technology (NEB, Frankfurt, Germany). Antibodies against BiP (H-129), ATF3 (C-19) and β-actin (C4) were from SantaCruz Biotech (Heidelberg, Germany). Secondary, alkaline phosphatase (AP)-coupled antibodies against the corresponding primary antibodies were from Dianova, Hamburg, Germany. AP detection was performed either by the chromogenic BCIP/NBT assay (Promega, Mannheim, Germany) or the chemiluminescent AP detection assay (Millipore, Germany) and analysed and documented using a BioRad QuantityOne Image Analyzer and documentation software (BioRad, Munich, Germany).

Determination of intracellular glutathione levels

To detect intracellular glutathione levels, cells were seeded in 96-well cell culture dishes and allowed to grow for 24 h under cell culture conditions. Cells were then incubated with the cytotoxic drugs for up to 5 h. Intracellular glutathione levels were quantified using the bioluminescent Promega GSH-Glo^{TM} glutathione assay (Promega, Mannheim, Germany), essentially as recommended by the supplier. In brief, adherent cells were directly dissolved in 100 µL GSH-Glo lysis and reaction buffer. After addition of 100 µL GSH-Glo Luciferin detection reagent, luminescence was detected using a MicroLumat LB 96P bioluminometer (EG&G Berthold, Bad Wildbad, Germany).

Determination of proteasomal activity

For the determination of cellular proteasome activity, cells were seeded in 96-well cell culture dishes, allowed to grow for 24 h under cell culture conditions, and then incubated with cytotoxic drugs for up to 5 h. Proteasomal activity was analysed using the bioluminescent Promega Proteasome-Glo^{TM} assay (Promega, Mannheim, Germany) as recommended by the supplier. Adherent cells were directly dissolved in 50 µL Proteasome-Glo lysis and reagent buffer, containing Suc-LLVY-aminoluciferin as a substrate. Leukemia cells were collected by centrifugation before lysis. Bioluminescence was detected using a MicroLumat LB 96P bioluminometer (EG&G Berthold, Bad Wildbad, Germany).

Statistical analysis

All experiments, except Western blots and FACScan analysis, were performed in quadruplicate. The results were evaluated using the non-parametric Wilcoxon sum test and the Mann-Whitney U rank-sum test where applicable (PASW Version 17.0, SPSS Inc., Chigaco, IL, USA). Values were plotted as means ± standard

deviation (SD) and significance was assumed at $p < 0.05$ by using the two-tailed test. Significant relations were indicated in the figures with an asterisk and the statistical test used was mentioned in the corresponding figure legends.

Ethical aspects

All experiments were performed on established cancer cell lines. Thus, no ethical approval or informed consent was needed.

Results

Nelfinavir reduces cell proliferation of breast cancer cells and is able to induce apoptosis in breast cancer cells when applied at higher concentrations

The human breast cancer cell lines T47D, MCF7, MDA-MB-453, and MDA-MB-435 were incubated with nelfinavir at various concentrations with various time intervals and analyzed for cell proliferation by counting of viable cells. Low doses of nelfinavir (5 µg/ml) reduced the cell proliferation of breast cancer cells and, at a concentration of 15 µg/mL, complete cell death was achieved **(Figure 1)**. Nelfinavir acted on estrogen receptor positive (T47D, MCF7) as well as on estrogen receptor negative (MDA-MB-453, MDA-MB-435) breast cancer cell lines.

Tamoxifen enhances the cytotoxic effect of nelfinavir

The mean plasma concentration of nelfinavir in HIV-infected persons taking oral doses of nelfinavir was determined to be 2.2 µg/mL, reaching maximal plasma concentrations of up to 4 µg/mL [23, 24]. These concentrations can achieve only a partial reduction of breast cancer cell proliferation **(Figure 1)**, and are not efficient in inducing apoptosis in breast cancer cells. However, the plasma concentration of nelfinavir can be significantly increased by administering higher oral doses of nelfinavir or by intravenous application of nelfinavir [25]. Still, a possible combination strategy of nelfinavir with other chemotherapeutic drugs that would allow a reduction of single drug concentrations would be advantageous. When combining nelfinavir with tamoxifen, we observed a substantially enhanced induction of cell death even at lower nelfinavir concentrations **(Figure 2).** For example, when used as a single agent, 6 µg/ml nelfinavir induced only a slight and non-significant reduction of cell viability by 6.1 % in MCF7 cells and by 6.4 % in T47D cells (Figure 2). Tamoxifen at 6 µg/ml reduced the cell viability by 26.5% in MCF7 cells and by 40 % in T47D cells (Figure 2; $p < 0.05$).

However, the combination of both significantly reduced cell viability of MCF7 and T47D cells by up to 77.0 % and 76.8%, respectively (Figure 2; $p < 0.05$). FACScan analysis confirmed that the combination of 6 µg/mL nelfinavir with 6 µg/mL tamoxifen efficiently induced apoptosis in breast cancer cells **(Figure 3; Table 1)**, although the same concentrations proved to be insufficient for the induction of apoptosis when used as single agents **(Figure 3; Table 1)**.

Nelfinavir exerts pleiotropic pro-apoptotic and anti-apoptotic effects in breast cancer cells

To gain a better insight into the cell death mechanism induced by nelfinavir alone and in combination with tamoxifen, Western blot analyses of drug-treated breast cancer cells were performed. First, the effects of nelfinavir as a single agent on breast cancer cells were analyzed. By using the cleavage of PARP as an indicator of apoptosis, the specific induction of apoptosis in breast cancer cells by high nelfinavir concentrations (15µg/mL) could be confirmed **(Figure 4)**. Western blotting further showed upregulation of BiP and ATF3 in nelfinavir-treated breast cancer cells, indicating induction of endoplasmic reticulum stress. However, upregulation of BiP following nelfinavir treatment was scarcely detectable in MDA-MB-453 cells. In these cells, strong upregulation of LC3B, a marker of autophagy could be observed instead. In all four cell lines, no change in the expression of the proteasome-regulated NFkappaB inhibitor IkappaB could be observed, suggesting no influence of nelfinavir on the proteasome activity in breast cancer cells. We have previously described an upregulation of the anti-apoptotic mcl-1 protein by nelfinavir in ovarian cancer cells [26], but this could only be observed in one (MDA-MB-435) of the four tested breast cancer cell lines. Nelfinavir has further been described as reducing AKT phosphorylation, resulting in enhanced radio-sensitivity [11, 16], which could be of special importance for

breast cancer treatment, for which radiotherapy can be applied. However, we observed no inhibition of AKT signaling in MCF7 and T47D cells by nelfinavir and even observed a markedly enhanced AKT phosphorylation level in nelfinavir-treated MDA-MB-453 and MDA-MB-435 cells **(Figure 4)**.

Tamoxifen enhances the pleiotropic effects of nelfinavir in breast cancer cells

The effects of tamoxifen on the pro- and anti-apoptotic pathways that were induced by nelfinavir **(Figure 4)** were further investigated. Cell lysates of breast cancer cells treated with 6 µg/mL nelfinavir or 6 µg/mL tamoxifen alone or in combination were subjected to Western blot analysis **(Figure 5)**. Tamoxifen enhanced upregulation of BiP in T47D and MCF7 cells, and to a lesser in MDA-MB-435 and MDA-MB-453 cells **(Figure 5)**. In MDA-MB-453 cells, expression of the autophagy marker LC3B was strongly enhanced. Tamoxifen, nelfinavir, and its combination had no effect on AKT or ERK phosphorylation in T47D and MCF7 cells but, in MDA-MB-435 and MDA-MB-453 cells, the combination of tamoxifen with nelfinavir markedly enhanced AKT phosphorylation **(Figure 5)**.

Nelfinavir reduces glutathione levels in breast cancer cells

Tamoxifen has been reported to induce oxidative stress [6]. Oxidative stress is reflected or can be facilitated by reduced intracellular glutathione levels, because glutathione serves as an endogenous anti-oxidant and reduced glutathione levels facilitate apoptosis [27]. When breast cancer cells were incubated with tamoxifen or nelfinavir, we observed that nelfinavir reduced glutathione levels in breast cancer cells even more pronounced than tamoxifen **(Figure 6A)**. At a concentration of 6 µg/ml, nelfinavir reduced the intracellular glutathione level of MCF7

and MDA-MB-435 cells by 21.6 % and 16.6 %, respectively ($p < 0.05$). A slight but statistically significant reduction of glutathione level by 7 % ($p < 0.05$) could be observed for MCF7 cells treated with 6 µg/ml tamoxifen (**Figure 6A**). However, the combination of 6 µg/ml nelfinavir with 6 µg/ml tamoxifen did not further reduce the glutathione levels in breast cancer cells in a significant manner (**Figure 6A**). Since the sub-toxic concentration of 6 µg/ml nelfinavir was already effective in reducing intracellular glutathione levels, we tested the effect of nelfinavir at higher concentrations (20 µg/ml), which proved to be toxic to all of the four breast cancer cell lines investigated. **Figure 6B** shows that 20 µg/ml nelfinavir reduced intracellular glutathione levels by up to 32.4 % in MCF7 cells and by 30.1 % in MDA-MB-435 cells.

The intracellular glutathione levels might vary not only because of external drug applications, but likewise due to differences in cell growth, nutrient concentrations (especially that of cysteine) and the redox state within the cells or of the surrounding medium. These factors can vary under cell culture conditions. Exogenously applied glutathione or cysteine derivatives such as N-acetyl-cysteine can enhance or replenish intracellular glutathione levels or support the anti-oxidative effect of glutathione. To analyze the involvement of glutathione depletion on nelfinavir-induced apoptosis, intracellular glutathione contents were replenished by the addition of externally applied glutathione or N-acetyl-cysteine. **Figure 6C** demonstrates that the addition of glutathione, and even more so, addition of N-acetyl-cysteine, can attenuate the cytotoxic effect of nelfinavir on breast cancer cells. In the absence of external glutathione or N-acetyl-cysteine, only 5 % of the MCF7 cells and 12 % of the MDA-MB-435 cells survived the application of 15 µg/ml nelfinavir (**Figure 6C**). Although the addition of 5 mM glutathione was not without negative effects on the cell viability of MCF7 cells (**Figure 6C**), the presence of external glutathione enhanced the remaining cell viability of MCF7 cells to 15 % and to 17 % in the case of MDA-MB-435 cells (**Figure 6C**). In the

presence of 5 mM N-acetyl-cysteine, 18 % of the MCF7 cells remained viable, and up to 34 % of the MDA-MB-435 cells survived the addition of nelfinavir (**Figure 6C**).

.

Nelfinavir has no effect on the chymotrypsin-like proteasome activity of breast cancer cells

Some previous studies have indicated the inhibition of proteasomal activity by HIV protease inhibitors, including nelfinavir [26, 28]. However, the fact that nelfinavir has no influence on tbe expression of the proteasome-regulated NF-kappaB inhibitor I-kappaB **(Figure 4)** indirectly suggests that nelfinavir has no effect on the proteasomal activity of breast cancer cells. To clearly detect any influence of nelfinavir on proteasomal activity in breast cancer cells, a direct, bioluminescent proteasome assay was performed. Bortezomib, a specific proteasome inhibitor clinically approved for the treatment of multiple myeloma, was used as a positive control. **Figure 7** shows that nelfinavir exerts no significant effect on the chymotryptic proteasome activity in MCF7 and MDA-MB-453 breast cancer cells. However, the effect of bortezomib on the tested breast cancer cells was likewise poor, and 15 ng/ml bortezomib reduced the chymotrypsin-like activity of the proteasome in MCF7 cells by 37.5 % and in T47D cells by 17.9% only (**Figure 7**). Under these conditions, but at even lower bortezomib concentrations (4.5 ng/ml), a marked 89.6% loss in cell viability could be observed in IM9 cells, a bortezomib-sensitive lymphoblastoid cell line. Still, even in IM9 cells, nelfinavir displayed no significant effect on the chymotrypsin-like proteasome activity (**Figure 7**).

Discussion

The HIV protease inhibitor nelfinavir is a prospective new anti-cancer drug, as shown by several *in vitro* as well as *in vivo* studies [8-13]. The concentrations of nelfinavir needed to induce cell death of cancer cells are higher than those applied for HIV-infected individuals for HIV suppression, but this may be achieved by the application of higher oral or intravenous doses of nelfinavir [25]. Still, the prospects of nelfinavir as an anti-cancer drug will rely less on its efficacy as a single drug and more on its ability to cooperate with or sensitize to other chemotherapeutic drugs or cancer treatment options. For example, we have recently demonstrated that nelfinavir cooperates with the multiple kinase inhibitor sorafenib to induce apoptosis in various cancer cell types [26, 29], and enhances TRAIL sensitivity in ovarian cancer cells [30]. The present results show that the cytotoxic effects of nelfinavir on breast cancer cells can be enhanced by combination with tamoxifen, thus allowing the effective concentration of nelfinavir to be reduced. Tamoxifen, although originally designed and applied as a selective estrogen receptor modulator, also represents a drug with several described pleiotropic anti-tumoral effects [6, 7], and two recent and independent studies observed that tamoxifen is able to induce the endoplasmic reticulum stress reaction [31, 32], thus explaining the synergistic effect of nelfinavir and tamoxifen on the induction of endoplasmic reticulum stress. The “nelfinavir-boosting” effect of tamoxifen was obviously independent of its ability to induce oxidative stress [6]. Instead, we observed that nelfinavir itself reduced cellular glutathione levels, indicating the occurrence of oxidative stress after nelfinavir treatment. Induction of oxidative stress occurs within few hours as an early effect of nelfinavir treatment and has so far been neglected as an additional mechanism of the pleiotropic anti-cancer effects of nelfinavir. The observation that the effect of nelfinavir can be attenuated by the addition of anti-oxidants (glutathione or N-acetyl-cysteine) could have an

impact on the efficacy of nelfinavir in cancer cells, as well as on the nelfinavir-induced adverse effects occurring in HIV-infected persons.

Nelfinavir has been reported to exert a radio-sensitizing effect by inhibiting proteasome activity and AKT signaling [16]. However, inhibition of proteasomal activity or AKT signaling in breast cancer cells was not observed in the present study. In contrast, nelfinavir markedly enhanced AKT phosphorylation in some breast cancer cell lines (MDA-MB-453 and MDA-MB-435). However, this observation is not surprising since we previously demonstrated activation of the cell-protective ERK1/2 signaling pathway by nelfinavir [26]. The endoplasmic stress reaction is primarily a cell-protective mechanism, aiming to rescue cells from transient stress-induced cell damage [21, 33]. Longer exposure to cell stress mechanisms or a cellular inability to cope with the stress-induced cell damage then finally induces a switch from cell protection to autophagy and apoptosis [21, 33]. It has repeatedly been shown that endoplasmic reticulum stress induces activation of both ERK1/2 and AKT signaling [34-37]. However, several studies have likewise shown that AKT activation, which can occur directly at the endoplasmic reticulum [38], primarily represents a short-term effect, and prolonged exposure of cells to endoplasmic reticulum stress indeed induces AKT inactivation [38, 39]. In fact, we observed a reduced AKT phosphorylation when breast cancer cells were treated with nelfinavir for more than 48 h (data not shown), although this indicates downregulation of AKT phosphorylation as a secondary event. Thus, the present data do not exclude the potential use of nelfinavir as a radio-sensitizer even for breast cancer patients, but a potential negative interaction between these two treatment options, especially shortly after nelfinavir application, should be kept in mind.

In addition to the data on AKT signaling, the present data revealed some other differences to previous studies performed by us and others on different cancer cell types. For example, upregulation of the anti-apoptotic mcl-1 protein by nelfinavir, as observed by us in ovarian cancer cells [26] and leukemia cells [29] could only be observed in a single breast

cancer cell line (MDA-MB-435), and only at high concentrations of nelfinavir (Figure 4). Further, we could not demonstrate proteasome inhibition by nelfinavir in breast cancer cells.

Although nelfinavir induced cell death in all four breast cancer cell lines tested, the data presented further indicate that the cell lines respond quite differently to nelfinavir, especially regarding the effect on cell stress, autophagy, and apoptosis. This might be due to the different hormone receptor status of the cells, but likewise due to the different malignancies of the tumours from which these cell lines have been derived. Therefore, we tried to include various types of breast cancer cell lines in this study, ranging from hormone receptor positive breast cancer cells of a high differentiation grade (T47D) to highly de-differentiated hormone receptor negative breast cancer cells (MDA-MB-435). Interestingly, especially when low doses of nelfinavir were applied, the de-differentiated hormone receptor negative breast cancer cell lines (MDA-MB-453, MDA-MB-435) appeared to react even better to nelfinavir than the T47D and MCF7 cells (**Figure 1**).

Despite, we observed that the combination of tamoxifen and nelfinavir was able to induce cell death in estrogen receptor positive as well as in estrogen receptor negative breast cancer cell lines. This indicates that both estrogen receptor positive as well as estrogen receptor negative breast cancer patients could benefit from a combination of these two drugs. However, since both nelfinavir and tamoxifen have to be used at concentrations higher than those used to inhibit the HIV protease in HIV-infected persons or the estrogen receptor in hormone receptor-positive breast cancer patients, care has to be taken that no unexpected adverse effects occur, especially when both drugs, although displaying moderate and tolerable adverse effects as single agents, are combined. Further, the observed reduction in glutathione levels by nelfinavir might cause an unexpected drug sensitization in other tissues.

Thus, clinical studies on breast cancer patients testing the described combination of nelfinavir and tamoxifen are of high interest in order to assess both efficacy and safety of this drug combination.

Conclusions

This study demonstrates the efficacy of nelfinavir in breast cancer cells as a single agent, and a possible combination treatment with tamoxifen. Both nelfinavir and tamoxifen are already-approved drugs with known pharmacokinetics and generally exhibit relative mild and well tolerable adverse effects even after long term application. However, since the concentrations of both drugs have to be increased for an efficient cancer therapy, and a combination of these two drugs has not yet been tested in humans, it is important to test the safety and tolerability of this combination in phase I studies first.

Abbreviations

AP: alkaline phosphatase; ATF3: activating transcription factor 3; BiP: binding protein; ERK: extracellular signal-regulated kinase: FACS: fluorescence-activated cell sorting; GSH: glutathione; HIV: human immunodeficiency virus; LC3B: autophagy marker light chain 3B; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP: poly [ADP-ribose] polymerase: PVDF: polyvinylidene fluoride; RIPA: radio immunoprecipitation buffer; TRAIL: tumor necrosis factor related apoptosis inducing ligand.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB designed and coordinated the experiments, KF and AB helped to draft the manuscript, and IM helped to draft the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

Acknowledgements

We are greatly indebted to Marianne Vogel, Reinhild Joswig, and Petra Burger for their excellent and highly motivated technical assistance. The generous supply of nelfinavir by Pfizer, Groton, CT is gratefully appreciated. This work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG BR 3641/1-1, DFG BR 3641/3-1).

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ: **Cancer statistics, 2009**. *CA Cancer J Clin* 2009, **59**:225-249.
2. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ: **Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009**. *Ann Oncol* 2009, **20**:1319-1329.
3. Fisher B, Redmond C: **Systemic therapy in node-negative patients: updated findings from NSABP clinical trials. National Surgical Adjuvant Breast and Bowel Project**. *J Natl Cancer Inst Monogr* 1992:105-116.
4. Howell A: **The endocrine prevention of breast cancer**. *Best Pract Res Clin Endocrinol Metab* 2008, **22**:615-623.
5. Harichand-Herdt S, Zelnak A, O'Regan R: **Endocrine therapy for the treatment of postmenopausal women with breast cancer**. *Expert Rev Anticancer Ther* 2009, **9**:187-198.
6. Gundimeda U, Chen ZH, Gopalakrishna R: **Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells**. *J Biol Chem* 1996, **271**:13504-13514.
7. Mandlekar S, Kong AN: **Mechanisms of tamoxifen-induced apoptosis**. *Apoptosis* 2001, **6**:469-477.
8. Pore N, Gupta AK, Cerniglia GJ, Maity A: **HIV protease inhibitors decrease VEGF/HIF-1alpha expression and angiogenesis in glioblastoma cells**. *Neoplasia* 2006, **8**:889-895.
9. Gills JJ, Lopiccolo J, Tsurutani J, Shoemaker RH, Best CJ, Abu-Asab MS, Borojerdi J, Warfel NA, Gardner ER, Danish M, Hollander MC, Kawabata S, Tsokos M, Figg WD, Steeg PS, Dennis PA: **Nelfinavir, A lead HIV protease inhibitor, is a broad-spectrum, anticancer agent that induces endoplasmic reticulum stress, autophagy, and apoptosis in vitro and in vivo**. *Clin Cancer Res* 2007, **13**:5183-5194.
10. Cohen J: **Biomedicine. HIV drug shows promise as potential cancer treatment**. *Science* 2007, **317**:1305.
11. Plastaras JP, Vapiwala N, Ahmed MS, Gudonis D, Cerniglia GJ, Feldman MD, Frank I, Gupta AK: **Validation and toxicity of PI3K/Akt pathway inhibition by HIV protease inhibitors in humans**. *Cancer Biol Ther* 2008, **7**:628-635.
12. Brunner TB, Geiger M, Grabenbauer GG, Lang-Welzenbach M, Mantoni TS, Cavallaro A, Sauer R, Hohenberger W, McKenna WG: **Phase I trial of the human immunodeficiency virus protease inhibitor nelfinavir and chemoradiation for locally advanced pancreatic cancer**. *J Clin Oncol* 2008, **26**:2699-2706.
13. Chow WA, Jiang C, Guan M: **Anti-HIV drugs for cancer therapeutics: back to the future?** *Lancet Oncol* 2009, **10**:61-71.
14. Pyrko P, Kardosh A, Wang W, Xiong W, Schonthal AH, Chen TC: **HIV-1 protease inhibitors nelfinavir and atazanavir induce malignant glioma death by triggering endoplasmic reticulum stress**. *Cancer Res* 2007, **67**:10920-10928.
15. Bruning A, Burger P, Vogel M, Rahmeh M, Gingelmaiers A, Friese K, Lenhard M, Burges A: **Nelfinavir induces the unfolded protein response in ovarian cancer cells, resulting in ER vacuolization, cell cycle retardation and apoptosis**. *Cancer Biol Ther* 2009, **8**:226-232.
16. Gupta AK, Li B, Cerniglia GJ, Ahmed MS, Hahn SM, Maity A: **The HIV protease inhibitor nelfinavir downregulates Akt phosphorylation by inhibiting proteasomal activity and inducing the unfolded protein response**. *Neoplasia* 2007, **9**:271-278.

17. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D: **Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response**. *Nat Cell Biol* 2000, **2**:326-332.
18. Lee AS: **The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress**. *Methods* 2005, **35**:373-381.
19. Fribley A, Wang CY: **Proteasome inhibitor induces apoptosis through induction of endoplasmic reticulum stress**. *Cancer Biol Ther* 2006, **5**:745-748.
20. Fels DR, Koumenis C: **The PERK/eIF2alpha/ATF4 module of the UPR in hypoxia resistance and tumor growth**. *Cancer Biol Ther* 2006, **5**:723-728.
21. Lin JH, Li H, Yasumura D, Cohen HR, Zhang C, Panning B, Shokat KM, Lavail MM, Walter P: **IRE1 signaling affects cell fate during the unfolded protein response**. *Science* 2007, **318**:944-949.
22. Boelens J, Lust S, Offner F, Bracke ME, Vanhoecke BW: **Review. The endoplasmic reticulum: a target for new anticancer drugs**. *In Vivo* 2007, **21**:215-226.
23. Regazzi MB, Villani P, Maserati R, Seminari E, Pan A, LoCaputo F, Gambarana E, Fiocchi C: **Clinical pharmacokinetics of nelfinavir combined with efavirenz and stavudine during rescue treatment of heavily pretreated HIV-infected patients**. *J Antimicrob Chemother* 2000, **45**:343-347.
24. Regazzi MB, Seminari E, Villani P, Carriero PL, Montagna M, Marubbi F, Maserati R: **Nelfinavir suspension obtained from nelfinavir tablets has equivalent pharmacokinetic profile**. *J Chemother* 2001, **13**:569-574.
25. Kaldor SW, Kalish VJ, Davies JF 2nd, Shetty BV, Fritz JE, Appelt K, Burgess JA, Campanale KM, Chirgadze NY, Clawson DK, Dressman BA, Hatch SD, Khalil DA, Kosa MB, Lubbehusen PP, Muesing MA, Patick AK, Reich SH, Su KS, Tatlock JH: **Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease**. *J Med Chem* 1997, **40**:3979-3985.
26. Bruning A, Burger P, Vogel M, Gingelmaier A, Friese K, Burges A: **Nelfinavir induces mitochondria protection by ERK1/2-mediated mcl-1 stabilization that can be overcome by sorafenib**. *Invest New Drugs* 2009, In press.
27. Franco R, Cidlowski JA: **Apoptosis and glutathione: beyond an antioxidant**. *Cell Death Differ* 2009, **16**:1303-1314.
28. Schmidtke G, Holzhutter HG, Bogyo M, Kairies N, Groll M, de Giuli R, Emch S, Groettrup M: **How an inhibitor of the HIV-I protease modulates proteasome activity**. *J Biol Chem* 1999, **274**:35734-35740.
29. Bruning A, Rahmeh M, Gingelmaier A, Friese K: **The mitochondria-independent cytotoxic effect of nelfinavir on leukemia cells can be enhanced by sorafenib-mediated mcl-1 downregulation and mitochondrial membrane destabilization**. *Mol Cancer* 2010, **9**:19.
30. Bruning A, Vogel M, Burger P, Rahmeh M, Gingelmaier A, Friese K, Lenhard M, Burges A: **Nelfinavir induces TRAIL receptor upregulation in ovarian cancer cells**. *Biochem Biophys Res Commun* 2008, **377**:1309-1314.
31. Scriven P, Coulson S, Haines R, Balasubramanian S, Cross S, Wyld L: **Activation and clinical significance of the unfolded protein response in breast cancer**. *Br J Cancer* 2009, **101**:1692-1698.
32. Andersson H, Helmestam M, Zebrowska A, Olovsson M, Brittebo E: **Tamoxifen-induced adduct formation and cell stress in human endometrial glands**. *Drug Metab Dispos* 2009, **38**:200-207.
33. Wek RC, Jiang HY, Anthony TG: **Coping with stress: eIF2 kinases and translational control**. *Biochem Soc Trans* 2006, **34**:7-11.

34. Hu P, Han Z, Couvillon AD, Exton JH: **Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death**. *J Biol Chem* 2004, **279**:49420-49429.
35. Matthews JA, Belof JL, Acevedo-Duncan M, Potter RL: **Glucosamine-induced increase in Akt phosphorylation corresponds to increased endoplasmic reticulum stress in astroglial cells**. *Mol Cell Biochem* 2007, **298**:109-123.
36. Jiang CC, Yang F, Thorne RF, Zhu BK, Hersey P, Zhang XD: **Human melanoma cells under endoplasmic reticulum stress acquire resistance to microtubule-targeting drugs through XBP-1-mediated activation of Akt**. *Neoplasia* 2009, **11**:436-447.
37. Price J, Zaidi AK, Bohensky J, Srinivas V, Shapiro IM, Ali H: **Akt-1 mediates survival of chondrocytes from endoplasmic reticulum-induced stress**. *J Cell Physiol* 2009, **222**:502-508.
38. Hosoi T, Hyoda K, Okuma Y, Nomura Y, Ozawa K: **Akt up- and down-regulation in response to endoplasmic reticulum stress**. *Brain Res* 2007, **1152**:27-31.
39. Dai R, Chen R, Li H: **Cross-talk between PI3K/Akt and MEK/ERK pathways mediates endoplasmic reticulum stress-induced cell cycle progression and cell death in human hepatocellular carcinoma cells**. *Int J Oncol* 2009, **34**:1749-1757.

Table 1: Quantitive analysis of apoptotic and necrotic cells after tamoxifen and nelfinavir treatment

T47D	LL	UL	UR	LR
control	98.78%	0.44%	0.77%	0.03%
TAM 6 µg/ml	92.71%	4.42%	2.71%	0.17%
NFV 6 µg/ml	95.50%	2.17%	2.31%	0.03%
TAM/NFV	11.18%	43.05%	44.44%	1.34%
MCF7	LL	UL	UR	LR
control	88.04%	3.10%	7.52%	1.33%
TAM 6 µg/ml	83.05%	7.16%	6.89%	2.90%
NFV 6 µg/ml	92.80%	2.46%	3.80%	0.95%
TAM/NFV	63.68%	14.63%	19.48%	2.21%

The relative cell distributions among the quadrants shown in Figure 2 were analysed using the BectonDickinson CellQuest software. LL: lower left (unstained; viable); UL: upper left (annexin positive; apoptotic); UR: upper right (annexin and PI positive; apoptotic/necrotic); LR: lower right (PI permeable; necrotic).

Figure legends

Figure 1: Effect of nelfinavir on the cell survival of breast cancer cells. A total of 2×10^4 cells per well of the four indicated breast cancer cell lines were seeded in quadruplicate in 24-well cell culture plates, incubated with the indicated nelfinavir concentrations, and the number of trypan blue-excluding cells was determined over a time period of four days (d1-d4). Values represent means ± SD.

Figure 2: Combination effect of nelfinavir and tamoxifen on the cell survival of breast cancer cells. A) A total of 5×10^3 MCF7 and T47D cells per well were seeded in quadruplicate in 96-well cell culture plates, incubated with the indicated nelfinavir and tamoxifen concentrations either alone or in combination, and analyzed for cell viability by an MTT assay after 72 h incubation. B) A similar experiment as shown in (A) was performed using different nelfinavir and tamoxifen concentrations, and MTT-derived staining intensities were analyzed by a photometer and shown as bar graphs. Values represent means ± SD. Significance was assumed at $p<0.05$ with the non-parametric Wilcoxon rank-sum test.

Figure 3: The combination of nelfinavir and tamoxifen enhances apoptosis in breast cancer cells. The breast cancer cell lines MCF7 and T47D were incubated with the indicated nelfinavir and tamoxifen concentrations either alone at 6 µg/mL or in combination (6 µg/mL nelfinavir (NFV) plus 6 µg/mL tamoxifen (TAM)), and, after 48 h incubation, analyzed by FACScan analysis for the occurence of apoptosis (FITC-annexin binding) and necrosis (propidium iodide permeability). FL-1 setting (propidium iodide; PJ): 575 nm; FL-2 setting (FITC): 530 nm).

Figure 4: The treatment of breast cancer cells with nelfinavir activates pleiotropic pathways. The indicated four breast cancer cell lines were incubated with or without 15 µg/mL nelfinavir for 48 h and analyzed by Western blot for the expression and modification of cell survival-related proteins and pathways.

Figure 5: The combination of nelfinavir and tamoxifen enhances the effect of nelfinavir-induced pathways. The estrogen receptor-positive breast cancer cell lines MCF7 and T47D and the estrogen receptor-negative breast cancer cell lines MDA-MB-435 and MDA-MB-453 were incubated with the indicated nelfinavir and tamoxifen concentrations for 48 h and analyzed by Western blotting. Staining intensities of selected bands were analyzed using a gel documentation system and program (BioRad Quantity One), and related to the corresponding β-actin expression value as an internal control. For LC3B expression, the lower, LC3B-II band was used for calculations.

Figure 6: Nelfinavir, but not tamoxifen, induces glutathione reduction in breast cancer cells. A) A total of 5×10^3 MCF7 and MDA-MB-435 cells per well were seeded in 96-well cell culture dishes and incubated for 5 h with either 6 µg/mL nelfinavir (NFV) or 6 µg/mL tamoxifen (TAM) alone or in combination (N/T). Intracellular glutathione levels were quantified using the bioluminescent Promega GSH-Glo glutathione assay. B) MCF7 and MDA-MB-435 cells were incubated for 5 h with the indicated nelfinavir concentrations and analyzed for the cellular glutathione level as described in (A). C) MCF7 and MDA-MB-435 cells were incubated with or without 15 µg/mL nelfinavir for 72 h in the presence or absence of 5 mM glutathione (GSH) or 5 mM N-acetyl-cysteine (NAC), and analyzed for cell survival

by an MTT assay. Values represent means ± SD. Significance was assumed at $p<0.05$ with the non-parametric Mann-Whitney U rank-sum test.

Figure 7: Nelfinavir has no effect on the chymotrypsin-like proteasome activity of breast cancer cells. A total of 5×10^3 MCF7 and MDA-MB-435 cells per well were seeded in quadruplicate in 96-well cell culture dishes and incubated for 5 h with either 15 µg/mL nelfinavir (NFV) or 15 ng/mL bortezomib (BTZ). Chymotryptic proteasomal activity was analyzed using the bioluminescent Promega Proteasome-Glo assay with Suc-LLVY-aminoluciferin as a substrate. The bortezomib-sensitive myeloic leukemia cell line IM9 was used as a bortezomib-responsive control and was treated with 8 µg/mL NFV and 4.5 ng/mL BTZ. Values represent means ± SD. Significance was assumed at $p<0.05$ with the non-parametric Mann-Whitney U rank-sum test.

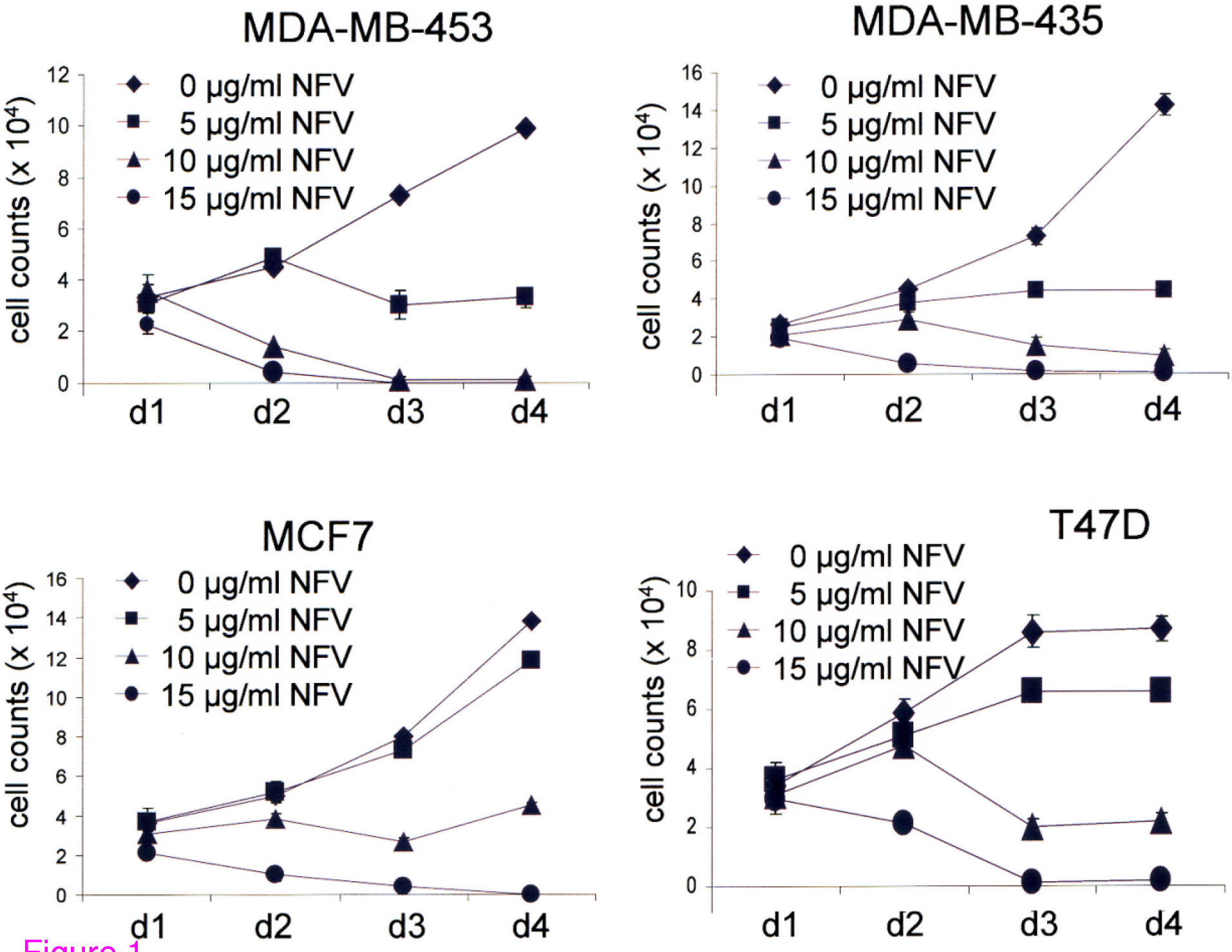

MDA-MB-453
MDA-MB-435
MCF7
T47D
0 µg/ml NFV
5 µg/ml NFV
10 µg/ml NFV
15 µg/ml NFV
cell counts (x 10⁴)
d1
d2
d3
d4

Figure 1

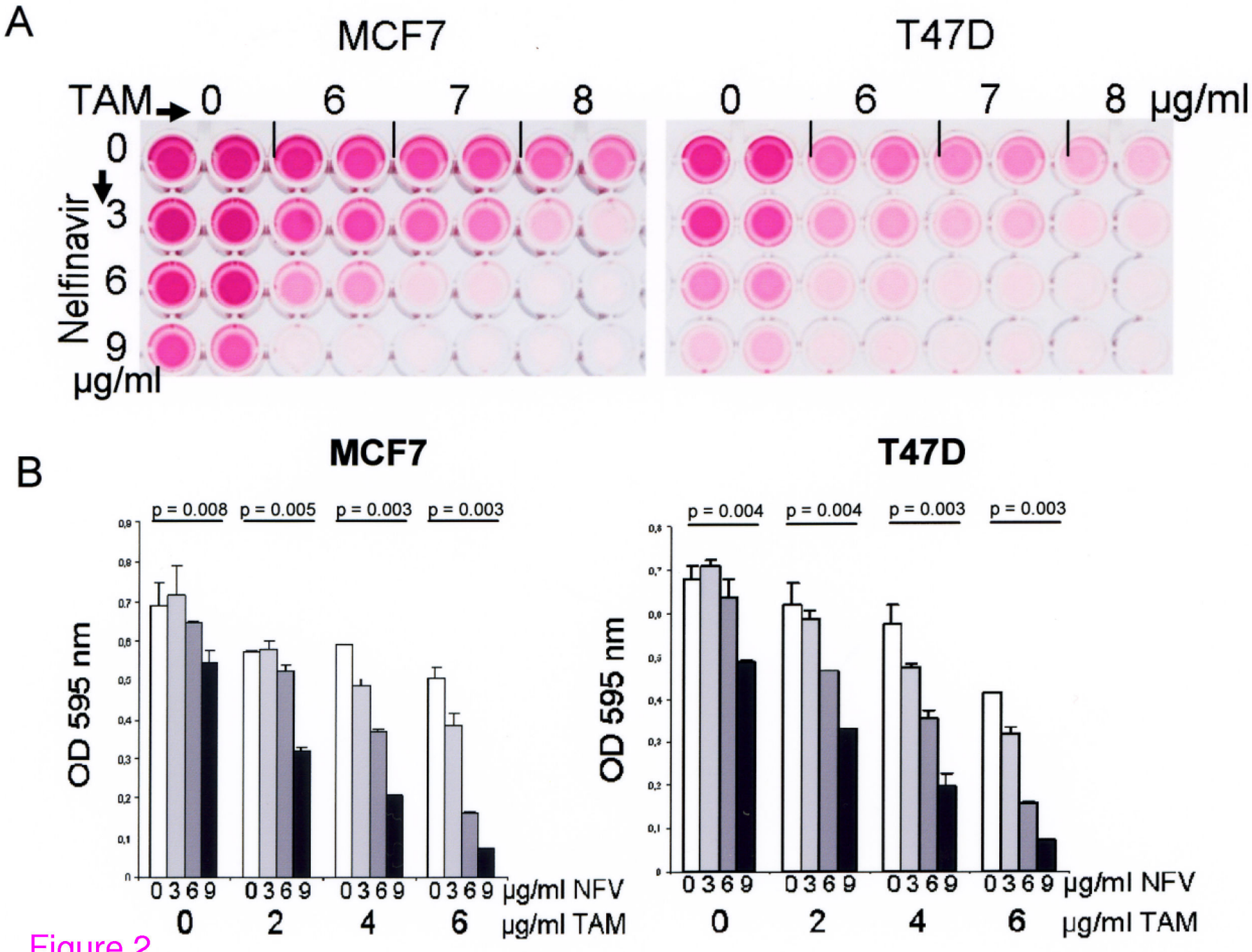

A
MCF7
T47D
TAM
0
6
7
8
µg/ml
Nelfinavir
0
3
6
9
µg/ml
B
MCF7
T47D
p = 0.008
p = 0.005
p = 0.003
p = 0.003
p = 0.004
p = 0.004
p = 0.003
p = 0.003
OD 595 nm
0 3 6 9 µg/ml NFV
0
2
4
6
µg/ml TAM

Figure 2

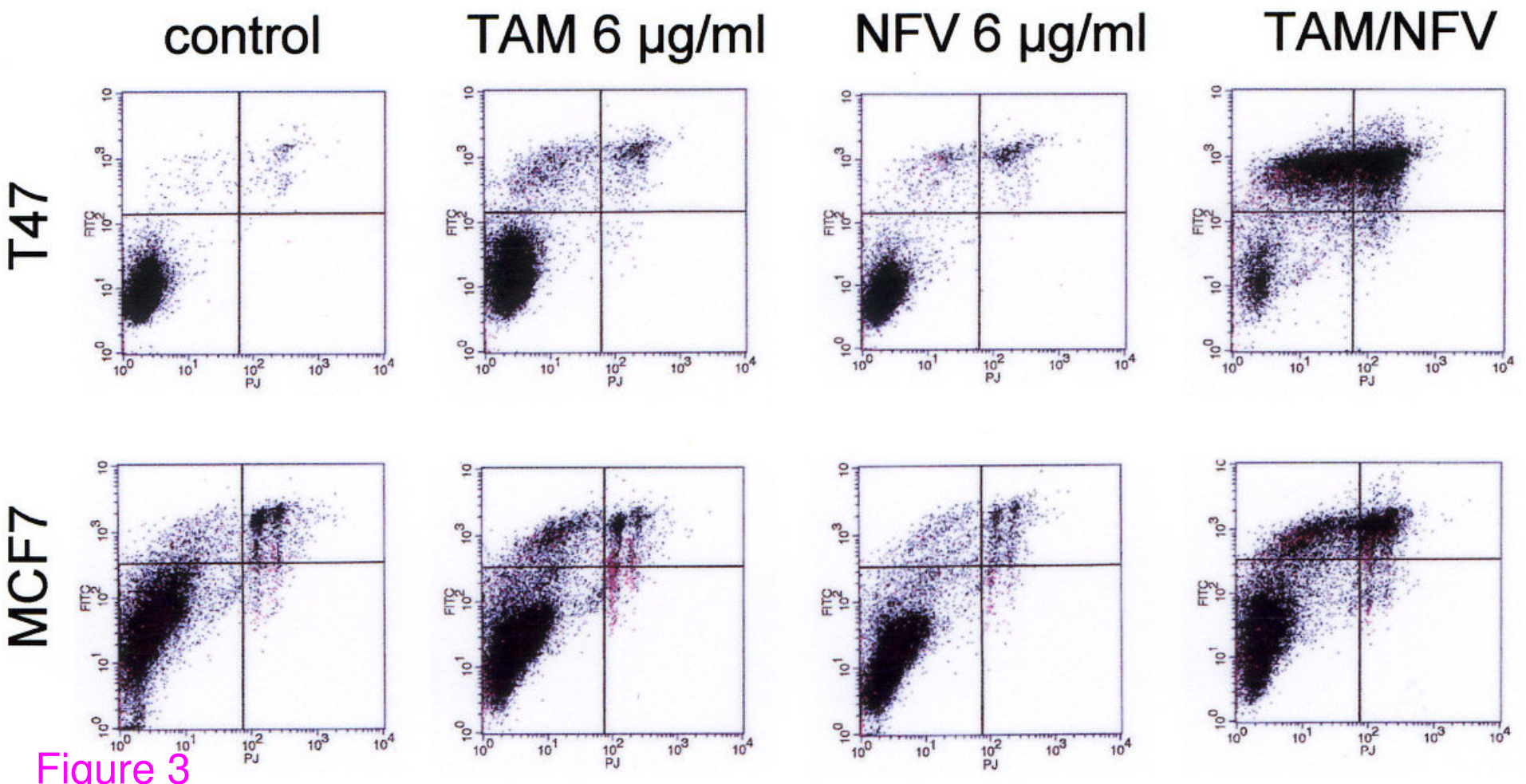


Figure 3

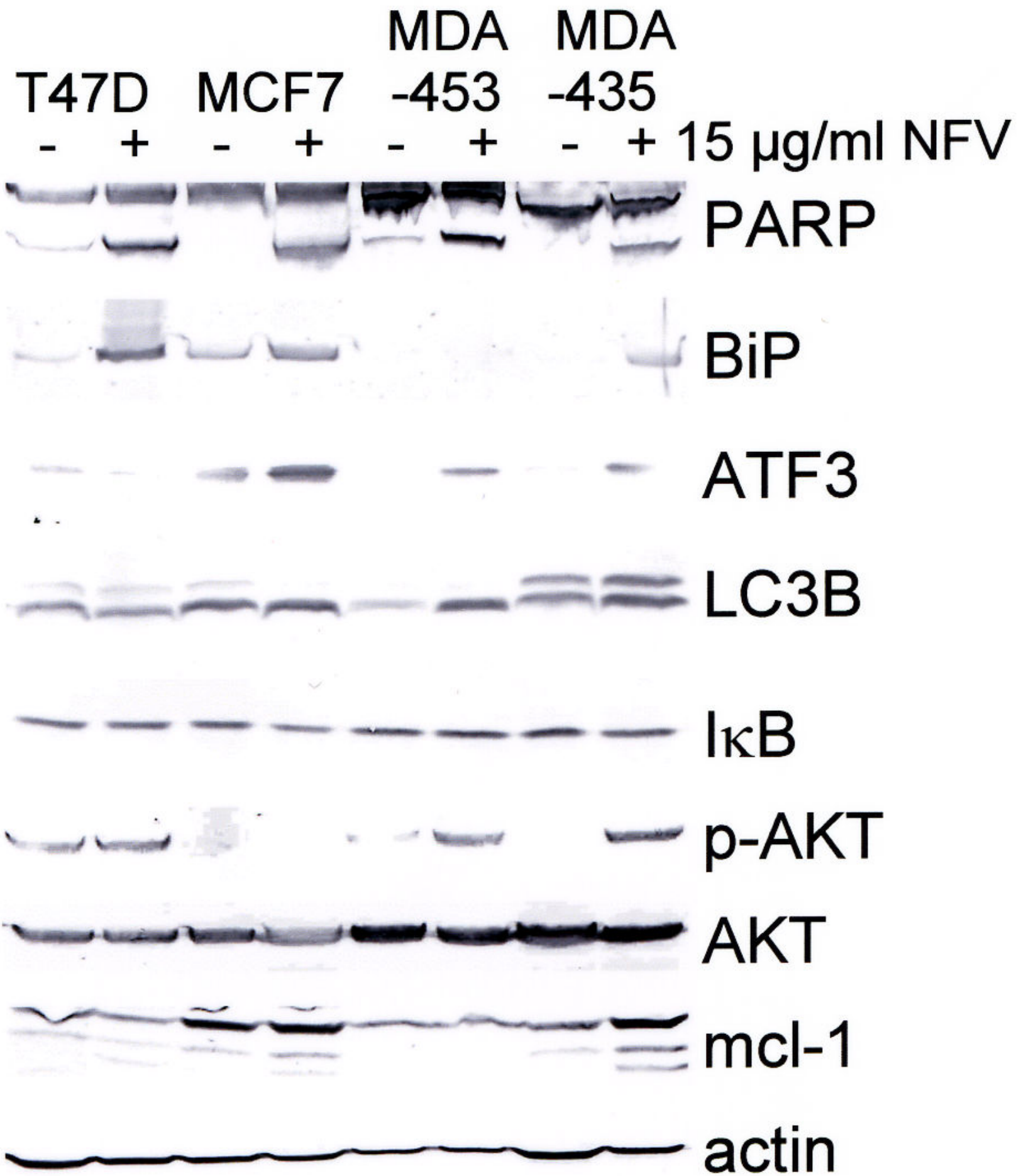
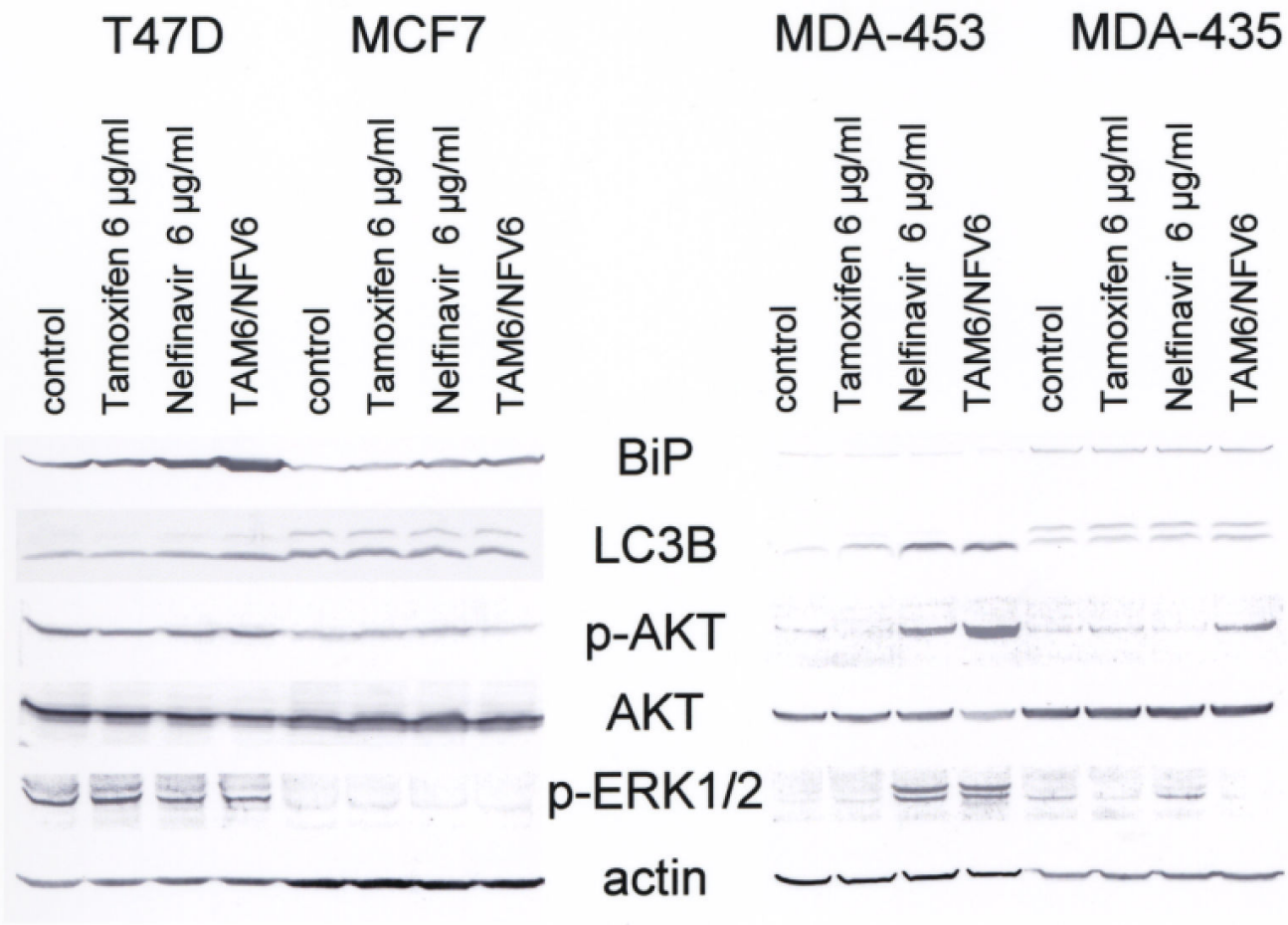

T47D
MCF7
MDA -453
MDA -435
- + - + - + - + 15 µg/ml NFV
PARP
BiP
ATF3
LC3B
IκB
p-AKT
AKT
mcl-1
actin

Figure 4


T47D
MCF7
MDA-453
MDA-435
control
Tamoxifen 6 µg/ml
Nelfinavir 6 µg/ml
TAM6/NFV6
BiP
LC3B
p-AKT
AKT
p-ERK1/2
actin

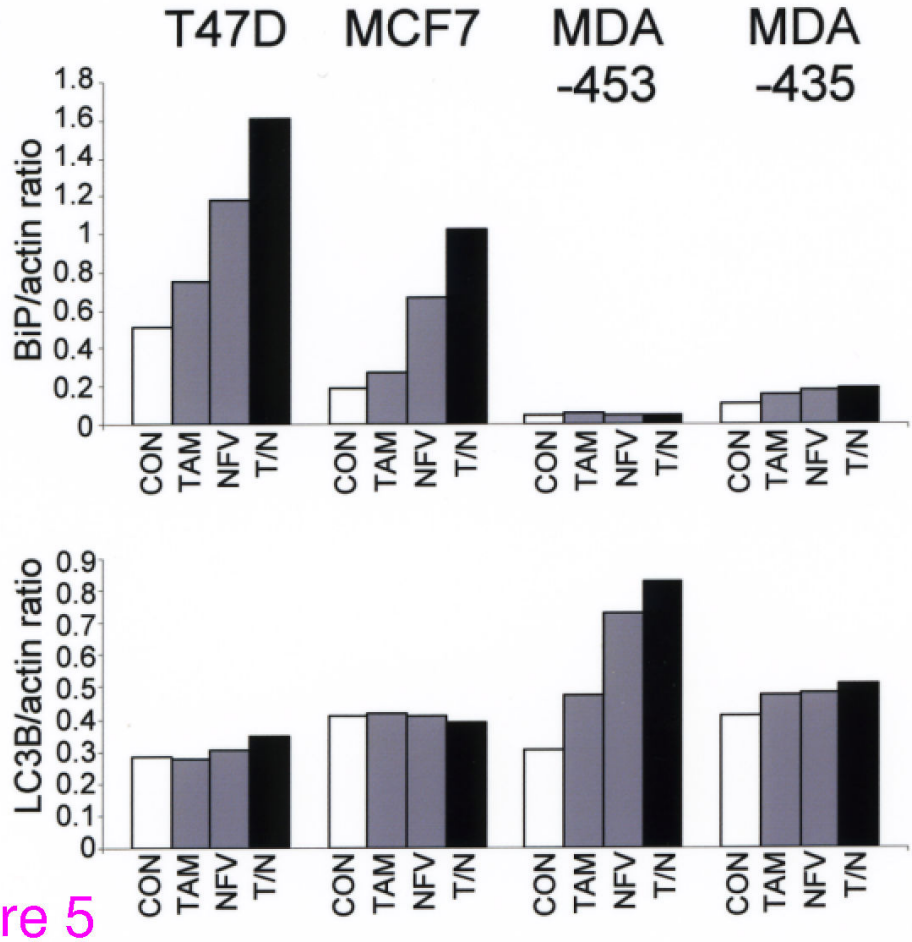

T47D
MCF7
MDA -453
MDA -435
BiP/actin ratio
LC3B/actin ratio
CON
TAM
NFV
T/N

Figure 5

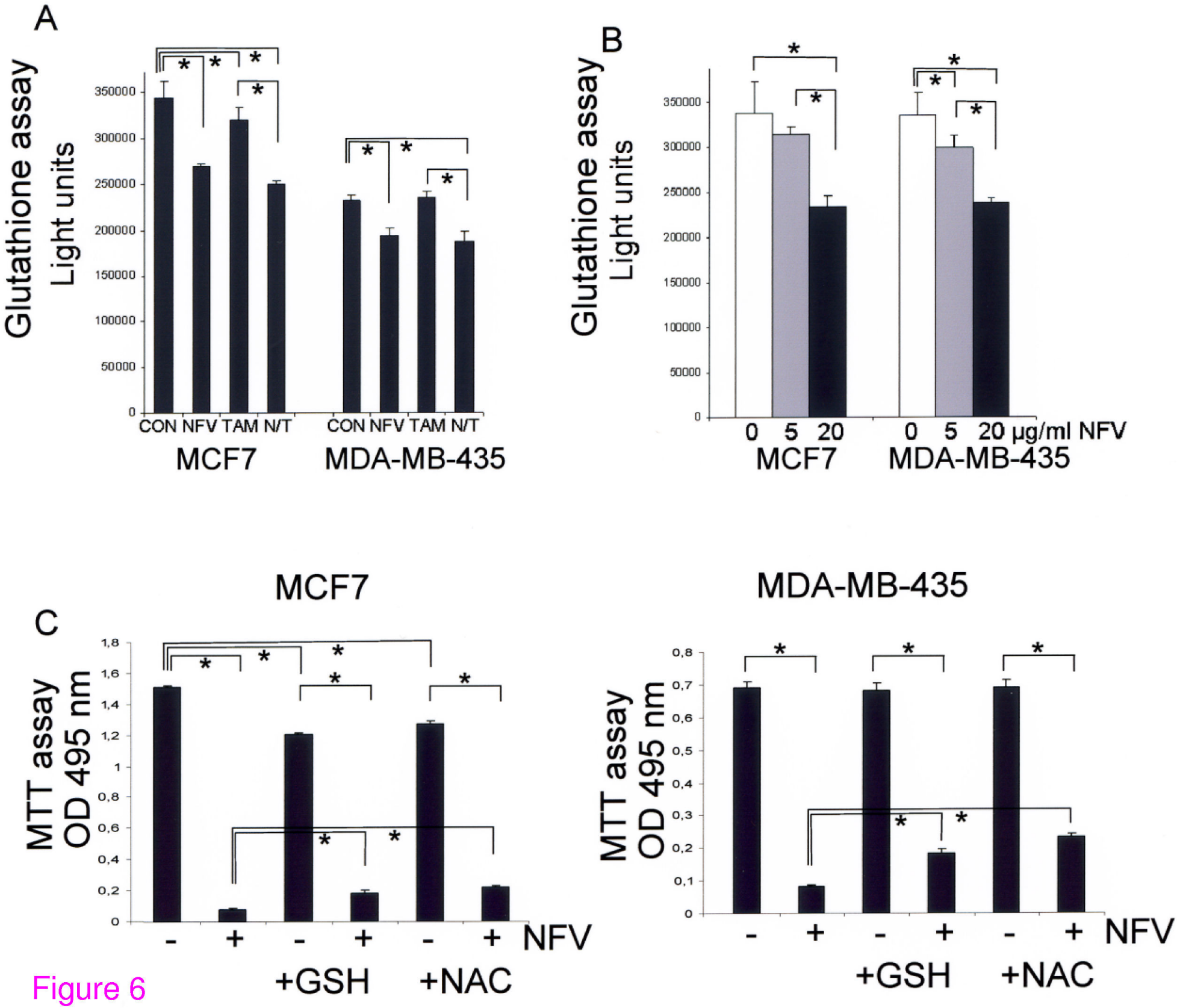

A
Glutathione assay
Light units
350000
300000
250000
200000
150000
100000
50000
0
CON NFV TAM N/T
CON NFV TAM N/T
MCF7
MDA-MB-435
B
Glutathione assay
Light units
0 5 20
0 5 20 µg/ml NFV
MCF7
MDA-MB-435
C
MCF7
MDA-MB-435
MTT assay
OD 495 nm
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0,7
0,5
0,3
0,1
- + - + - + NFV
+GSH +NAC

Figure 6

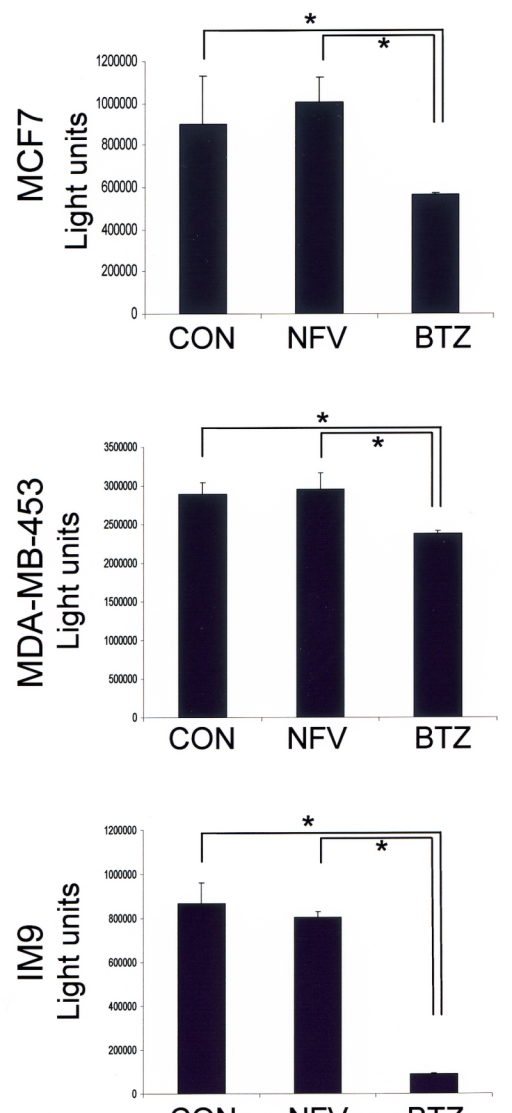

MCF7
Light units
1200000
1000000
800000
600000
400000
200000
0
*
*
CON
NFV
BTZ
MDA-MB-453
Light units
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
*
*
CON
NFV
BTZ
IM9
Light units
1200000
1000000
800000
600000
400000
200000
0
*
*
CON
NFV
BTZ

Figure 7