The antipsychotic drug chlorpromazine enhances the cytotoxic effect of tamoxifen in tamoxifen-sensitive and tamoxifen-resistant human breast cancer cells

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Tamoxifen resistance is a major clinical problem in the treatment of estrogen receptor α-positive breast tumors. It is, at present, unclear what exactly causes tamoxifen resistance. For decades, chlorpromazine has been used for treating psychotic diseases, such as schizophrenia. However, the compound is now also recognized as a multitargeting drug with diverse potential applications, for example, it has antiproliferative properties and it can reverse resistance toward antibiotics in bacteria. Furthermore, chlorpromazine can reverse multidrug resistance caused by overexpression of P-glycoprotein in cancer cells. In this study, we have investigated the effect of chlorpromazine on tamoxifen response of human breast cancer cells. We found that chlorpromazine worked synergistically together with tamoxifen with respect to reduction of cell growth and metabolic activity, both in the antiestrogen-sensitive breast cancer cell line, MCF-7, and in a tamoxifen-resistant cell line, established from the MCF-7 cells. Tamoxifen-sensitive and tamoxifen-resistant cells were killed equally well by combined treatment with chlorpromazine and tamoxifen. This synergistic effect could be prevented by addition of estrogen, suggesting that chlorpromazine enhances the effect of tamoxifen through an estrogen receptor-mediated mechanism. To investigate this putative mechanism, we applied biophysical techniques to simple model membranes in the form of unilamellar liposomes of well-defined composition and found that chlorpromazine interacts strongly with lipid bilayers of different composition leading to increased permeability. This implies that chlorpromazine can change influx properties of membranes hence suggesting that chlorpromazine may be a promising chemosensitizing compound for enhancing the cytotoxic effect of tamoxifen. *Anti-Cancer Drugs* 20:723–735 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Antiestrogen therapy is used for treating breast cancer patients with tumors that express estrogen receptor α (ERα). Antiestrogens work by blocking ERα, thus antagonizing estrogen-mediated growth and survival of breast cancer cells. The antiestrogen tamoxifen has been used for more than 30 years as first-line adjuvant treatment for breast cancer patients, and is still widely used. Unfortunately, after a period, most patients with advanced disease develop resistance toward tamoxifen such that breast cancer tumors become unresponsive to treatment and continue to grow in the presence of tamoxifen [1,2]. The exact molecular mechanisms involved in the development of tamoxifen resistance have not been completely elucidated yet. However, growing evidence suggests that resistance involves multiple molecular changes, resulting in aberrant activation of growth factor signaling cascades, thus making antiestrogen-resistant tumors grow independently of estrogen signaling [3].

Chlorpromazine (CPZ) belongs to the family of phenothiazines, which is a group of structurally related amphiphilic compounds, recognized for their anti-dopamine activity. CPZ was first described in the 1950s where it was widely prescribed for psychotic patients. Today, the drug is still used for treating psychotic diseases, such as schizophrenia [4]. Drug dose of CPZ is typically 100–300 mg/day in schizophrenic patients [5]. This is estimated to result in a serum concentration of approximately 5 μmol/l [6,7]. However, major differences in measured drug serum levels in patients receiving comparable doses might reflect differences in absorption

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and metabolism of the compound. The treatment with CPZ is associated with some side effects, such as sedation, slurred speech, dry mouth, and urinary retention. More rarely, akathisia (restlessness) and dystonias (involuntary muscle contractions) are observed, but these effects can usually be relieved by the addition of anticholinergics to the medication regimen [5].

Apart from their activity in neurons, phenothiazines have also been reported to display antiproliferative properties. CPZ was shown to inhibit proliferation of leukemia cells in culture without affecting normal lymphocyte viability [8], and CPZ inhibits proliferation and induces apoptosis in different types of cultured cells including melanoma cells [9]. Most investigations on effects of CPZ on normal cells indicate that CPZ either causes minimal effect, or acts in a cytostatic rather than cytotoxic manner (reviewed by Nordenberg *et al.* [10]). These data suggest a potential use of CPZ in cancer therapy.

Moreover, phenothiazines, such as CPZ, thioridazine and trifluopherazine, have been shown to possess the ability of reversing antibiotics resistance in bacteria [11] and multidrug resistance (MDR) in cancer [12]. These different types of resistances have in common that they are likely to be mediated by efflux pumps, which decrease intracellular drug levels. The mode of action used by phenothiazines to circumvent antibiotic resistance is suggested to involve phenothiazine-mediated inhibition of antibiotic efflux pumps. However, direct cytotoxic effects might also play a role in phenothiazine-mediated killing of bacteria [11]. MDR in cancer is often caused by monodrug treatment and characterized by unresponsiveness to a variety of other unrelated drugs. It has long been known that overexpression of the drug transporter P-glycoprotein can result in MDR [13]. The mechanism behind CPZ-mediated reversal of MDR has been suggested to involve either direct inhibition of P-glycoprotein by CPZ, or interaction of CPZ with membrane phospholipids, thereby indirectly influencing P-glycoprotein activity [12,14]. Although tamoxifen can bind to the P-glycoprotein transporter, it is unclear whether it is indeed a substrate of the pump [15]. However, tamoxifen has been shown to decrease drug efflux and, similarly to CPZ, to reverse MDR in cancer cells [16].

To investigate the effect of CPZ on tamoxifen response, we have used a breast cancer cell model consisting of a tamoxifen-sensitive cell line, MCF-7, and a tamoxifen-resistant cell line, MCF-7/TAM^R-1. The MCF-7 cell line has been adapted to grow in medium with only 1% fetal calf serum, which results in an estrogen level comparable with that in serum from postmenopausal women [17]. The tamoxifen-resistant cell line MCF-7/TAM^R-1 (for simplicity referred to as TAM^R-1) was established from this low serum-adapted MCF-7 cell line by long-term treatment of the cells with tamoxifen. After several weeks proliferating cell clones emerged, giving rise to the resistant cell line, TAM^R-1. These cells survive and proliferate in the presence of tamoxifen, and resistance is retained after withdrawal of antiestrogen [18]. Interestingly, TAM^R-1 display increased expression of P-glycoprotein from the MDR-1 gene, as compared with MCF-7 cells [19].

In this cell culture model system, we could show that treatment of the breast cancer cells with a combination of CPZ and tamoxifen efficiently inhibits proliferation and results in cell killing, an effect that was independent of initial tamoxifen sensitivity. To shed some light on the possible mechanism behind the mode of action of CPZ, we have undertaken a series of biophysical investigations aimed at quantifying the effect of CPZ on the lipid-bilayer component of cell membranes. To obtain clear-cut results, we have performed these investigations in the simplest possible setting by using well-defined lipid layers in the form of monolayers on air–water interfaces and in the form of unilamellar vesicles (ULVs) composed of mixtures of different types of charged and uncharged lipids and cholesterol. The results suggest that CPZ action on cell membranes is likely to involve interaction of CPZ with the phospholipid bilayer, resulting in a drastic increase in the membrane permeability.

The finding that CPZ enhances the cytotoxic effect of tamoxifen in breast cancer cells indicates that CPZ could potentially be used in combination with tamoxifen in breast cancer patients for increasing intracellular tamoxifen concentrations, thereby hopefully achieving enhanced tamoxifen-mediated growth inhibition and cytotoxicity.

Methods

Cell culture

The MCF-7 cell line was originally obtained from the Breast Cancer Task Force Cell Culture Bank (Mason Research Institute, Worcester, Massachusetts, USA). The tamoxifen-resistant cell line MCF-7/TAM^R-1 (TAM^R-1) was established from MCF-7 cells as described earlier [17]. Cells were propagated in growth medium consisting of phenol-red-free Dulbecco's modified Eagle's medium/F12 (1 : 1) (Gibco, Invitrogen, Carlsbad, California, USA) with 1% fetal calf serum (Gibco), 2.5 mmol/l L-glutamine (Gibco), and 6 ng/ml insulin (Novo-Nordisk, Bagsvaerd Copenhagen, Denmark). TAM^R-1 cells were propagated in the presence of 1 µmol/l tamoxifen (Sigma-Aldrich, St. Louis, Missouri, USA). Cells were maintained at 37°C in humidified air containing 5% CO_2. One week before the onset of experiments, tamoxifen was withdrawn from the medium of TAM^R-1 cells.

Cell viability tests

The WST-1 test was performed by seeding cells in 96-well plates. Cells were treated with tamoxifen, CPZ (Sigma-Aldrich), or the two in combination for 48 h.

WST-1 reagent (Roche Applied Science, Basel, Switzerland) was added to the cells according to the manufacturer's instructions. Conversion of WST-1 to formazan by mitochondrial dehydrogenases, as indicative of metabolically active cells, was measured 1 h after the addition of the WST-1 reagent in a microtiter plate reader (VERSAMAX, Molecular Devices Ltd., Sunnyvale, California, USA). Experiments were performed thrice obtaining similar results.

For measuring the effect of estradiol in combination with CPZ and tamoxifen on cell growth, the cells were seeded in 24-well plates. One day after seeding, treatment with 5 µmol/l CPZ, 1 µmol/l tamoxifen, and 10 nmol/l estradiol (Sigma-Aldrich) was initiated. After 3 days, experimental medium was renewed. After an additional 2 days (resulting in total treatment time of 5 days), cells present in the multiwell plate were washed once with PBS, stained with 0.5% crystal violet in 25% methanol and destained with H_2O. The dye was dissolved in 0.1 mol/l sodium citrate dihydrate in 50% ethanol, and the absorbance was measured at 570 nm to give a relative indication of cell number [20]. The crystal violet data shown are representatives from three independent experiments each performed in triplicate.

Clonogenic survival assay

MCF-7 cells (2000) and TAM^R-1 cells (2500) were seeded in six-well plates and grown for 4 days to allow formation of small cell clusters. Fresh medium was added containing tamoxifen and CPZ, and cells were grown for an additional 10 days. Subsequently, cells were stained with crystal violet as described above. Colonies (containing >30 cells) were quantified using the UVIdoc software (UVItec Ltd., Cambridge, UK) by setting the detection limit such that small cell clusters (containing <30 cells) were not counted. The settings for colony counting were confirmed by microscopy. The results from the colony formation assay were based on six independent experiments.

Flow cytometry

Cells were seeded in 12-well plates 24 h before treatment with tamoxifen, CPZ, or a combination of the two. The broad range caspase inhibitor, z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk; Bachem, Torrance, California, USA), was used at a concentration of 5 µmol/l and the cathepsin inhibitor, z-Phe-Ala-fmk (zFA-fmk; MP Biomedicals, Solon, Ohio, USA), was used at a concentration of 85 µmol/l as described earlier [21]. For determination of cell death, cells were incubated with 0.5 µmol/l SYTOX green nucleic acid stain (Molecular Probes, Invitrogen) for 15 min in the dark. Cells were harvested by trypsination and combined with floating cells from the medium. Flow cytometric analyses were performed using a FACSCalibur (Becton-Dickinson, Franklin Lakes, New Jersey, USA) flow cytometer. The acquired data were analyzed using the Cell Quest Pro software (Becton Dickinson). For each measurement, 10 000 cells were analyzed. FACS analyses were performed three times with similar results.

Western blot analysis

Cell lysates were prepared in lysis buffer (50 mmol/l Tris–HCl, pH 7.5; 150 mmol/l NaCl; 1% Triton X-100; 10% glycerol; 1 mmol/l dithiothreitol; 30 mmol/l: sodium pyrophosphate; and 10 mmol/l NaF, supplied with 1 mmol/l activated Na_3VO_4 and protease inhibitor cocktail; Complete, Roche Applied Science). Western blot analysis was performed as described before [21]. Polyvinylidene fluoride membranes were blocked in PBS with 0.2% casein and 0.1% Tween-20, and incubated with primary antibodies (mouse anti β-actin, Sigma-Aldrich, 1 : 10 000; mouse anti-poly(ADPribose)polymerase (PARP), BD Biosciences, San Jose, California, USA, 1 : 1,400) at 4°C over night. Membranes were incubated with secondary Goat-anti-Mouse antibody (Jackson, West Grove, Pennsylvania, USA, 1 : 20 000) for 1 h at room temperature. Protein bands were visualized using the chemiluminescence CDP-star (Tropix, Bedford, Massachusetts, USA).

Preparation of unilamellar vesicles and differential scanning calorimetry

ULVs were prepared by dissolving lipids, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-*sn*-3-phosphatidylglycerol (DMPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), and cholesterol (Avanti Polar Lipids, Alabaster, Alabama, USA) in chloroform, which was then evaporated under nitrogen flow for 2 h. Complete removal of chloroform was performed for 20 h under vacuum. The dried lipids were hydrated in buffer [10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mmol/l NaCl, pH 7.5] to give a stock solution of 25 mmol/l and extruded 12 times through 100 nm filters (Whatman, Clifton, New Jersey, USA) to generate ULVs. Storage and handling of ULVs were carried out above melting temperature of the membranes in an oven at 37°C. A 0.25-mmol/l stock of CPZ was prepared. The ULV and the CPZ stocks were mixed to give a fixed ratio between CPZ and lipid.

Differential scanning calorimetry (DSC) experiments using ULVs composed of a series of different lipid mixtures indicated in each of the figures were performed by means of a Science Corp N-DSCII Calorimeter

(Lindon, Utah, USA). The scan rate was 0.25°C/min and the pressure was set at 3 atm. Six scans were performed in the increasing temperature interval 15–45°C. Data analysis was performed using Origin 7.0 (Becton Dickinson) to find the melting temperature of the ULVs, the melting enthalpy and the broadness of the melting process. Data from one representative scan is shown.

Isothermal titration calorimetry

ULVs were made as described above. Isothermal titration calorimetry (ITC) experiments were performed by adding a CPZ solution into a 5 mmol/l ULV solution during an injection time of 8 s. The time between each injection was 350 s and in total 35 injections were carried out. The temperature was set to 37°C. Data analysis was performed using Origin 7.0 to determine the partition coefficient K of CPZ from water into the ULVs and the change in standard enthalpy H associated with that process. To determine the partition coefficient, the model of Heerklotz *et al.* [22] was used. The partition coefficient is a function of the ratio between the concentration of bound and free CPZ, formally defined as $K = C_{CPZ,bound}/(C_{CPZ,free} \cdot C_{lipid})$, where C_{lipid} is the concentration of lipids. When K is determined, the change in standard Gibbs free energy associated with the partition process can be found from $\Delta G = -RT \ln [55.5 \text{ (mol/l)}\ K]$, where R is the universal gas constant, T the absolute temperature and 55.5 mol/l (the concentration of pure water) is the so-called cratic contribution to the free energy from water. The change in standard entropy can then be calculated as $\Delta S = (\Delta H - \Delta G)/T$.

Calcein-enclosed vesicles and permeability assay

A self-quenching calcein solution was prepared (50 mmol/l calcein in 10 mmol/l HEPES, pH 7.5; 30 μmol/l $CaCl_2$; 10 μmol/l EDTA) as described in Ref. [23]. ULVs were prepared of POPC and cholesterol (Avanti Polar Lipids) as described above, but hydrated in the calcein solution at a final concentration of 5 mmol/l lipid. Untrapped calcein was removed by dialysis in a dialysis cassette (Pierce, Rockford, Illinois, USA) with a cut-off of 10 kDa against HEPES release buffer (10 mmol/l HEPES, 150 mmol/l KCl) of same osmolarity as the calcein solution. The vesicles, composed of lipid mixtures indicated in the figure, were diluted to 4 μmol/l in HEPES release buffer in a cuvette, and placed in a temperature-regulated cuvette holder at 37°C within the fluorometer. The fluorescence measurements were performed using a fluorometer (ISS, Chronos, Illinois, USA) with the excitation light produced by a laser diode with an excitation wavelength of 471 nm and the emission channel was set to 515 nm. The fluorescence was recorded as a function of time. The baseline fluorescence level was recorded for 5 min, then CPZ (2–40 μmol/l) was added and the calcein release was monitored for 45 min. Complete release was obtained by adding Triton X-100 to a final concentration of 0.3%.

Surface pressure/molecular area isotherms

Surface pressure/molecular area isotherms were obtained at 20°C by using a Langmuir-Blodgett mini-trough (Kibron μ-Trough S) and the software Filmware 2.41 (Becton Dickinson). The trough was filled with HEPES buffer (10 mmol/l HEPES, 150 mmol/l NaCl) with or without CPZ (at concentrations of 2.5, 10, or 25 μmol/l). The surface was swept and impurities, if any, were removed from the water/air interface with a pipette. A solution of 0.25 mg/ml dipalmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids) in hexane/MeOH (98 : 2) was carefully spread over the surface in small droplets from a Hamilton syringe and the solvent was allowed to evaporate for 10 min before the measurement was started. The compressions were performed at 2 $Å^2$/chain/min while the surface tension was recorded with a platinum rod. The surface tension of the film-free solution was taken as a reference. For each concentration of CPZ, at least five surface pressure/molecule area isotherms were recorded.

Statistical analysis

To determine whether differences between treatments of the cell cultures were statistically significant, the two-tailed t-test (Student's t-test) was performed. Statistical significance was concluded when a P value was less than 0.05.

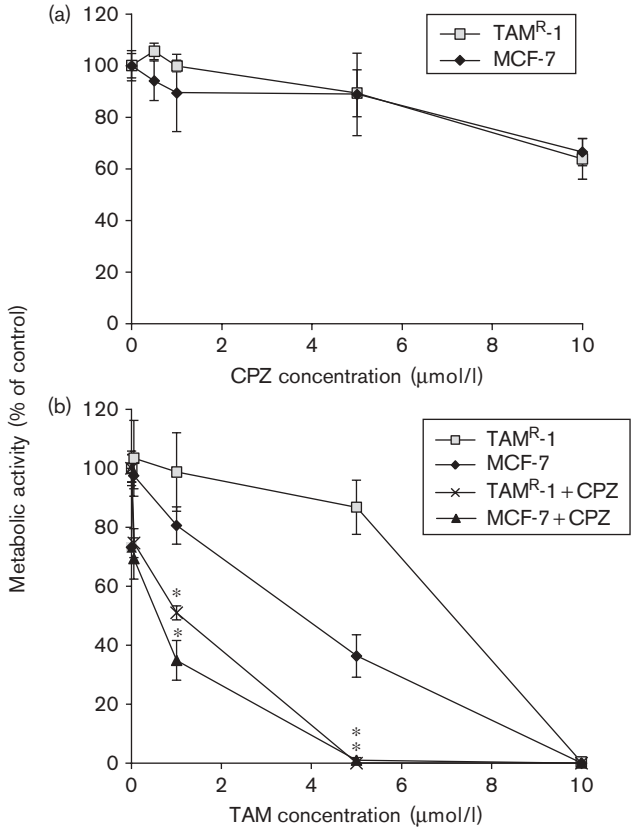
Results

Chlorpromazine and tamoxifen reduce cell metabolic activity and proliferation in a synergistic manner in both tamoxifen-sensitive and tamoxifen-resistant breast cancer cells

For studying the effect of CPZ on tamoxifen sensitivity, we used two different cell lines; the antiestrogen-sensitive cell line, MCF-7, and the tamoxifen resistant cell line, TAM^R-1. It has been shown that CPZ can directly affect cell proliferation and induce apoptosis in some cell types [10]. Therefore, we analyzed the effect of treatment with CPZ for 48 h on MCF-7 and TAM^R-1 cells by the WST-1 viability test, which provides a measure of the metabolic activity of the cells. The lowest concentrations of CPZ tested (0.5, 1, and 5 μmol/l) did not result in any decrease in cell metabolic activity (Fig. 1a). However, at 10 μmol/l CPZ a relatively small decrease in cell metabolic activity of approximately 30% was observed. MCF-7 cells and TAM^R-1 cells responded similarly toward CPZ treatment.

Then, we investigated the effect of tamoxifen on MCF-7 cells and TAM^R-1 cells measured by the WST-1 assay as described above. We could confirm that MCF-7 cells were more sensitive toward tamoxifen than TAM^R-1 cells, as 0.05, 1, and 5 μmol/l tamoxifen, respectively, reduced the metabolic activity of MCF-7 cells in a dose-dependent manner after 48 h of treatment. The metabolic activity of TAM^R-1 cells was only

Fig. 1



Treatment with tamoxifen (TAM) in combination with chlorpromazine (CPZ) results in reduced metabolic activity of MCF-7 and TAM^R-1 breast cancer cells. (a) Metabolic activity of MCF-7 and TAM^R-1 cells was measured after treatment for 48 h with increasing concentrations of CPZ (0.5–10 μmol/l). The WST-1 colorimetric test was performed as described in the Methods and the metabolic activity was calculated relative to the respective untreated cells. (b) MCF-7 and TAM^R-1 cells were treated with TAM (0.05–5 μmol/l) alone or in combination with 10 μmol/l CPZ for 48 h. TAM was added such that the amount of ethanol was 0.1% in all samples. Metabolic activity was measured using WST-1 as described above and presented in percentage of control cells treated with 0.1% ethanol. *Statistical difference when comparing the combined CPZ and TAM treatment (calculated relative to treatment with 10 μmol/l CPZ alone) with TAM treatment (Student's *t*-test, $P<0.05$).

significantly affected by the highest concentration of tamoxifen (i.e. 10 μmol/l), which is toxic to both cell lines (Fig. 1b). When combining 10 μmol/l CPZ with 1 and 5 μmol/l tamoxifen, a statistically significant ($P < 0.05$) decrease in the number of metabolically active cells was observed as compared with treatment with either CPZ or tamoxifen alone (Fig. 1b). Interestingly, this effect was observed for both cell lines, indicating that CPZ enhances the effect of tamoxifen independently of initial antiestrogen sensitivity.

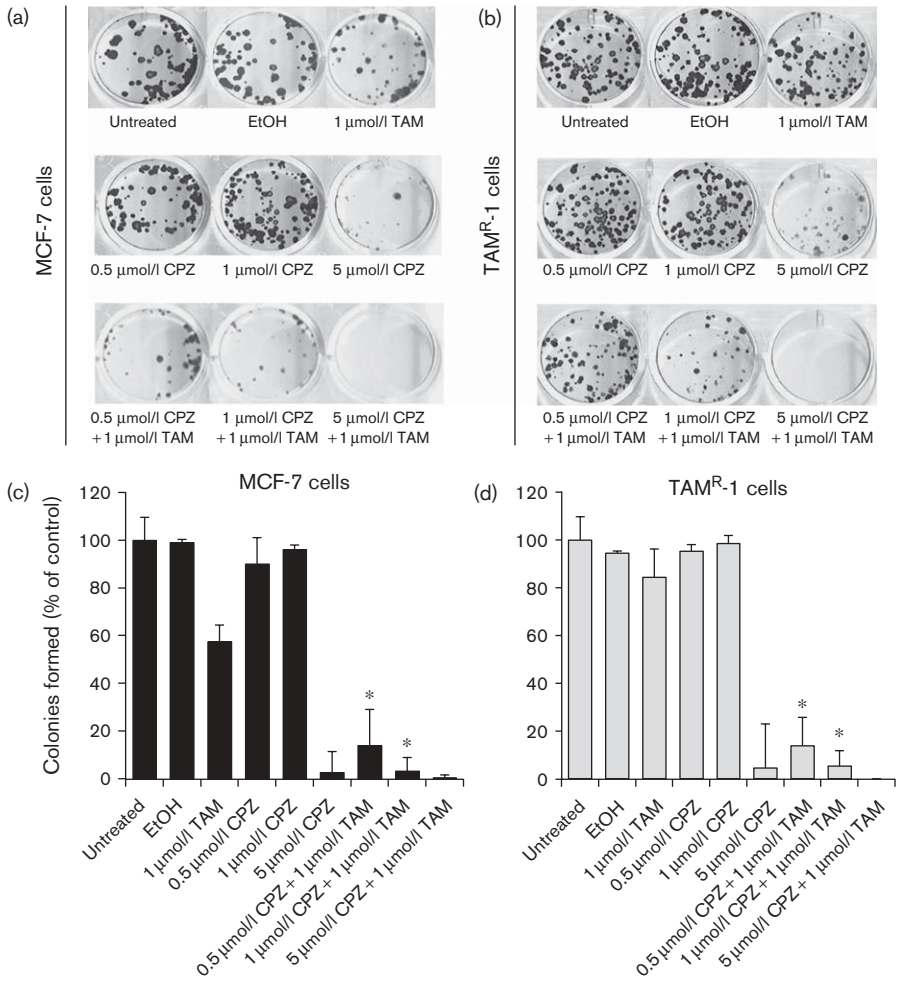
To analyze the effect of CPZ and tamoxifen treatments on the ability of cells to form colonies, when treated for a longer time period, a clonogenic survival assay was applied. Cells were seeded at low density and allowed to form small cell clusters before treatment for 10 days with 0.5, 1, and 5 μmol/l CPZ; 1 μmol/l tamoxifen; or the two drugs in combination. Cells were stained with crystal violet and representative cell culture wells are shown in Fig. 2a (MCF-7 cells) and Fig. 2b (TAM^R-1 cells). The number of cell colonies formed is shown in Figs. 2c and d for MCF-7 and TAM^R-1 cells, respectively. MCF-7 cell clonogenicity (Fig. 2a and c) was reduced by approximately 40% by treatment with 1 μmol/l tamoxifen. The two lowest concentrations of CPZ (0.5 and 1 μmol/l) did not affect clonogenic survival of MCF-7 cells. However, 5 μmol/l of CPZ resulted in almost complete loss of the ability of MCF-7 cells to form colonies. When CPZ was used in combination with 1 μmol/l tamoxifen, a statistically significant reduction in the number of colonies formed from MCF-7 cells was observed, as compared with treatment with similar concentrations of tamoxifen alone ($P < 0.05$) (Fig. 2c). The same was true for the tamoxifen-resistant cell line, TAM^R-1; CPZ in combination with tamoxifen significantly reduced colony formation (Fig. 2b and d). The finding that 0.5 and 1 μmol/l CPZ, which have no growth inhibitory effect when added to the cell lines, in combination with 1 μmol/l tamoxifen reduced colony formation significantly compared with tamoxifen alone, shows that CPZ and tamoxifen work in a synergistic manner. This suggests that CPZ enhances the effect of tamoxifen and, in the case of tamoxifen-resistant cells, can overcome resistance toward tamoxifen.

The synergistic effect of chlorpromazine and tamoxifen is estrogen receptor-dependent

Then, we performed growth assays, where cells were treated with tamoxifen or tamoxifen in combination with CPZ for a total of 5–6 days. After treatment, cell number was determined by manual counting, Fig 3a (Bürker-Türk chamber) or by the measurement of optical density of crystal violet stained cells, Fig 3b. First, the optimal concentration of tamoxifen was determined in a dose–response growth assay using increasing concentrations of tamoxifen for treatment of MCF-7 and TAM^R-1 cells (Fig. 3a). As expected, MCF-7 cells were more sensitive to 0.1 and 1 μmol/l tamoxifen than TAM^R-1 cells. TAM^R-1 cells were not affected by treatment with 0.1 μmol/l tamoxifen. In both MCF-7 and TAM^R-1 cells, treatment with 2.5 μmol/l tamoxifen resulted in drastic reduction in cell number, and after treatment with 5 μmol/l tamoxifen, a complete reduction in cell number was observed in this assay (Fig. 3a). We, therefore, chose to perform the following experiments using 1 μmol/l of tamoxifen for 5 days to obtain an intermediate response of tamoxifen in both cell lines.

Under these conditions, treatment of cells with 1 μmol/l tamoxifen alone resulted in a reduction of cell number by approximately 50% in the case of the MCF-7 cell

Fig. 2



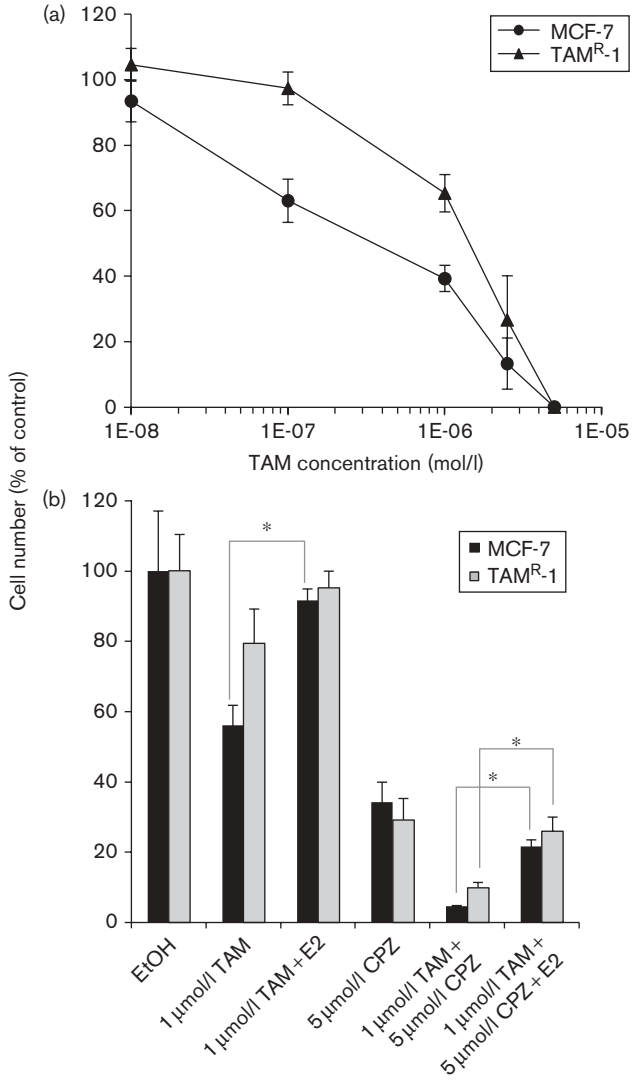
Clonogenic survival of MCF-7 and TAM^R-1 cells is decreased by combined treatment with tamoxifen (TAM) and chlorpromazine (CPZ). MCF-7 and TAM^R-1 cells were allowed to form small cell clusters (<30 cells) before treatment for 10 days with CPZ (0.5, 1, and 5 μmol/l, respectively), TAM (1 μmol/l), or the two drugs in combination (1 μmol/l TAM combined with 0.5, 1, and 5 μmol/l CPZ, respectively). Ethanol (EtOH)-treated cells were incubated with 0.1% EtOH, the same amount of EtOH present in the TAM-treated samples. Colonies were stained by a crystal violet staining method as described in the Methods. Representative pictures of MCF-7 (a) and TAM^R-1 (b) cell colonies after crystal violet staining are shown. Cell colonies were quantified and average values from six independent experiments are shown for MCF-7 (c) and TAM^R-1 (d) cells, respectively. The colony counts are expressed relative to untreated control. *Statistical significant difference in number of colonies formed after treatment with a combination of 0.5 and 1 μmol/l CPZ with 1 μmol/l TAM as compared with the treatment with 1 μmol/l TAM alone (Student's *t*-test, $P<0.05$). CPZ, 5 μmol/l, in combination with 1 μmol/l TAM was tested against 5 μmol/l CPZ alone (no significant difference).

line and by approximately 20% in the case of TAM^R-1 cells (Fig. 3b). Both in case of MCF-7 cells and TAM^R-1 cells, the tamoxifen-induced growth inhibition could be reversed by addition of 10 nmol/l estradiol (Fig. 3b), confirming that the effect of 1 μmol/l tamoxifen is mediated through an estrogen receptor-dependent mechanism. After 5 days of treatment with 5 μmol/l CPZ alone, cell growth of both cell lines was inhibited by approximately 70% (Fig. 3b), consistent with the results obtained by the colony formation assay (Fig. 2, treatment for 10 days). The combination of CPZ with tamoxifen resulted in almost complete loss of cells both in the case of MCF-7 and TAM^R-1 cell lines. Addition of 10 nmol/l estradiol reduced the effect of combined CPZ and tamoxifen to a level almost corresponding to the effect of 5 μmol/l CPZ alone (Fig. 3b, no statistical significant difference was found between treatment with 5 μmol/l CPZ vs. combined treatment with 1 μmol/l tamoxifen, 5 μmol/l CPZ, and 10 nmol/l estradiol). This indicates that the synergism between CPZ and tamoxifen occurs through an estrogen receptor-mediated mechanism in the tamoxifen-sensitive MCF-7 cells and in the tamoxifen-resistant TAM^R-1 cells as well.

Chlorpromazine enhances tamoxifen-induced cell death

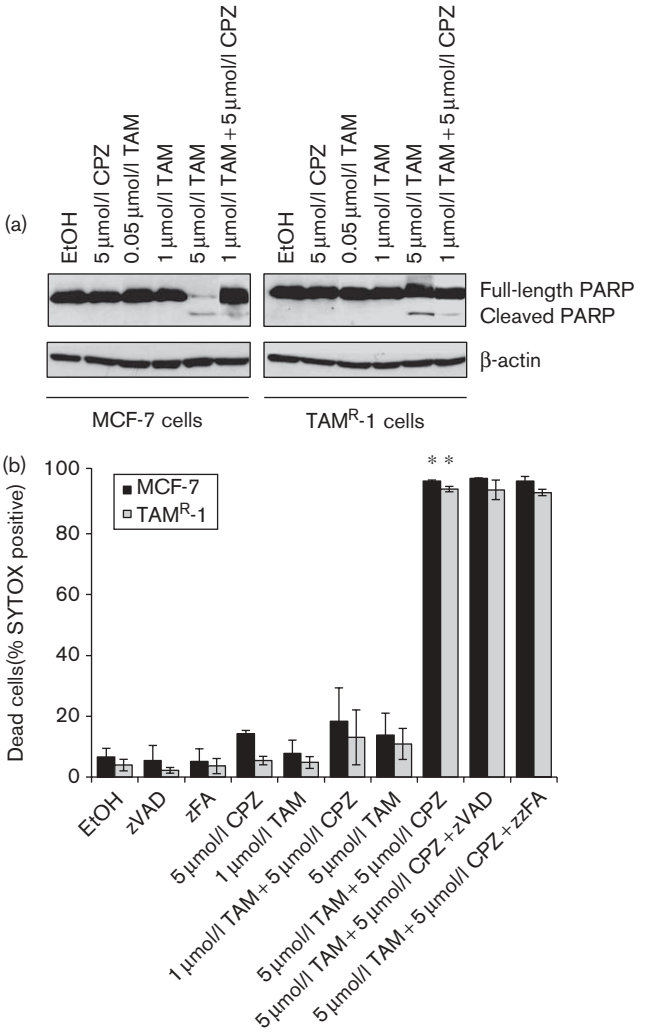
To study whether CPZ affects the cytotoxicity of tamoxifen, we first investigated cleavage of PARP as a measure of early-stage apoptosis. MCF-7 and TAM^R-1

Fig. 3



Combined chlorpromazine (CPZ)–tamoxifen (TAM) treatment induces an estrogen receptor-dependent response. (a) MCF-7 and TAM^R-1 cells were treated for 6 days with increasing concentrations of TAM, and the number of cells was counted. Cell number is given in the percentage of control cells (treated with 0.1% ethanol, EtOH). (b) Cells were treated for 5 days with 5 µmol/l CPZ, 1 µmol/l TAM or 1 µmol/l TAM in combination with: 10 nmol/l estradiol (E2), 5 µmol/l CPZ or 5 µmol/l CPZ + 10 nmol/l E2. Control cells were incubated with 0.1% EtOH. Cell number was determined by staining cells with crystal violet and expressed as percentage of EtOH-treated control cells. *Statistical significant difference in cell number when comparing TAM versus TAM + E2 and TAM + CPZ versus TAM + CPZ + E2 treatments, respectively ($P < 0.05$). In the case of MCF-7 cells, but not TAM^R-1 cells, statistical significant difference was also found when comparing 1 µmol/l TAM treatment versus control (not shown). Comparison of control treatment versus TAM + E2 or treatment with CPZ versus TAM + CPZ + E2 showed no statistical significant difference.

Fig. 4



Chlorpromazine (CPZ) and tamoxifen (TAM) activate late-stage apoptotic or necrotic cell death. (a) MCF-7 and TAM^R-1 cells were treated with CPZ and TAM as indicated in the figure, and western blot analysis for detection of full-length (116 kDa) and cleaved (85 kDa) poly(ADPribose)polymerase (PARP) were performed as described in the Methods. (b) Cells were treated with 1 or 5 µmol/l TAM alone or in combination with 5 µmol/l CPZ. A broad-range caspase inhibitor (85 µmol/l zVAD-fmk) or cathepsin inhibitor (5 µmol/l zFA-fmk) was added as indicated in the figure. After 3 days, cells were incubated with SYTOX green nucleic stain and analyzed by flow cytometry. The percentage of late apoptotic/necrotic cells (SYTOX green-positive cells) is indicated. *Statistical significant difference when comparing the combined CPZ and TAM treatment with SYTOX-positive cells from the respective TAM and CPZ treatments (Student's *t*-test, $P < 0.05$).

cells were treated with 0.05, 1, and 5 µmol/l tamoxifen for 3 days. As shown in Fig. 4a, only the highest concentration of tamoxifen (5 µmol/l) resulted in PARP cleavage in both cell lines. Thus, cell death mediated by treatment with 5 µmol/l tamoxifen appears to occur through caspase activation (presumably caspase-7), which is an indicator of early-stage classical apoptosis. When combining 1 µmol/l tamoxifen with 5 µmol/l CPZ, a slight but not very pronounced induction of PARP cleavage was observed (Fig. 4a). In the case of 5 µmol/l tamoxifen in combination with 5 µmol/l CPZ, cells were severely affected by treatment making it impossible to perform

the western blot analysis for the detection of PARP. We, therefore, suspected that the addition of CPZ could change the type of cell death, induced by tamoxifen, into a late-stage apoptotic or necrotic type. To analyze this, we stained cells with SYTOX green, a fluorescent dye only penetrating cells with compromised plasma membrane, a feature of late-stage apoptosis/necrosis, and the percentage of SYTOX-positive cells was determined by flow cytometry (Fig. 4b). Tamoxifen treatment for 3 days (1 or 5 μmol/l) did not result in late-stage apoptosis or necrosis, and only a slight (but not statistical significant) increase in the percentage of SYTOX-positive cells was measured in case of combining 1 μmol/l tamoxifen with 5 μmol/l CPZ. However, the combination of 5 μmol/l CPZ and 5 μmol/l tamoxifen resulted in nearly complete cell killing, an effect that was statistically significant both in MCF-7 and TAM^R-1 cells (Fig. 4b). These results suggest that CPZ in concert with tamoxifen kills MCF-7 and TAM^R-1 by inducing a late-stage apoptotic or necrotic type of cell death. The massive cell death after combined CPZ and tamoxifen treatment was not attenuated by the addition of the broad-range caspase inhibitor zVAD-fmk, nor the cathepsin inhibitor zFA-fmk, respectively (Fig. 4b), indicating that cell death was more likely necrotic rather than apoptotic.

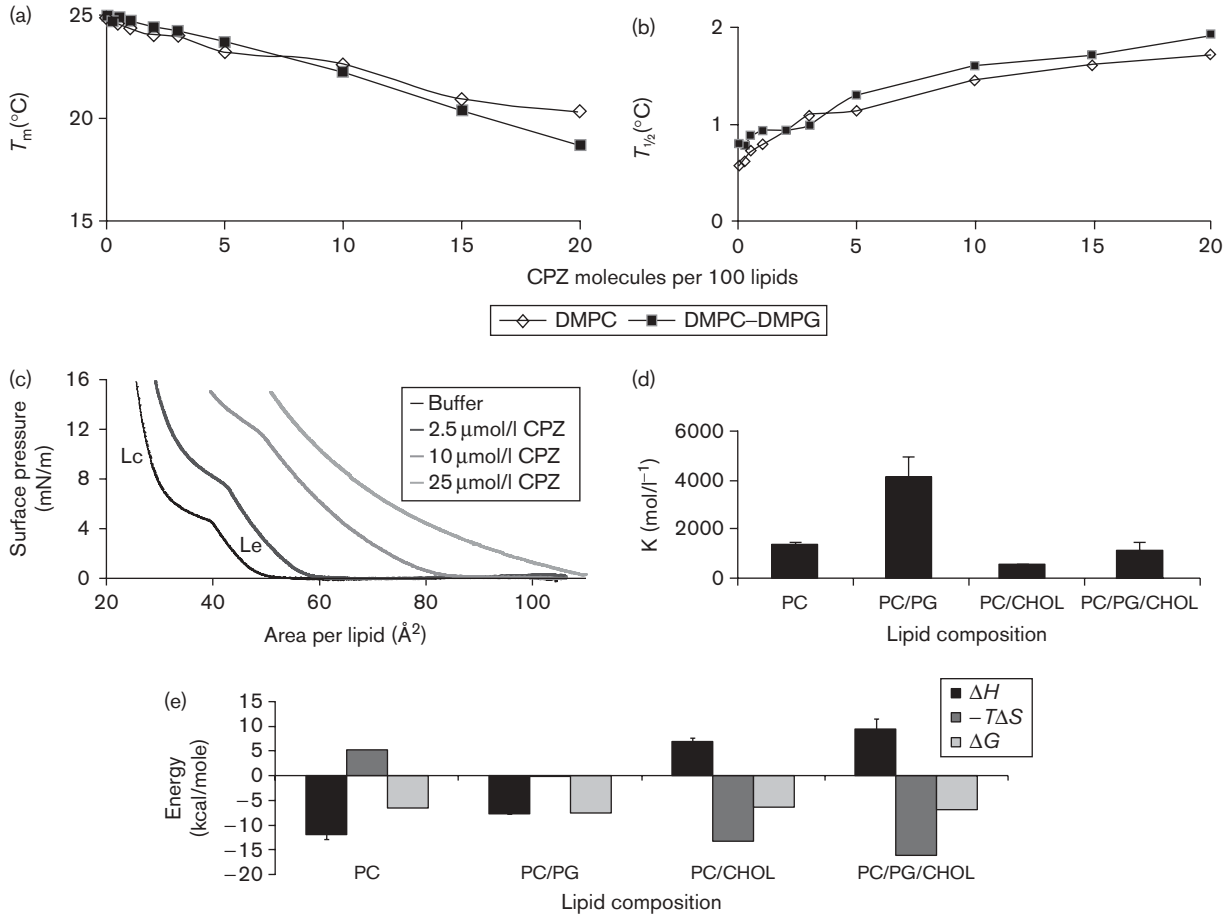
Chlorpromazine interacts with model membranes by intercalating into hydrophobic regions causing freezing point depression and by binding to charged surfaces

As our cell culture studies proposed that CPZ enhanced the cytotoxic effect of tamoxifen by a mechanism that was dependent on estrogen signaling but not related to initial tamoxifen sensitivity, one hypothesis was that CPZ increased the cellular levels of tamoxifen. It has been suggested earlier that reversal of MDR by CPZ involves direct interaction of CPZ with the cell membrane [24]. To confirm that CPZ has an effect on membranes, DSC experiments were performed on mixtures of model membranes by adding increasing concentrations of CPZ (defined by number of CPZ per lipid). Moreover, we investigated the main phase transition of lipid membranes by determining the temperature where membranes make a transition from a gel phase to a fluid phase, that is, the melting temperature T_m for one-component bilayers or the midpoint temperature of the coexistence region for the lipid mixtures. We found that CPZ lowered the transition temperature for increasing concentrations (Fig. 5a), suggesting that CPZ intercalates in the hydrophobic region of the bilayers or interface toward the lipid head groups. We also found that the width of the melting peak (the interval of temperature at which the melting occurs), defined by $T_{1/2}$, increased as the CPZ concentration increased (Fig. 5b), which is typical behavior of a system to which compounds are added that interact strongly with the system. As T_m is related to the structural arrangement of the acyl chains of lipids in the membrane interior and ultimately to the positive change in entropy, accompanied by the structural change at the transition, we can conclude that CPZ partitions into the membranes and is positioned in a way that affects the lipid acyl chains in the interior. As the melting enthalpy is lowered at increasing CPZ concentration (data not shown), we can expect CPZ to bind close to the surface of the membrane acting as substitutional impurity and not interstitial impurity [25], and thus CPZ competes with lipids for space.

Complementary to the DSC experiments, monolayer studies were conducted to investigate the interaction of CPZ with a lipid layer. A monolayer of DPPC was compressed on a subphase of buffer containing various concentrations of CPZ until a surface pressure of approximately 15 mN/m was reached (Fig. 5c). The phase transition between the liquid-expanded (Le) and the liquid-condensed (Lc) phase is represented by a plateau in the compression isotherm, and the location of this phase transition is used as an indication of the intermolecular forces acting in the monolayer. The phase transition between the Le and Lc phases was shifted toward higher surface pressure and higher molecule area with increasing concentration of CPZ (Fig. 5c). The isotherm on a subphase of 25 μmol/l CPZ did not show a phase transition under a surface pressure of 15 mN/m, but subsequent studies showed a transition above that (data not shown). The fact that a higher surface pressure is needed for inducing the phase transition indicates that CPZ is counteracting this pressure in the monolayer favoring the Le phase by disturbing the forces that tend to condense the monolayer. This suggests that CPZ enters the lipid monolayer thereby increasing the area of the monolayer. In the region up to the phase transition (i.e. the Le phase) the slope is decreasing as the concentration of CPZ is increased indicating that a small increase in pressure decreases the area more when CPZ is present, and therefore the monolayer is easier to compress (Fig. 5c). In accordance with the DSC results, this suggests that the lipid layer is becoming more fluid when CPZ interacts with it. These results are also in agreement with studies by Agasøsler *et al.* [26] who studied the interaction of CPZ with lipid monolayers composed of different types of lipids, including charged and unsaturated phospholipids.

CPZ has a pK_a of 9.3, which renders the molecule positively charged at pH = 7.5. Hence, it could be expected that CPZ would interact more strongly with negatively charged bilayers compared with zwitterionic ones. By using DSC, we found that adding negatively charged lipids (DMPG) did not affect the reduction of T_m (Fig. 5a), which indicates that electrostatic forces do not pull CPZ into the interior of the membrane. However, by using isothermal calorimetry and ITC, we

Fig. 5

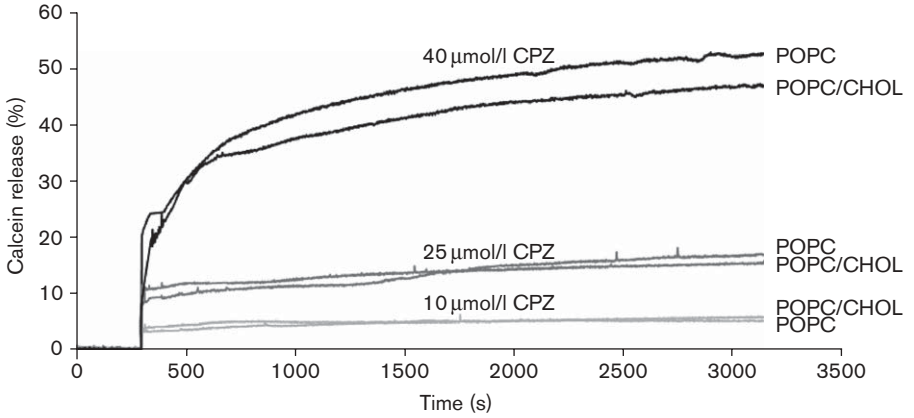


Chlorpromazine (CPZ) interacts with lipid membranes. Phase transition studies were performed by differential scanning calorimetry on 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) and DMPC–DMPG (1,2-dimyristoyl-*sn*-3-phosphatidylglycerol) membranes (a, b) as described in the Methods. The phase transition temperature, T_m, (a) and the width of the melting peak, $T_{½}$, (b) are shown after addition of increasing concentrations of CPZ. The surface pressure versus the compression of monolayers was measured by monolayer compression on DPPC monolayers (c). The liquid-expanded (Le) and the liquid-condensed (Lc) phases are indicated in the figure (corresponding to compression of dipalmitoylphosphatidylcholine monolayer on the buffer solution). The phase transition occurs at the plateau between the two phases. The buffer isotherm has the most significant phase transition while the area per lipid (at a given pressure) is increased with increasing amounts of CPZ. Binding studies by isothermal calorimetry on DMPC, DMPC–DMPG, DMPC–cholesterol (CHOL), and DMPC–DMPG–CHOL, respectively, (d, e) provides the lipid/water partition constant, K, of CPZ for different lipid compositions (d) and shows the change in the driving force behind the binding, when CHOL is added (the change in standard enthalpy, ΔH, the product of the absolute temperature, T, and the change in standard entropy, ΔS, and the change in standard Gibbs free energy, ΔG, are shown) (e).

did find that the partition coefficient increased if negatively charged DMPG lipids were present in the membrane (Fig. 5d), indicating that surface charge increases the number of CPZ molecules that binds to the membranes. Therefore, CPZ might bind to membranes as two subpopulations: one population that partitions into the acyl chain region of the membrane and consequently affect T_m – this process being driven by hydrophobic interaction; and a second population that does not affect T_m by only binding to the surface of the polar head group region of the lipid layer. The negatively charged lipids would cause electrostatic attraction of CPZ to the surface and increase the second population.

As biological membranes are rich in cholesterol, we performed analysis of membranes containing cholesterol. When cholesterol was present, the partition coefficient decreased as compared with membranes without cholesterol (Fig. 5d), indicating that fewer CPZ molecules partitioned into the membranes. This is in agreement with the idea that cholesterol tends to push out foreign hydrophobic molecules that otherwise partition into membranes, because cholesterol causes closer packing of the acyl chains, thus leaving less space for impurities [27,28]. Cholesterol not only reduced the partition coefficient, but also changed the thermodynamic nature of the partitioning process as well (Fig. 5e). The balance between the enthalpic and entropic contribution to the

Fig. 6



Chlorpromazine (CPZ) causes increased membrane permeabilization. Increasing concentrations of CPZ (10, 25, and 40 μmol/l) was added at time of 300 s to liposomes made of POPC or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC)/cholesterol (CHOL) (70 : 30) (indicated in the figure). Liposomes were prepared containing calcein as described in the Methods. The percentage of release of calcein is given compared with total release when 0.3% Triton X-100 was added.

change in Gibbs free energy shows that the partitioning process is driven by enthalpy, which favors the process, whereas entropy does not (at this temperature, 37°C) in the case where no cholesterol is present in the membranes. The enthalpic part is likely to arise from electrostatic forces, which are in agreement with the increase in enthalpy, if DMPG is present in the membranes. However, if cholesterol is present, the partition process is now driven and favored by entropy, whereas enthalpy will counteract the partitioning. This can be explained by the fact that cholesterol causes ordering of the acyl chains, giving them fewer degrees of freedom, which might in part be regained if CPZ is inserted into the membrane, causing a perturbation and increased structural entropy of the system. However, although the entropy is increased, if CPZ partitions into the membrane, the partition constant is still lower than for membranes without cholesterol because of the large enthalpic part that opposes partitioning.

Chlorpromazine works by increasing membrane permeability

To clarify whether the interaction of CPZ with lipid layers could cause a change in the permeability of a membrane, a permeability assay was applied as described in the Methods. The fluorophor calcein was introduced in POPC vesicles in a self-quenching concentration. Upon release from the vesicles, calcein is diluted and the percentage of release compared with the total release is determined from the increase in fluorescence. It was shown that the addition of CPZ did, in fact, cause a release of calcein in a concentration-dependent manner with an increasing degree of release with a higher concentration of CPZ (Fig. 6). At the highest CPZ concentration (i.e. 40 μmol/l) almost 50% of the calcein was released. No change in the release could be observed by the presence of cholesterol for the lower CPZ concentrations (i.e. 25 and 10 μmol/l), whereas at a concentration of 40 μmol/l CPZ there was a decreased CPZ-induced release in the presence of cholesterol (Fig. 6), supporting the notion that cholesterol competes with CPZ. These results clearly show that the interaction between CPZ and membrane lipids leads to a drastic increase in membrane permeability.

Discussion

Tamoxifen is an antiestrogen, which is widely used for endocrine therapy of ERα-positive breast cancer. The drug was introduced almost 30 years ago, resulting in prolonged disease-free survival and increased overall survival of patients. Tamoxifen is a selective estrogen receptor modulator, displaying a relatively complex mode of action. It has both antagonistic and agonistic properties with respect to estrogen signaling, where the outcome of tamoxifen binding to ERα is presumably determined by posttranslational modifications of ERα, the specific cellular content of coactivators, corepressors, and other factors binding to and influencing ERα activation as well [29]. Furthermore, tamoxifen may bind directly to DNA, causing DNA adducts, which is believed to occur independently of ERα [30], and it has been shown earlier that treatment of cultured breast cancer cells with a high concentration of tamoxifen (10 μmol/l) can cause double-stranded DNA breaks [31]. One side effect of tamoxifen is an increased risk of developing endometrial cancer [2,32,33], and it has been discussed whether this is caused by the potential role of tamoxifen as a carcinogen, causing DNA damage, or because of tamoxifen acting as an agonist in the endometrium. Tamoxifen is a nonsteroidal triphenylethylene, but is able

to adopt a structure mimicking a steroid hormone, which is presumed to be the mechanism for crossing biomembranes and thereby entering the cytoplasm of the cells. Tamoxifen interacts directly with cellular membranes and has been shown earlier to decrease cell membrane fluidity [34], which may play a role in processes such as cell contact inhibition and metastasis [35]. Thus, membrane interaction properties of tamoxifen may contribute to its anticancer action.

It seems that the concentration of tamoxifen is highly important for determining which response is triggered in the cell. It has been shown that MCF-7 cells undergo an autophagic cell death at low tamoxifen concentrations (i.e. 1 μmol/l), which can be prevented by simultaneous addition of estradiol. In contrast, higher tamoxifen concentrations (i.e. 10 μmol/l) induce lytic cell death (necrosis), which is not abrogated by estradiol [36]. The physiological serum concentration of tamoxifen in patients, treated with 20 mg tamoxifen twice daily, is approximately 1 μmol/l. However, as tamoxifen is thought to accumulate in cells (especially cells in the hepatic tissue) because of partition of the predominantly hydrophobic tamoxifen in biomembranes, the intracellular tamoxifen concentration is estimated to be several times higher than the serum concentration [37].

In this study, we present data showing that the antipsychotic drug CPZ enhances the cytotoxic effect of tamoxifen in antiestrogen-sensitive MCF-7 cells and tamoxifen-resistant cells derived from MCF-7. The basis for this conclusion is that 5 μmol/l tamoxifen alone seemed to induce classical mitochondrial apoptotic cell death as measured by cleavage of the caspase substrate PARP and very low necrotic or necrotic-like cell death. In contrast, treatment with 5 μmol/l tamoxifen in combination with 5 μmol/l CPZ resulted in nearly complete cell killing, which could not be abrogated by inhibitors of caspases or cathepsins, supporting a cytotoxic effect inducing necrosis or necrosis-like cell death.

The synergistic effect of CPZ and tamoxifen on inhibition of cell growth/viability could be prevented by addition of 10 nmol/l estradiol, indicating the involvement of the ERα. This led us to the hypothesis that CPZ might alter tamoxifen influx allowing more tamoxifen to enter the cells. The tamoxifen-resistant cells used in this study are not dependent on estrogen for growth. However, they express the ERα, which is able to bind both estradiol and tamoxifen [18]. Thus, the estrogen receptor may be involved in sustaining a high intracellular tamoxifen concentration leading to a cytotoxic cell death in both MCF-7 and TAM^R-1 cells.

It has been published earlier that expression of the MDR-1 gene is increased five-fold in the TAM^R-1 breast cancer cell line, compared with MCF-7 cells, indicating that P-glycoprotein efflux of tamoxifen could be part of the resistance mechanism [19]. Another hypothesis is, therefore, that CPZ interferes with P-glycoprotein-mediated efflux of tamoxifen in TAM^R-1 cells. However, this would not explain why MCF-7 cells are also killed by the combination therapy, and a putative effect of CPZ on P-glycoprotein is, therefore, not a likely explanation for the synergy between CPZ and tamoxifen.

Altered activity of the ErbB receptor family, and of hence the protein kinases Akt and ERK1/2, seem to play important roles in mediating cell growth and survival of the antiestrogen-resistant cell line [38]. It has been shown earlier that targeting specific growth-inducing signaling pathways [39,40] causes preferential inhibition of antiestrogen-resistant cells, but not of parental, antiestrogen-sensitive MCF-7 cells, indicating that the efficiency of these compounds in killing resistant breast cancer cells is related to specific alterations in the resistant cell line. In contrast, our results show that CPZ when used in combination with tamoxifen works equally well in killing tamoxifen-sensitive and tamoxifen-resistant MCF-7 cells, pointing to a mechanism unrelated to the tamoxifen resistance.

It has been shown before that a variety of the so-called chemosensitizers, compounds with the ability to reverse resistance, including CPZ, can alter membrane properties [24,26,41,42]. By in-vitro experiments, we confirmed that CPZ interacts strongly with the lipid membrane. On the basis of these experiments, we suggest that CPZ binds to the membrane in two subpopulations – one where the interaction is primarily with the acyl chains of the lipids and another were the charged state of the head group seems to be dominating for the binding. Agasøsler *et al.* [26] observed an effect similar to what we observed on monolayers of zwitterionic lipids, but found a much greater change in area when investigating monolayers of negatively charged lipids. In our case, we obtained a more than two-fold higher partition constant when incorporating 20% of DPPG by ITC. Furthermore, we showed that this effect results in increased membrane permeability measured by transfer of the fluorophore calcein. As calcein is a molecule of considerable size (i.e. 623 Da) not only compared with many drugs, such as tamoxifen, but also compared with different metabolites in the cell (e.g. cAMP, glucose, citrate, and ATP), it is plausible that CPZ can cause a change in the metabolic state of the cells. The increased permeability of the membrane vesicles can be because of either pore formation, local or global destabilization of the membrane, or a detergent-like effect of CPZ. In either way, CPZ causes destabilization of some kind of structure that allows molecules to pass the membrane. Hence, this suggests that CPZ might cause tamoxifen to accumulate in cells, and this could explain the efficiency of the

combined treatment of CPZ and tamoxifen in killing both tamoxifen-sensitive and tamoxifen-resistant breast cancer cells. Further investigations, however, are required to fully clarify the exact mechanism behind enhancing the cytotoxic effect of tamoxifen by CPZ.

The clinical prospect of CPZ enhancing the cytotoxic effect of tamoxifen is that CPZ could potentially be used in combination with tamoxifen in breast cancer patients for enhancing the effects of tamoxifen, and hopefully, the combination would also work in tamoxifen-resistant tumors, which still express the ERα.

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