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The antidepressants maprotiline and fluoxetine induce Type II autophagic cell death in drug-resistant Burkitt's lymphoma

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Resistance to chemotherapy is a major obstacle for the success of cancer therapy and is most commonly attributed to the inability of cancer cells to die by apoptosis, the archetypal programed cell death (PCD) response. The development of anticancer drugs that can overcome this resistance to apoptosis and induce other forms of cell death is therefore paramount for efficient cancer therapy. We report that the antidepressants maprotiline and fluoxetine induce autophagic PCD in the chemoresistant Burkitt's lymphoma (BL) cell line DG-75, which does not involve caspases, DNA fragmentation or PARP cleavage, but is associated with the development of cytoplasmic vacuoles, all consistent with an autophagic mode of PCD. Autophagic PCD was confirmed by transmission electron microscopy, upregulation of Beclin-I and the extent of PCD being reduced by the autophagic inhibitor 3-MA. In contrast, these compounds induced apoptotic PCD in the biopsy-like chemosensitive BL MUTU-I cell line. We provide evidence that the chemoresistant DG-75 cells do not express the proapoptotic Bcl-2 proteins Bax and Bak, show diminished levels of stored intracellular calcium and display shortened rod-like mitochondria, all of which are known to be associated with a defective "apoptotic" response in cancer cells. PCD in the two cell lines has different Ca^{2+} responses to maprotiline and fluoxetine, which may also account for their differential PCD responses. Our study, therefore, supports a new mechanistic role for maprotiline and fluoxetine as novel proautophagic agents in the treatment of resistant BL, and thus an alternative therapeutic application for these compounds.

When compared to the normal cells of the body, cancer cells survive and flourish based on their unique ability to obstruct "normal" cell death signaling. Current anticancer drug development is focused on reversing this obstruction, re-educating the cancer cell to die by a receptive programed cell death (PCD) pathway. Numerous pathways of PCD have been documented including apoptosis, autophagy, parapoptosis, mitotic catastrophe and senescence.[1] In the past, the efficacy of potential anticancer drugs was held synonymous with apoptosis with the majority of drugs relying on reactivating a receptive apoptotic pathway in the cancer cell for activity. However, there is increasing evidence that cancers with an aggressive phenotype have lost their accessible apoptotic pathways and so possess or develop an inherent resistance to standard therapy.[2,3] Resistance may already be present at the onset of therapy where patients initially fail to respond, or it may emerge over time during chemotherapy (acquired chemoresistance), even after a dramatic initial response. For this reason, there is great interest in investigating alternative ways by which aggressive cancer cells die.

Type II PCD or autophagic cell death is a caspase-independent form of cell death that has been observed in cells that cannot die by normal apoptotic mechanisms such as in cells deficient in Bax/Bak, in the absence of functional caspase-dependent pathways such as APAF-1 and other Bcl-2 proteins.[4] Type II PCD has been observed in cell lines from various tissues in response to a range of cancer therapies.[3] It can also be induced by starvation, pathogens, toxins as well as during development.[3,4] Type II PCD is morphologically distinct from apoptosis and is associated with the formation of large cytoplasmic autophagic vacuoles.

The development of drugs that induce autophagic cell death in cancer cells is becoming an increasingly popular alternative therapeutic approach. With a number of proautophagic drugs including mTOR inhibitors such as rapamycin (sirolimus) and its related derivatives temsirolimus (CCI-779), everolimus (RAD001) and AP23573 under evaluation

Key words: apoptosis, antidepressants, autophagic cell death, calcium, Bax/Bak

Abbreviations: 3-MA: 3-methyladenine; BAPTA-AM: 1,2-bis(2 aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); BFA1: bafilomycin A1; BL: Burkitt's lymphoma; BSA: bovine serum albumin; DAPI: 4′-6-diamidino-2-phenylindole; EBV: Epstein-Barr virus; ECL: electrochemiluminescence; EGTA: ethyleneglycotetraacetic acid; FACS: fluorescent-activated cell sorting; FBS: fetal bovine serum; FURA: 5-oxazolecarboxylic acid, 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acid; HIV: human immunodeficiency virus; NAC: *N*-acetylcysteine; PARP: poly(ADP-ribose) polymerase; PBS: phosphate-buffered saline; PCD: programed cell death; PI: propidium iodide; ROS: reactive oxygen species

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in clinical trials[3] either alone or in combination with other chemotherapeutic agents, these rapamycin analogs as well as arsenic trioxide and temozolomide bring great hope of combating apoptosis-resistant cancers.[3]

MYC-driven tumors, like Burkitt's lymphoma (BL), often display resistance to chemotherapeutics because of acquisition of mutations that impair the apoptotic pathway regulated by the Bcl-2 protein family.[5] BL accounts for 30–50% of lymphomas in children and remains a serious health problem in those areas where it is endemic: namely the malarial belts of equatorial Africa, Northeastern Brazil and Papua New Guinea.[6] In addition, because of its growing association with HIV infection, BL is becoming a more common malignancy in humans making up the largest group of HIV-associated non-Hodgkin lymphomas (35–50% of these neoplasms).[7] A variety of chemotherapeutic drugs are used in the treatment of BL tumors including DNA intercalating agents and topoisomerase inhibitors. Such drugs are usually successfully supplemented with the monoclonal antibody rituximib, which sensitizes B-cell lymphomas and leukemias to chemotherapy[8] allowing for survival rates of up to 60% in children affected with the disease. However, for older adults and HIV-infected patients, the long-term survival rates in response to chemotherapy are only 25%[9,10] with reoccurrence and resistance common. In addition, in parts of the developing world where BL is endemic, access to chemotherapeutic regimens is limited. Consequently, there is a need to develop more selective, potent, economical alternatives for the treatment of BL.

Antidepressants have been proposed to offer a novel therapeutic approach in the treatment of BL.[11] Antidepressants of the uptake inhibitor type were initially developed to treat depression by inhibiting serotonin reuptake, increasing synaptic concentrations of the monoamines noradrenaline, serotonin and dopamine. However, the recent discovery that some antidepressants act as proapoptotic agents in the treatment of cancer adds greatly to their diverse pharmacological application.[11] Reuptake inhibitor–type antidepressants have been shown to induce apoptosis in a range of malignant cell lines, including BL.[12–19] Such cell death has been shown to display many of the features of classical apoptosis including caspase-3 activation and cytochrome c release.[10,20] However, the mechanisms by which these antidepressants can induce apoptosis in BL are still unclear.

In our study, we provide evidence that the antidepressants maprotiline and fluoxetine induce Type II autophagic cell death in a resistant BL cell line. We propose that these agents have the potential to overcome the "apoptotic block" usually associated with chemoresistant cancers and encourage the use of these agents either alone or in combination with other therapies in the treatment of cancers that are resistant to radiotherapy and proapoptotic-related chemotherapy.

Material and Methods

Cell lines

DG-75 and MUTU-I (c179) Burkitt's lymphoma cell lines were gifts from Dr. Dermot Walls (School of Biotechnology, Dublin City University, Ireland) and Prof. Martin Rowe (Division of Cancer Studies, The University of Birmingham, UK). H929 and CEM cell lines were gifts from Prof. Mark Lawler (School of Medicine, Trinity College Dublin (TCD)) and Dr. Daniela Zisterer (School of Biochemistry and Immunology, TCD), respectively.

Cell culture

The DG-75 cell line is a B-lymphocyte, BL line derived from a metastatic pleural effusion (lung) of a sporadic case of BL.[21] The MUTU-I (c179) cell line is an isogenic stable group-I BL cell line derived from a BL-biopsy.[22] The H929 cell line is a multiple myeloma line derived from a malignant effusion in a patient with myeloma,[23] and the CEM-Neo is an acute peripheral blood T-lymphoblastic leukemia cell line.[24] The above cell lines were cultured in RPMI-1640 medium containing phenol red and supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine (2 mM) and penicillin and streptomycin (100 μg/ml). The MUTU-I c179 cell line required the additional supplements of alpha-thioglycerol (5 mM in PBS with 20 μM bathocuprione disulfonic acid), sodium pyruvate (100 mM) and HEPES (1 mM). Cells were maintained in 72-cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 95% oxygen and 5% carbon dioxide.

Detection of apoptotic bodies by PI FACS analysis

Propidium iodide (PI) flow cytometry analysis exploits the phenomenon that end-stage PCD produces fragmented pieces of DNA; such fragments bind to PI easily and give a characteristic fluorescent peak. Annexin V staining was avoided in our study as it has been previously shown to cross react the B-cell receptor.[25] A total of 750,000 cells were seeded in 5 ml of media and treated with the appropriate amount of compound and incubated for a specified time at 37°C in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. Cells were harvested by centrifugation, washed with 5 ml of ice-cold PBS and the pellet was resuspended in 200 μl of PBS and 2 ml of ice-cold 70% ethanol. The cells were left to fix overnight at 4°C. After fixation, the pellet was resuspended in 400 μl of PBS with 25 μl of RNAse A (10 mg/ml stock) and 75 μl of PI (1 mg/ml). Cells were incubated at 37°C in the dark for 30 min. Cell cycle analysis was performed using appropriate gates counting 10,000 cells and analyzed using CELLQUEST software package.

Detection of DNA fragmentation by agarose gel electrophoresis

A total of 1×10^7 cells were treated with the relevant compound for 48 hr, harvested by centrifugation at 500*g* for 5 min and the pellet was resuspended in 1 ml of lysis buffer (20 mM EDTA, 100 mM Tris pH 8.0 and 0.8% (w/v) sodium lauryl sarcosinate). Cells were incubated at 37°C for 1 hr before the addition of 0.5 mg/ml of RNAse A. After 2 hr at 37°C, 6 mg/ml of proteinase-K was added, and cells were

incubated overnight at 37°C. Aliquots of DNA (45 μl) were mixed with 5 μl of loading dye (0.25% bromophenol blue, 30% glycerol in Tris borate EDTA (TBE) and samples). Samples were loaded on a 1.5% agarose gel in TAE running buffer (40 mM Tris acetate and 1 mM EDTA) and electrophoresed at a constant voltage of 55 V for 4 hr. The DNA laddering pattern was visible under UV light using a UVP gel documentation system.

Western blot analysis

A total of 5×10^6 cells were harvested by centrifugation at 500*g* for 5 min, and the pellet was washed with ice-cold PBS. Cells were resuspended in 60 μl PBS and 60 μl lysis buffer (Laemmli buffer; 62.5 mM Tris-HCl, 2% w/v SDS, 10% glycerol, 0.1% w/v bromophenol Blue supplemented with protease inhibitors). The protein concentration of each sample was determined; samples were diluted in lysis buffer (with 5% DTT), heated to 95°C for 5 min, loaded and resolved using 10% SDS gel electrophoresis. Proteins were transferred onto the PVDF membrane using a wet transfer apparatus at 60 V (150 mA) for 90–120 min at RT. The membrane was soaked in blocking solution [5% (w/v) Marvel Milk in 0.1% TBS (10 mM Tris and 150 mM NaCl)] with 1% Tween (TBST) overnight at 4°C, washed twice in TBST for 3 min at a time and incubated for 1 hr at room temperature with the primary antibody of interest (in TBST containing 5% dry milk). The membrane was then washed again in 0.1% TBST before incubating in the appropriate associated IgG-HP secondary antibody for 1 hr at room temperature. Membranes were developed using electrochemiluminescence (ECL) detection, results recorded on Kodak X-Omat LS film and developed using an X-ray processor.

Transmission electron microscopy

A total of $10–20 \times 10^6$ cells treated with the indicated compound were harvested by centrifugation and fixed with 4% glutaraldehyde in media for 1 hr. Pellets were washed twice in 0.5 M phosphate. An equivalent volume of 2% warm agarose solution (2 g in H_2O) was added to each pellet and allowed to solidify at 4°C for 30 min. The solid pellet was removed and cut into small slices. Slices were suspended in 0.05 M phosphate buffer, and each sample was washed on a rotor for 10 min. A total of 2% osmium tetroxide (O_2O_4) solution (2% solution in 0.05 M potassium phosphate buffer) was added to each sample and left to fix for 45 min. O_2O_4 was aspirated off the samples and cells were dehydrated using an increasing alcohol series (30–95% aqueous ethanol solutions for 10-min intervals) before incubating overnight in 100% ethanol. Ethanol was removed and each pellet was subsequently washed twice (15 min at RT) in propylene oxide solution (100%). Pellets were embedded in a 50% resin solution for 2–3 hr at room temperature on a rotor. Removing the 50% solution, pellets were then embedded in 100% epoxy resin and left for 2–3 hr at room temperature before a final embedment in 100% fresh epoxy resin. Each pellet was subsequently transferred into a mold, and each sample was covered by fresh 100% epoxy resin. The mold was subsequently placed in a 50°C oven and degassed for 1 hr, before placing in a 60°C oven overnight. Ultrathin sections were cut on an ultramicrotome and collected on copper grids. Each grid was counterstained with uranyl acetate and lead citrate and stored for ultrastructural examination. Ultrastructural examination was carried out in a JOEL 1210 electron microscope. Images were taken with a 1,500–3,000× objective (2 μM scale bars). A number of images were obtained as a representative of each sample.

Morphological examination of mitochondria by confocal microscopy

Cells were seeded at 750,000 cells in 5 ml and treated with the appropriate drug for the desired time. After incubation with drug, cells were washed in medium before resuspending in fresh medium containing 100 nM Mitotracker Red CMX ROS (Invitrogen). After 15–30 min incubation, cells were pelleted and washed twice in prewarmed fresh media. Cells were then fixed in 4% paraformaldehyde (in PBS) at 37°C with gentle agitation. Cells were then rinsed three times in PBS before final suspension in PBS with 15 mM sodium azide. Cells were viewed using an Olympus FV1000 point scanning microscope. Images were taken using a 60× oil immersion lens with an NA (numerical aperture) of 1.42. The software used to collect images is FluoView Version 7.1 software. The sample was excited with a 405 nm laser diode and the 543 nm line of a Green Helium-Neon laser.

Measurements of cytosolic calcium levels using FURA-2

Cytosolic Ca^{2+} concentration was measured by a previously described method.[26] A total of 1.5×10^5 cells/well were seeded and left for 18–24 hr. Plates were centrifuged at 500*g* for 5 min, and the media were removed. A total of 200 μl of FURA-2 loading solution (4 μM FURA-2) was added to each well and left for 2–3 hr in the dark at RT. Cells were washed twice in prewarmed HBS-BSA [140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose and 20 mM HEPES containing 1 mg/ml bovine serum albumin (BSA)] and left to incubate for 30 min at 37°C. Plates were read on a GEMINI SpectraMAX plate reader at 340 and 380 nm emission at 495 nm. The $[Ca^{2+}]_i$ was calculated from the 340/380 nm fluorescence ratio using FL Winlab software according to Ref. 26.

Results

The relative apoptotic sensitivity of MUTU-I and DG-75 cell lines to various chemotherapeutic agents

Two BL cells lines were used in our study; a chemosensitive, Group I biopsy-derived cell line (MUTU-I c179) and a cell line derived from a metastatic pleural effusion thought to represent the sporadic, more resistant form of BL (DG-75).

When the two cell lines were exposed to a number of chemotherapeutic agents (etoposide, taxol, vincristine, 17AAG, combrestatin, tamoxifen and PBOX-6) and apoptosis measured by PI-FACS analysis, it was seen (Figs. 1*a* and 1*b*) that considerably more apoptosis occurred over a shorter time scale for MUTU-I cells (24 hr) compared to DG-75 cells (72 hr).

Maprotiline and fluoxetine have been previously shown by our laboratory to induce apoptosis in the MUTU-I cell line from 2 hr, reaching 50% apoptosis after 12 hr and ~60–70% at 24 hr.[18] In the DG-75 cell line, a delayed apoptotic effect for both maprotiline (30 hr) and fluoxetine (20 hr) was observed with maximum effects of 50–60% at 72 hr.[18] Maprotiline and fluoxetine failed to induce apoptosis in the MUTU-I cell line in the presence of the general caspase inhibitor zVAD-fmk (Fig. 1*c*) consistent with caspases having a role in this cell death. DNA from MUTU-I cells treated with maprotiline and fluoxetine had the characteristic DNA laddering effect of DNA fragmentation by endonucleases, and the effect was comparable to HL-60 cells treated with etoposide, which acted as the positive control for apoptotic DNA cleavage (Fig. 1*e*). Cleavage of poly(ADP-ribose) polymerase (PARP), a protein involved in DNA repair strongly activated by DNA strand breaks, was also shown to occur on treatment with these two compounds (Fig. 1*f*) again comparable in effect to that seen in HL-60 cells.[18]

Maprotiline and fluoxetine induce autophagy (Type II PCD) in the DG-75 cell line

DG-75 cells showed less sensitivity to apoptosis than MUTU-I cells. However, even at exposure times of 4–8 hr, DG-75 cells showed signs of cell death such as cell shrinkage, irregular plasma membranes, cell "ghosts" and intracellular vacuoles/vesicles (data not shown), and inhibition of proliferation was apparent.[18] DG-75 cells treated with either drug did not show DNA fragmentation (Fig. 1*e*), caspase dependence of death (Fig. 1*d*) nor PARP cleavage (Fig. 1*f*), thus confirming the absence of a classical apoptotic pathway for antidepressant-induced PCD in these cells. These results suggested that an alternative mechanism of PCD, possibly autophagy, was occurring in the DG-75 cell line in response to maprotiline and fluoxetine. It had also previously been reported that the DG-75 cell line can undergo an alternative form of cell death with a delayed apoptotic response in reaction to cytotoxic stimuli (betulinic acid).[27]

To characterize further the mode of cell death of DG-75 cells induced by maprotiline and fluoxetine, levels of Beclin I, a Bcl-2-interacting protein, were investigated. Beclin-I is a Class III phosphatidylinositol 3-kinase-interacting protein that has been shown to promote autophagy *in vitro*.[28] Western blot analysis revealed that DG-75 cells treated with maprotiline and fluoxetine show increased levels of Beclin-I over an extended time period consistent with autophagic cell death (Fig. 2*a*). The increase in Beclin 1 levels after 48 hr implies that autophagosome formation coincides with the PCD response to maprotiline and fluoxetine shown by PI FACS analysis to be most apparent between 50 and 72 hr exposure.

This result is in contrast to MUTU-I cells, where it was found that after 24 hr of treatment, levels of Beclin-I protein were reduced in response to maprotiline and fluoxetine treatment (Fig. 2*b*), consistent with maprotiline- and fluoxetine-inducing apoptosis in MUTU-I cells through the activation of caspases, which have been previously reported to cleave Beclin-I.[29] Indeed, in the presence of the general caspase inhibitor z VAD-fmk, the amount of cell death induced by maprotiline and fluoxetine in DG-75 cells was increased in agreement with previous observations that caspases cleave Beclin-I inactivating its autophagic inducing activity and thus overall cell death (Fig. 1*d*).[29]

Ultrastructural morphological examination of maprotiline- and fluoxetine-treated DG-75 cells revealed the presence of vacuole-like structures in the cytoplasm consistent with autophagic vesicles (Figs. 3*c*–3*h*) and intact nuclei with evidence of damaged mitochondria. Ultrastructural examination of MUTU-I cells treated with fluoxetine or maprotiline did not show any evidence of such autophagosomes (data not shown).

3-Methyladenine (3-MA) is an inhibitor of phosphatidylinositol 3-kinase previously shown to inhibit autophagy *in vitro*.[30] In the presence of 3-MA, the amount of cell death in DG-75 cells induced by maprotiline and fluoxetine after 72 hr was significantly reduced (~40% reduction) (Fig. 2*d*). In contrast, MUTU-I cells when preincubated with 3-MA before treatment with maprotiline and fluoxetine showed no effect on the extent of cell death (Fig. 2*c*).

Bafilomycin A1 (BFA1) is a vacuolar H^+-ATPase inhibitor thought to be involved in the fusion between the autophagosomes and the lysosome in the normal autophagic process.[31] It was found that in the presence of BFA1, there was no change in the amount of DG-75 cell death induced by maprotiline and fluoxetine after 72 hr (Fig. 2*d*). MUTU-I cells preincubated with BFA1, before treatment with maprotiline and fluoxetine also, showed no effect on the extent of cell death (Fig. 2*c*). These results imply that (*i*) late-endosome early lysosome formation is not involved in the mechanism of cell death or (*ii*) after 72 hr, BFA1 has no inhibitory effect and is required at 24, 48 and 72 hr to exert a consistent effect.

The above results are therefore consistent with maprotiline- and fluoxetine-inducing autophagy (Type II PCD) in the chemoresistant DG-75 BL cell line.

Absence of some Bcl-2 family members in DG-75 cells

Emerging evidence that the Bcl-2 proteins are involved in autophagy and mitochondrial morphology is of great importance to the understanding of PCD pathways. The DG-75 cell line has been reported to lack a number of apoptotic pathway controls and mediators like Bax[32] or APAF-I-related pathway.[33] The lack of such classical apoptotic pathways

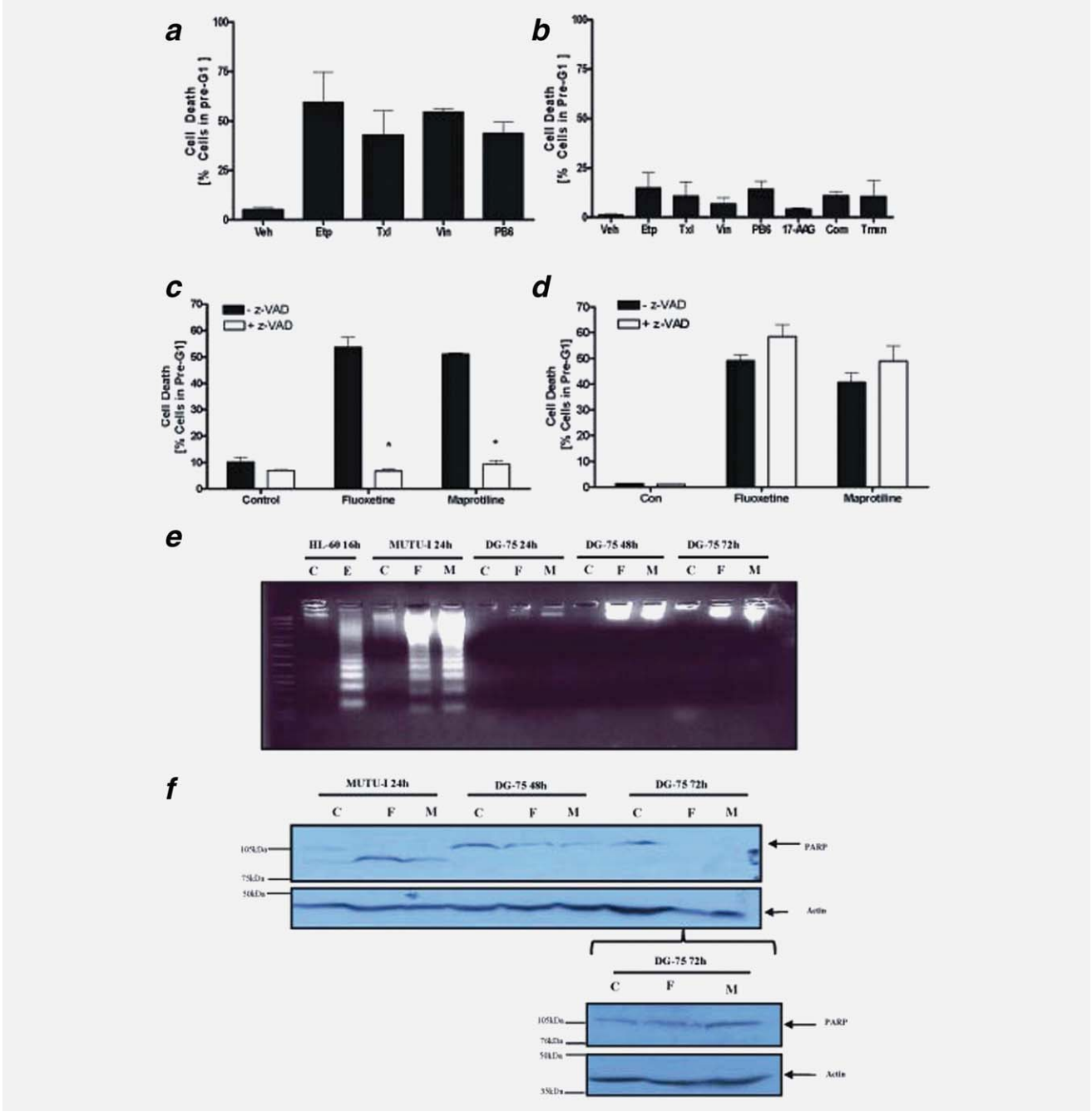

Figure 1. Differential programed cell death effects of fluoxetine and maprotiline on two Burkitt's lymphoma cell lines. (*a*, *b*) MUTU-I (*a*) and DG-75 (*b*) cells were seeded at a density of 7×10^5 cells/5 ml and treated with etoposide (Etp; 10 μM) taxol (Txl; 10 μM), vinblastine (Vin; 10 μM), 17AAG (10 μM), combrestatin (Com; 10 μM), tamoxifen (Tmxn; 10 μM) and PBOX-6 (PB6; 10 μM) for 24 (a) or 72 hr (*b*). (*c*, *d*) A total of 7×10^5 MUTU-I (*c*) and DG-75 (*d*) cells were pretreated for 1 hr with the general caspase inhibitor z-VAD-fmk (150 μM) followed by treatment with fluoxetine (50 μM) or maprotiline (50 μM) for 24 (*c*) or 72 hr (*d*). Cells were harvested by centrifugation and fixed overnight in 70% ethanol. FACS analysis was carried out upon incubation with propidium iodide and RNase A. A total of 10,000 cells were counted using appropriate gates. Values represent the mean + SEM of three independent experiments. $*p < 0.05$ [Based on a one-way ANOVA test followed by the Bonferroni multiple comparison test, comparing all columns]. (*e*) HL-60, MUTU-I and DG-75 cells were treated with etoposide (E) (20 μM), fluoxetine (F) or (50 μM) maprotiline (M) (50 μM) for 24, 48 and 72 hr. Whole cell lysates were isolated by centrifugation, lysed, DNA resolved on an agarose gel and visualized using UV light. Results are representative of three separate experiments. (*f*) MUTU-I and DG-75 cells were treated with maprotiline (M) (50 μM) or fluoxetine (F) (50 μM) for 24, 48 and 72 hr. Whole cell lysates were prepared, protein resolved by SDS-PAGE and probed with PARP-antibody. Blots were stripped and reprobed with β-actin as a loading control. Results are representative of three separate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

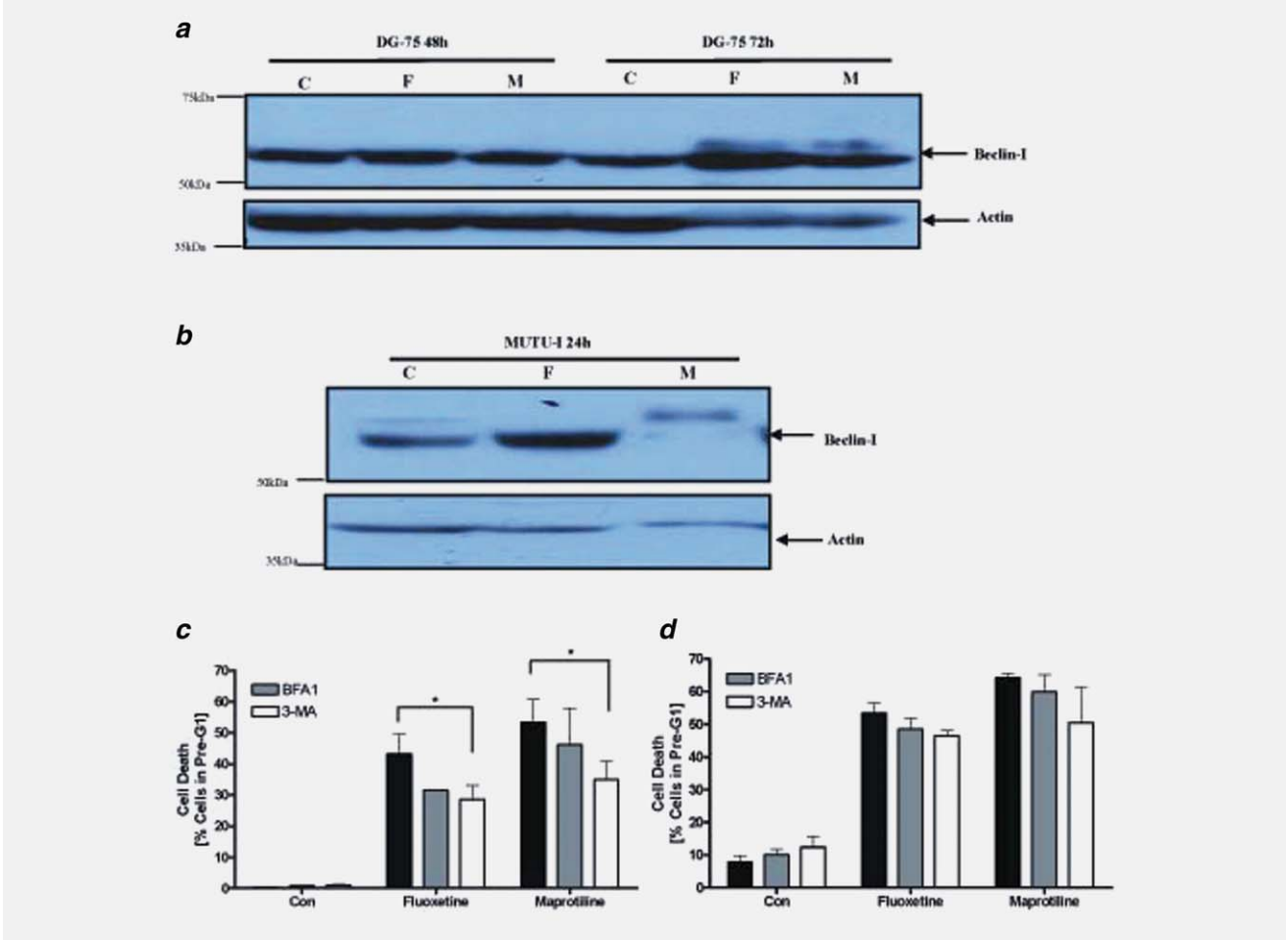

Figure 2. Maprotiline and fluoxetine induce autophagic-Type II programed cell death in a chemoresistant BL cell line. (*a*, *b*) Whole cell lysates were prepared from subconfluent DG-75 9 (*a*) and MUTU-I (*b*) cell lines treated with maprotiline (M) (50 μM) and fluoxetine (F) (50 μM) for 24 (*b*), 48 (*a*) and 72 hr (*a*). Protein was resolved by SDS-PAGE and probed with anti-Beclin-I antibody. Blots were stripped and reprobed with β-actin as a loading control. Results are representative of three separate experiments. (*c*, *d*) DG-75 and MUTU-I cells were pretreated with 3-MA (1 mM) or 3-BFA1 (10 μM) followed by treatment with fluoxetine (50 μM) or maprotiline (50 μM) for 24 or 72 hr. Cells were harvested by centrifugation and fixed overnight in 70% ethanol. FACS analysis was carried out upon incubation with propidium iodide and RNase A. A total of 10,000 cells were counted using appropriate gates. Values represent the mean + SEM of three independent experiments. $*p < 0.05$ [Based on a one-way ANOVA test followed by the Bonferroni multiple comparison test, comparing all columns]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

could be responsible for the unique response of DG-75 cells to maprotiline and fluoxetine.

Western blot analysis revealed that Bax, Bak and Bcl-2 are not expressed in DG-75 cells compared to MUTU-I cells, which express the 21-kDa Bax and 23-kDa Bak proteins but not Bcl-2 (Figs. 4*a*–4*c*).

DG-75 cells have punctate rod-like mitochondria compared to MUTU-I cells, which have filamentous networks of mitochondria

Mitochondrial morphology is cell type–dependent and reliant on the metabolic demands of a cell, where mitochondria can be found as filamentous or thread-like structures (long form), forming an integrated network or as small grain or tubular-like (short form) structures. The long and short forms of mitochondria are in dynamic equilibrium controlled by the balance between the two processes of fusion and fission. The fission/fusion process appears to function in the repair of defective mitochondria, proper segregation of mitochondria into daughter cells during cell division as well as the efficiency of oxidative phosphorylation and intramitochondrial calcium signaling.[34] It is not clear how these processes occur or are controlled in the cell; however, it is known that during apoptosis mitochondrial networks become fragmented into smaller more numerous organelles in a caspase-independent manner, and inhibition of the mitochondrial fission machinery also inhibits cell death.[35] The process of mitochondrial fission is thought to be important in the programed demise

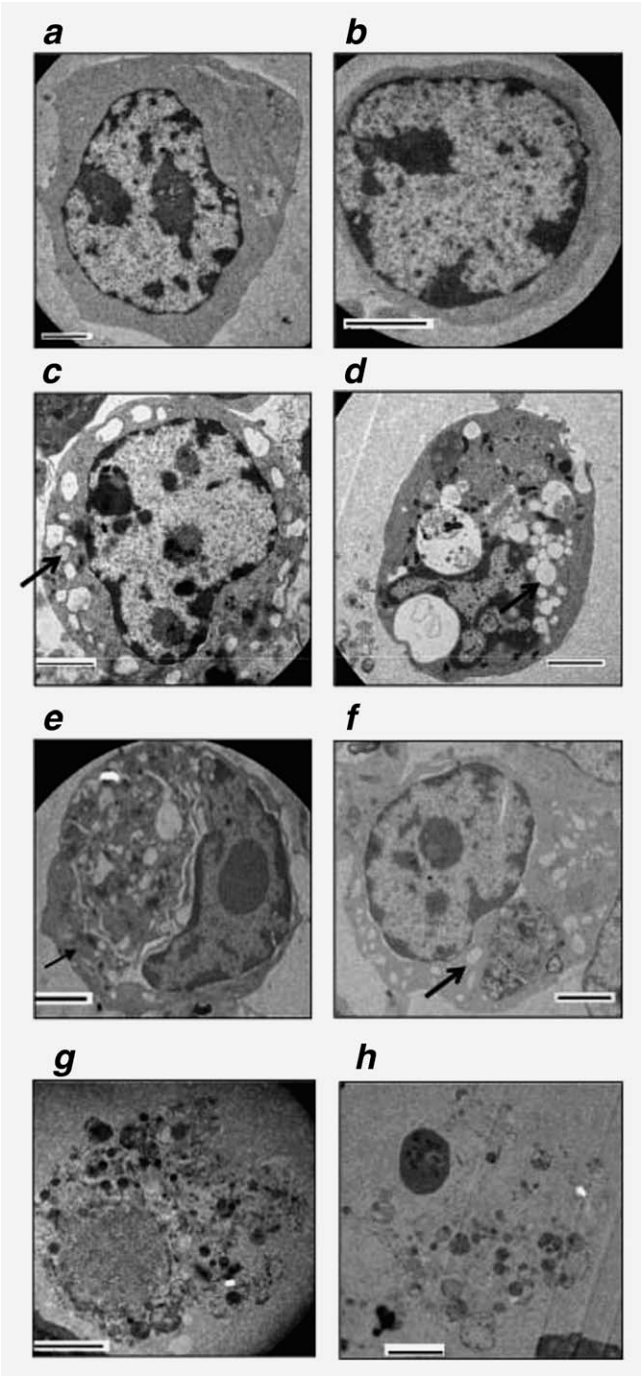

Figure 3. Identification of cytoplasmic vacuoles in fluoxetine- and maprotiline-treated DG-75 cells by transmission electron microscopy. Ultrastructural transmission images of DG-75 cells untreated (*a*, *b*) or treated with fluoxetine (50 μM) (*c*, *e*, *g*) or maprotiline (50 μM) (*d*, *f*, *h*) for 72 hr. Cells were fixed, dehydrated, embedded in epoxy resin, ultra-sliced, mounted on a copper grid and stained with uranyl acetate and lead citrate before ultrastructural examination on a JEM-2100 transmission electron microscope. Images were taken with a ×1,500–3,000 objective (2 μM scale bars). Arrows indicate the presence of cytoplasmic vacuoles and cell organelles surrounded by membrane-enclosed autophagosomes.

of the cells occurring early in the cell death pathway, around the time that Bax translocates from the cytosol to the mitochondria but before caspase activation.

Confocal microscopy was carried out using the mitochondria-specific dye Mitotracker Red CMX ROS with cell nuclei being stained with 4′-6-diamidino-2-phenylindole (DAPI). After such 3D imaging, it was seen that the DG-75 cell line contains constitutively punctate rod-like mitochondria (Figs. 5*b* and 5*d*) constant with an overactive fission process. It was also observed that the distribution of such rod-like mitochondria within in the cell was localized or polarized to one side of the cell. In contrast, MUTU-I cells were found to contain a filamentous network of mitochondria, which were distributed equally throughout the cell (Figs. 5*a* and 5*c*).

Reactive oxygen species are not involved in fluoxetine- and maprotiline-mediated cell death in BL

Reactive oxygen species (ROS) have previously been implicated in the carcinogenesis of BL.[36] To determine if the production of ROS is involved in fluoxetine- and maprotiline-mediated cell death in BL, cells were preincubated with *N*-acetylcysteine (NAC, 5 mM) before treatment with fluoxetine or maprotiline. Analysis by PI FACS revealed that the extent of maprotiline- and fluoxetine-mediated cell death did not change significantly in both the biopsy-like and chemoresistant BL cell lines (Figs. 5*e* and 5*f*) in the presence of NAC consistent with ROS having no role in both process.

Different dependency on calcium changes of cells for PCD responses

Ca^{2+} signaling is important in the activation and execution of cell death[37] where increases in $[Ca^{2+}]_i$ have been observed during apoptosis and in some cases have been necessary for apoptosis to take place.[38] Autophagy appears to depend on intracellular calcium stores rather than an increase in cytosolic calcium *per se*.[39]

The majority of antidepressants have been shown to increase cytoplasmic calcium levels. In particular, maprotiline increases $[Ca^{2+}]_i$ in prostate cancer cells[40] and has inhibitory effects on the GABA-, NMDA- and high-K^+-induced $[Ca^{2+}]_i$ increases.[41] Fluoxetine has been shown to inhibit voltage-gated Ca^{2+} channels,[42–44] calcium-activated potassium channels[45] and has been shown to inhibit the ATP-induced $[Ca^{2+}]_i$ increase in PC-12 cells by inhibiting both extracellular Ca^{2+} influx and Ca^{2+} release from intracellular stores.[46] As the DG-75 and MUTU-I cell lines have different cell death pathway responses to maprotiline and fluoxetine and these agents have been associated with calcium signaling, the role of calcium in DG-75 and MUTU-I cell death was investigated.

To evaluate if calcium influx and/or release was involved in fluoxetine- and maprotiline-mediated cell death of MUTU-I cells and DG-75 cells, cells were incubated with the extracellular calcium chelator, EGTA (1 mM) and with the

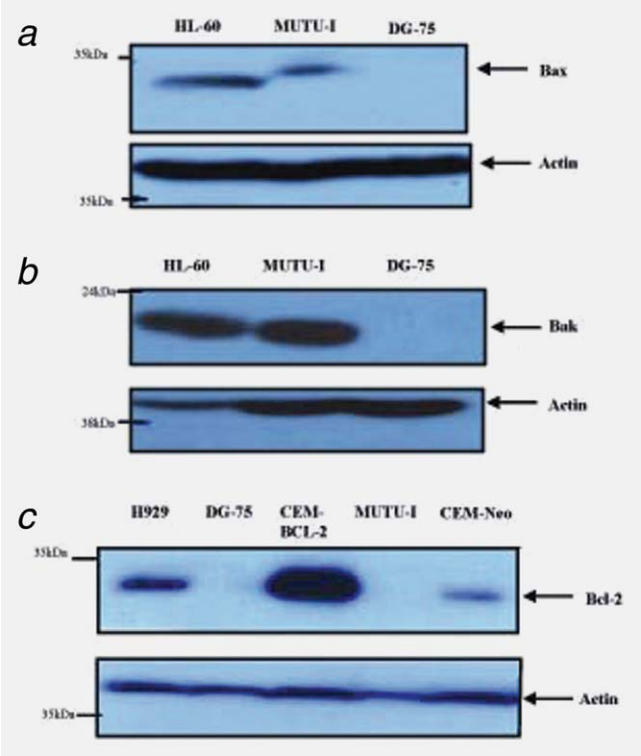

Figure 4. The chemosensitive cell line expresses the proapoptotic Bcl-2 proteins, Bax and Bak, whereas the chemoresistant BL cell line does not. (*a–c*) Whole cell lysates were prepared from subconfluent HL-60, DG-75, MUTU-I, H929, CEM-Neo and CEM-Bcl-2 (cells overexpressing BCL-2) cell lines. Protein was resolved by SDS-PAGE and probed with anti-Bax (*a*), anti-Bak (*b*) or anti-Bcl-2 (*c*) antibodies. Blots were stripped and reprobed with β-actin as a loading control. Results are representative of three separate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

intracellular calcium chelator, BAPTA-AM (25 μM) before the addition of fluoxetine or maprotiline.

Exposure of DG-75 cells, for 72 hr (to coincide with maximal cell death activity), showed that in the presence of EGTA, fluoxetine or maprotiline was unable to induce PCD (Fig. 6*b*) consistent with extracellular calcium influx being important for PCD of these chemoresistant cells. In the presence of BAPTA-AM, maprotiline and fluoxetine had a more potent effect on the DG-75 cells, killing ~70% of these cells.

In contrast, exposure of MUTU-I cells, for 24 hr incubation (to coincide with maximal cell death activity), showed that in the presence of BAPTA-AM neither fluoxetine nor maprotiline was able to induce PCD in the MUTU-I cell line (Fig. 6*a*) consistent with intracellular calcium release being important in antidepressant-mediated cell death. EGTA had no effect on fluoxetine- and maprotiline-mediated cell death of MUTU-I cells consistent with Ca^{2+} influx having no role.

To determine if maprotiline and fluoxetine treatment of DG-75 or MUTU-I cells resulted in a release of internal calcium, cells were loaded with the fluorescent calcium indicator FURA-2. From these experiments, it was found that maprotiline and fluoxetine increased $[Ca^{2+}]_i$ levels in MUTU-I cells (Fig. 6*c*) but not in DG-75 cells (Fig. 6*d*).

These results are consistent with intracellular release rather than influx of calcium being important for apoptosis in MUTU-I cells, whereas for DG-75 cells calcium influx is important for autophagic responses, although the dependence on intracellular release of calcium seems complex.

Discussion

Our study reports for the first time that the antidepressants maprotiline and fluoxetine induce Type II autophagic cell death in a chemoresistant BL cell line, DG-75. Several lines of evidence support this autophagic cell death pathway including Beclin-I upregulation (Fig. 2*a*), development of excessive cytoplasmic vacuoles and the amount of cell death inhibited by the autophagic inhibitor 3-MA (Fig. 3). The cell death was found to be cell type specific as maprotiline and fluoxetine induced classical apoptosis in a biopsy-like BL cell line (MUTU-I) consistent with previous reports.[10,15,16,20,47,48]

Type II autophagic cell death in DG-75 cells required extracellular calcium influx and was insensitive to thapsigargin, a known stimulant of ER calcium release, consistent with the calcium dependence of autophagic cell death reported for other cells.[39,49] Conversely, antidepressant-induced apoptosis in the MUTU-I cell line did not require extracellular calcium influx but did require increased intracellular cytoplasmic calcium release, consistent with previous reports that increased $[Ca^{2+}]_i$ is essential for apoptosis.[38]

In both cases, it appears that calcium plays an important role, albeit a complex one in fluoxetine- and maprotiline-induced cell death of BL. There have been previous reports where fluoxetine and maprotiline have been shown to trigger calcium fluxes in cancer cells *in vitro*.[10,40] To elucidate the precise role of calcium in each PCD response will require further extensive investigations.

Calcium released into the cytoplasm from intracellular stores is thought to be taken up by the mitochondria who are in turn responsible for eliciting a range of prosurvival or prodeath signals, depending on the nature of the calcium signal itself.[38] Uptake of calcium into a responsive mitochondrial network like that of the "normal" filamentous mitochondrial network observed in the MUTU-I cell line (Figs. 5*a* and 5*c*) is thought to sensitize the mitochondria, resulting in the dimerization of the proapoptotic Bcl-2 proteins, Bax and Bak, on the mitochondrial outer membrane, inducing cytochrome c release and initiating the classical apoptotic caspase-cascade.

In our study, MUTU-I cells are shown to express Bax and Bak, display filamentous mitochondria and die by "classical" apoptosis in response to fluoxetine or maprotiline. In

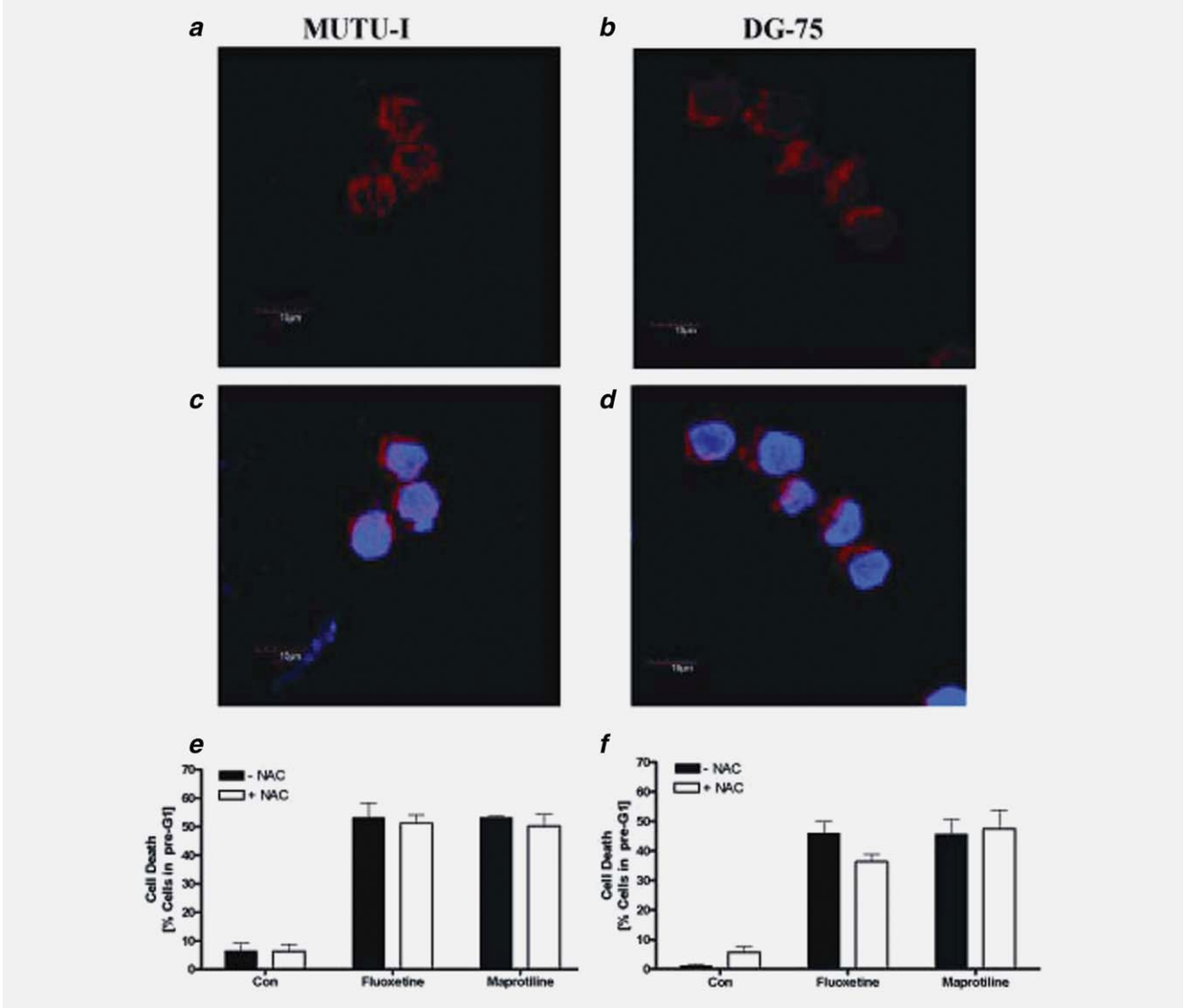

Figure 5. Mitochondrial morphology in chemoresistant cells is different to the mitochondrial morphology found in chemosensitive cells, and ROS are not involved in fluoxetine-or maprotiline-induced cell death. (*a–d*) MUTU-I (*a*, *c*) and DG-75 (*b*, *d*) cells were incubated with Mitotracker Red CMX ROS (100 nM), fixed and mounted in 0.2 μg/ml DAPI onto poly-L-lysine-coated slides. Images were taken using a ×60 oil immersion lens with an NA of 1.42 using an Olympus FV1000 point scanning microscope (excited with a 405 nm laser diode and the 543 nm line of a Green Helium-Neon laser). Images represent cells stained with Mitotracker (*a*, *b*) and DAPI (*c*, *d*). The software used to collect images was FluoView Version 7.1 software. (*e*, *f*) MUTU-I (*e*) and DG-75 (*f*) cells were pretreated with NAC (5 mM) followed by treatment with fluoxetine (50 μM) or maprotiline (50 μM) for 24 (*e*) or 72 hr (*f*). Cells were harvested by centrifugation and fixed overnight in 70% ethanol. FACS analysis was carried out upon incubation with propidium iodide and RNase A. A total of 10,000 cells were counted using appropriate gates. Values represent the mean + SEM of three independent experiments. $*p < 0.05$ [Based on a one-way ANOVA test followed by the Bonferroni multiple comparison test, comparing all columns using GRAPHPAD Prism4 software]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

contrast, the chemoresistant DG-75 cells do not express Bax or Bak display a more fragmented "abnormal" mitochondrial network and die by an autophagic (Type II) mode of PCD. This is consistent with previous reports showing that Bax and Bak are essential to mitochondrial morphogenesis in normal healthy cells by promoting mitochondrial fission,[35] and that cells deficient in Bax and Bak display constitutive defects in mitochondrial morphology (mitochondria that are shorter than normal).[35]

We propose that the lack of Bax and Bak in the DG-75 cell line leaves DG-75 cells with short rod-like mitochondria that are unable to elicit the release of specific proapoptotic proteins from the mitochondria and thus undergo PCD by an alternative Type II autophagic

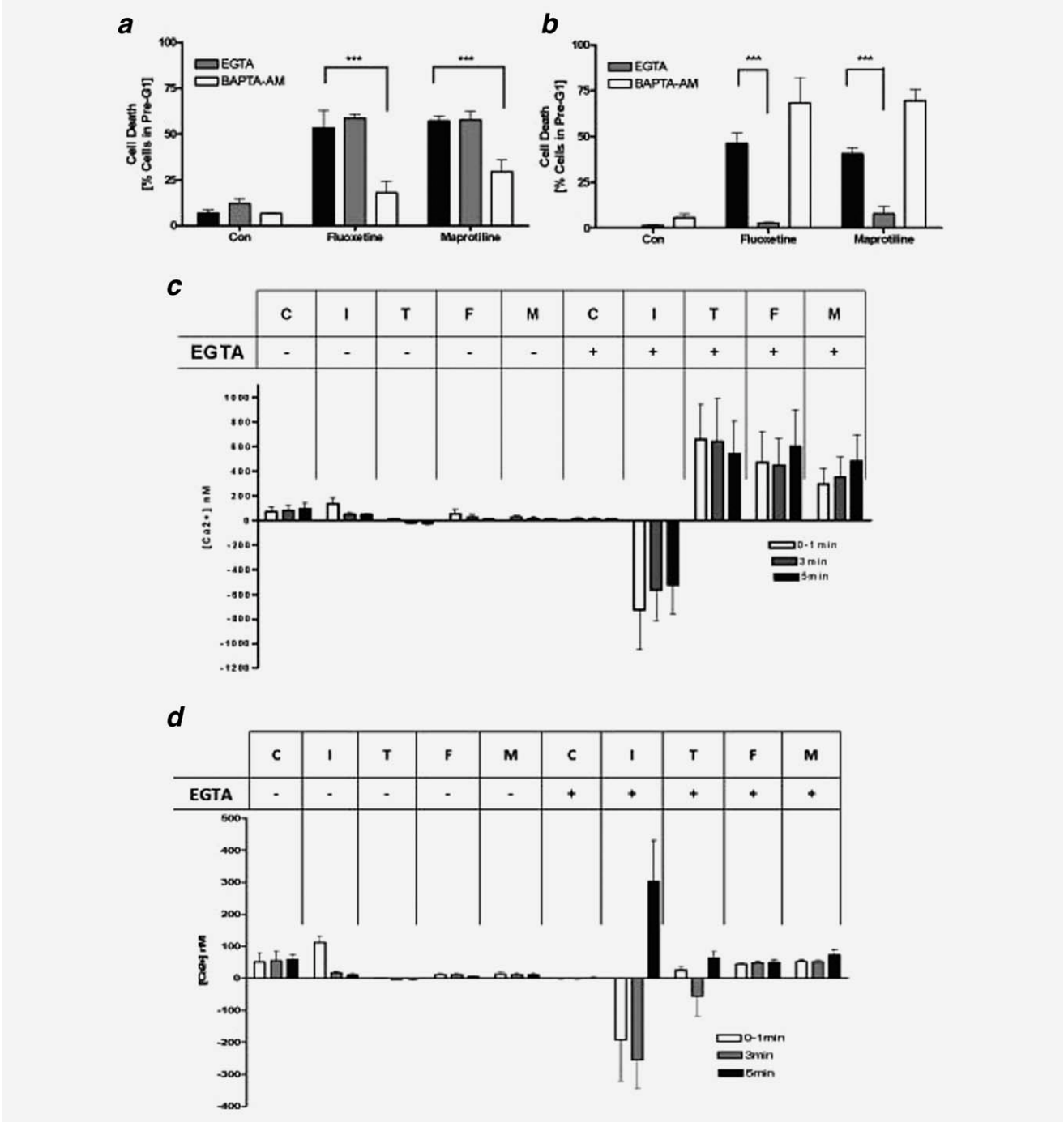

Figure 6. The differential role of calcium in fluoxetine- and maprotiline-induced programed cell death. (*a*, *b*) MUTU-I (*a*) and DG-75 (*b*) cells were pretreated for 1 hr with EGTA (1 mM) or BAPTA-AM (25 μM) followed by treatment with fluoxetine (50 μM) or maprotiline (50 μM) for 24 (*a*) or 72 hr (*b*). Cells were harvested by centrifugation and fixed overnight in 70% ethanol. FACS analysis was carried out upon incubation with propidium iodide and RNase A. A total of 10,000 cells were counted using appropriate gates. Values represent the mean + SEM of three independent experiments. ***$p < 0.001$ [Based on a one-way ANOVA test followed by the Bonferroni multiple comparison test, comparing all columns using GRAPHPAD Prism4 software]. (*c*, *d*) A total of 1.5×10^5 MUTU-I (*c*) and DG-75 (*d*) cells/well were loaded with FURA-2 for 2–3 hr. Cells were washed in HBS-BSA and left to incubate for 30 min at 37°C. Plates were read at 0 min before the addition of EGTA (3 mM), $CaCl_2$ (1 mM), ionomycin (I) (1 μM), thapsigargin (T) (1 μM) fluoxetine (F) (50 μM), maprotiline (M) (50 μM) and then read again at 1, 3 and 5 min on a GEMINI SpectraMAX plate reader at 340 and 380 nm excitation and emission at 510 nM. The $[Ca^{2+}]i$ was calculated from the 340/380 nM fluorescence ratio calibrated using standard techniques. Values represent the mean + SEM of two independent experiments with each value in triplicate.

mechanism.[50] These results are also consistent with the resistance of DG-75 cells to the ER calcium-releasing effects of thapsigargin (Fig. 6*d*) as Bax/Bak-deficient cells have also been shown to display reduced resting ER calcium resulting in a decreased uptake of Ca^{2+} by mitochondria from the ER.[49]

In summary, in response to fluoxetine and maprotiline, autophagy is initiated in DG-75 cells overcoming their apoptotic block resulting in Type II or autophagic PCD response. As autophagy represents an alternative mechanism to overcome at least partly the dramatic resistance of many cancers to radiotherapy and proapoptotic-related chemotherapy, the work presented herein provides evidence for the potential application of the antidepressants fluoxetine and maprotiline for use as proautophagic agents either alone or in combination with other agents in the treatment of resistant forms of BL, such as those cases of BL associated with patients suffering from HIV infection and other forms of immunosuppression as well as for cases of relapsed or refractory BL. This work also builds a useful model for studying the crosstalk between apoptosis and autophagy, investigating the role of the Bcl-2 proteins and calcium in these systems and helping to delineate and eventually exploit their respective regulatory controls.

As fluoxetine and maprotiline can easily reach *in vivo*, the *in vitro* concentrations used in our study[18,51] are already currently in use for another purpose and hence have a plethora of information regarding their toxicities and pharmacological actions, the time-consuming bench-to-bedside transition could be reduced allowing for the use of these agents in the treatment of BL, especially in those areas where an oral, economical alternative to invasive, expensive chemotherapy is greatly needed.

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References

1. Dimri GP. What has senescence got to do with cancer? *Cancer Cell* 2005;7:505–12.
2. Okada H, Mak TW. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* 2004;4:592–603.
3. Lefranc F, Facchini V, Kiss R. Proautophagic drugs: a novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. *Oncologist* 2007;12:1395–403.
4. Degterev A, Yuan J. Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* 2008;9:378–90.
5. Mason KD, Vandenberg CJ, Scott CL, Wei AH, Cory S, Huang DC, Roberts AW. In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *Proc Natl Acad Sci USA* 2008; 105:17961–6.
6. Serafeim A, Grafton G, Chamba A, Gregory CD, Blakely RD, Bowery NG, Barnes NM, Gordon J. 5-Hydroxytryptamine drives apoptosis in biopsylike Burkitt lymphoma cells: reversal by selective serotonin reuptake inhibitors. *Blood* 2002;99:2545–53.
7. Grogg KL, Miller RF, Dogan A. HIV infection and lymphoma. *J Clin Pathol* 2007;60:1365–72.
8. Spreafico F, Massimino M, Luksch R, Casanova M, Cefalo GS, Collini P, Ferrari A, Polastri D, Terenziani M, Gasparini M, Fossati-Bellani F. Intensive, very short-term chemotherapy for advanced Burkitt's lymphoma in children. *J Clin Oncol* 2002; 20:2783–8.
9. Evens AM, Gordon LI. Burkitt's and Burkitt-like lymphoma. *Curr Treat Options Oncol* 2002;3:291–305.
10. Serafeim A, Holder MJ, Grafton G, Chamba A, Drayson MT, Luong QT, Bunce CM, Gregory CD, Barnes NM, Gordon J. Selective serotonin reuptake inhibitors directly signal for apoptosis in biopsy-like Burkitt lymphoma cells. *Blood* 2003;101:3212–19.
11. Meredith EJ, Holder MJ, Chamba A, Challa A, Drake Lee A, Bunce CM, Drayson MT, Pilkington G, Blakely RD, Dyer MJS, Barnes NM, Gordon J. The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential antitumor target for psychotropics. *FASEB J* 2005;19:1187–9.
12. Arimochi H, Morita K. Characterization of cytotoxic actions of tricyclic antidepressants on human HT29 colon carcinoma cells. *Eur J Pharmacol* 2006;541: 17–23.
13. Xia Z, Bergstrand A, DePierre JW, Nassberger L. The antidepressants imipramine, clomipramine, and citalopram induce apoptosis in human acute myeloid leukemia HL-60 cells via caspase-3 activation. *J Biochem Mol Toxicol* 1999;13: 338–47.
14. Stepulak A, Rzeski W, Sifringer M, Brocke K, Gratopp A, Kupisz K, Turski L, Ikonomidou C. Fluoxetine inhibits the extracellular signal regulated kinase pathway and suppresses growth of cancer cells. *Cancer Biol Ther* 2008;7:1685–93.
15. Karlsson H, Gu Y, DePierre J, Nassberger L. Induction of apoptosis in proliferating lymphocytes by tricyclic antidepressants. *Apoptosis* 1998;3:255–60.
16. Spanova A, Kovaru H, Lisa V, Lukasova E, Rittich B. Estimation of apoptosis in C6 glioma cells treated with antidepressants. *Physiol Res* 1997;46:161–4.
17. Abdul M, Logothetis CJ, Hoosein NM. Growth-inhibitory effects of serotonin uptake inhibitors on human prostate carcinoma cell lines. *J Urol* 1995;154: 247–50.
18. Cloonan S, Drozgowska A, Fayne D, Williams DC. The antidepressants maprotiline and fluoxetine have potent selective antiproliferative effects against Burkitt's lymphoma independently of the norepenephrine and serotonin transporters. *Leuk Lymphoma* 2010;51:523–39.
19. Reddy KK, Lefkove B, Chen LB, Govindarajan B, Carracedo A, Velasco G, Carrillo CO, Bhandarkar SS, Owens MJ, Mechta-Grigoriou F, Arbiser JL. The antidepressant sertraline downregulates Akt and has activity against melanoma cells. *Pigment Cell Melanoma Res* 2008;21: 451–6.
20. Levkovitz Y, Gil-Ad I, Zeldich E, Dayag M, Weizman A. Differential induction of apoptosis by antidepressants in glioma and neuroblastoma cell lines: evidence for p-c-Jun, cytochrome c, and caspase-3 involvement. *J Mol Neurosci* 2005;27: 29–42.
21. Ben-Bassat H, Goldblum N, Mitrani S, Goldblum T, Yoffey JM, Cohen MM, Bentwich Z, Ramot B, Klein E, Klein G. Establishment in continuous culture of a new type of lymphocyte from a "Burkitt like" malignant lymphoma (line D.G.-75). *Int J Cancer* 1977;19:27–33.
22. Gregory CD, Rowe M, Rickinson AB. Different Epstein-Barr virus-B cell interactions in phenotypically distinct

clones of a Burkitt's lymphoma cell line. *J Gen Virol* 1990;71(Part 7):1481–95.
23. Gazdar AF, Oie HK, Kirsch IR, Hollis GF. Establishment and characterization of a human plasma cell myeloma culture having a rearranged cellular myc proto-oncogene. *Blood* 1986;67:1542–9.
24. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA, McCarthy RE. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* 1965;18:522–9.
25. Holder MJ, Barnes NM, Gregory CD, Gordon J. Lymphoma cells protected from apoptosis by dysregulated bcl-2 continue to bind annexin V in response to B-cell receptor engagement: a cautionary tale. *Leuk Res* 2006;30:77–80.
26. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–50.
27. Karpova MB, Sanmun D, Henter JI, Smirnov AF, Fadeel B. Betulinic acid, a natural cytotoxic agent, fails to trigger apoptosis in human Burkitt's lymphoma-derived B-cell lines. *Int J Cancer* 2006;118: 246–52.
28. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 1999; 402:672–6.
29. Cho DH, Jo YK, Hwang JJ, Lee YM, Roh SA, Kim JC. Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. *Cancer Lett* 2009; 274:95–100.
30. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci USA* 1982;79:1889–92.
31. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line H-4-II-E cells. *Cell Struct Funct* 1998;23:33–42.
32. Gutierrez MI, Cherney B, Hussain A, Mostowski H, Tosato G, Magrath I, Bhatia K. Bax is frequently compromised in Burkitt's lymphomas with irreversible resistance to Fas-induced apoptosis. *Cancer Res* 1999;59:696–703.
33. Sun Y, Orrenius S, Pervaiz S, Fadeel B. Plasma membrane sequestration of apoptotic protease-activating factor-1 in human B-lymphoma cells: a novel mechanism of chemoresistance. *Blood* 2005;105:4070–77.
34. Chan DC. Mitochondria: dynamic organelles in disease, aging, and development. *Cell* 2006;125:1241–52.
35. Sheridan C, Delivani P, Cullen SP, Martin SJ. Bax- or Bak-induced mitochondrial fission can be uncoupled from cytochrome C release. *Mol Cell* 2008;31:570–85.
36. Cerimele F, Battle T, Lynch R, Frank DA, Murad E, Cohen C, Macaron N, Sixbey J, Smith K, Watnick RS, Eliopoulos A, Shehata B, et al. Reactive oxygen signaling and MAPK activation distinguish Epstein-Barr Virus (EBV)-positive versus EBV-negative Burkitt's lymphoma. *Proc Natl Acad Sci USA* 2005;102:175–9.
37. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* 2003;4:552–65.
38. Roderick HL, Cook SJ. Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival. *Nat Rev Cancer* 2008;8:361–75.
39. Gordon PB, Holen I, Fosse M, Rotnes JS, Seglen PO. Dependence of hepatocytic autophagy on intracellularly sequestered calcium. *J Biol Chem* 1993;268: 26107–12.
40. Hsu SS, Chen WC, Lo YK, Cheng JS, Yeh JH, Cheng HH, Chen JS, Chang HT, Jiann BP, Huang JK, Jan CR. Effect of the antidepressant maprotiline on Ca^{2+} movement and proliferation in human prostate cancer cells. *Clin Exp Pharmacol Physiol* 2004;31:444–9.
41. Takebayashi M, Kagaya A, Inagaki M, Kozuru T, Jitsuiki H, Kurata K, Okamoto Y, Yamawaki S. Effects of antidepressants on gamma-aminobutyric acid- and N-methyl-D-aspartate-induced intracellular $Ca(^{2+})$ concentration increases in primary cultured rat cortical neurons. *Neuropsychobiology* 2000;42:120–6.
42. Hahn SJ, Choi J-S, Rhie D-J, Oh C-S, Jo Y-H, Kim M-S. Inhibition by fluoxetine of voltage-activated ion channels in rat PC12 cells. *Eur J Pharmacol* 1999;367: 113–18.
43. Deak F, Lasztoczi B, Pacher P, Petheo GL, Valeria K, Spat A. Inhibition of voltage-gated calcium channels by fluoxetine in rat hippocampal pyramidal cells. *Neuropharmacology* 2000;39:1029–36.
44. Wang SJ, Su CF, Kuo YH. Fluoxetine depresses glutamate exocytosis in the rat cerebrocortical nerve terminals (synaptosomes) via inhibition of P/Q-type Ca^{2+} channels. *Synapse* 2003;48:170–7.
45. Terstappen GC, Pellacani A, Aldegheri L, Graziani F, Carignani C, Pula G, Virginio C. The antidepressant fluoxetine blocks the human small conductance calcium-activated potassium channels SK1, SK2 and SK3. *Neurosci Lett* 2003;346:85–8.
46. Kim HJ, Choi JS, Lee YM, Shim EY, Hong SH, Kim MJ, Min DS, Rhie DJ, Kim MS, Jo YH, Hahn SJ, Yoon SH. Fluoxetine inhibits ATP-induced $[Ca(^{2+})](i)$ increase in PC12 cells by inhibiting both extracellular $Ca(^{2+})$ influx and $Ca(^{2+})$ release from intracellular stores. *Neuropharmacology* 2005;49:265–74.
47. Xia Z, DePierre JW, Nassberger L. Modulation of apoptosis induced by tricyclic antidepressants in human peripheral lymphocytes. *J Biochem Mol Toxicol* 1998;12:115–23.
48. Chang HC, Huang CC, Huang CJ, Cheng JS, Liu SI, Tsai JY, Chang HT, Huang JK, Chou CT, Jan CR. Desipramine-induced apoptosis in human PC3 prostate cancer cells: activation of JNK kinase and caspase-3 pathways and a protective role of $[Ca^{2+}]i$ elevation. *Toxicology* 2008;250:9–14.
49. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ. BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. *Science* 2003;300: 135–9.
50. Lindsten T, Thompson CB. Cell death in the absence of Bax and Bak. *Cell Death Differ* 2006;13:1272–6.
51. Gordon J, Barnes N, Meredith E, Drayson M. In response to "selective serotonin reuptake inhibitors—a new modality for the treatment of lymphoma/leukaemia?" by C. Schuster, et al. [Biochem. Pharmacol. 74 (9) 2007 1424-1435]. *Biochem Pharmacol* 2008;75:e1–3; author reply e4-5.