Functional inactivation of the oestrogen receptor by the antioestrogen, ZM 182780, sensitises tumour cells to reactive oxygen species

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Abstract

Reactive oxygen species (ROS) play a fundamental role in both apoptotic and necrotic cell death. Their importance is highlighted by studies showing that they mediate cell death in response to radiotherapy and to some forms of chemotherapy. Here we provide the first evidence for a role of ROS in response to an antiendocrine agent currently undergoing clinical trials. Using the oestrogen receptor (ER) containing rat pituitary GH_3 cell line, we show that cell death is induced by the pure steroidal antioestrogen, ZM 182780, and that this is blocked by the antioxidant, *N*-acetyl cysteine (NAC). By flow cytometry, we show that, prior to the onset of DNA breakdown measured by ELISA, ZM 182780 exposure has no significant effect on intracellular oxidant concentrations. In contrast, ZM 182780 exposure greatly increases sensitivity to oxidants generated by blocking cellular antioxidant pathways and from exogenous administration of hydrogen peroxide (H_2O_2). As both necrosis and apoptosis are controlled by mitochondrial function, further experiments conducted to determine mitochondrial membrane potential ($\Delta\Psi_m$) have indicated that the ZM 182780-induced loss of ER function increases the ease with which oxidants collapse mitochondrial activity and, as a consequence, cell death.

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Introduction

The oestrogen receptor is a ligand activated transcription factor that, in a number of oestrogen target tissues such as the breast (Katzenellenbogen *et al.* 1979) and the pituitary (Wiklund & Gorski 1982, Nowakowski & Maurer 1994), is responsible for altering growth and function. It is the ability of oestrogens acting via the ER to induce growth of breast tumour cell lines that has provided the drive for the development of ligands that block the transcriptional function of the ER. Tamoxifen was first identified as an antioestrogen over 3 decades ago and is now one of the most widely used medical therapies for breast cancer. Studies over the years have demonstrated that tamoxifen will act as a tissue- and gene-specific oestrogen/antioestrogen (Ramkumar & Adler 1995). Indeed, in recent years, this agonist/antagonist rheostat property of the ER has been exploited by the synthesis of selective oestrogen receptor modulators (SERMs), compounds that are organ- and gene-specific oestrogens (McDonnell 1995). The ability of tamoxifen and other SERMs to act as tissue- and gene-specific oestrogens may in part stem from the presence of a recently identified second form of the ER, the ERβ (Paech *et al.* 1997, Kuiper & Gustafsson 1997). This receptor is promiscuous and in addition to binding to oestrogen response element, in the presence of tamoxifen, it will activate transcription of genes containing the activator protein-1 response element (Paech *et al.* 1997). For some ER containing organs such as the endometrium, this is an undesirable consequence of 'antioestrogen' action (Decensi *et al.* 1996). In contast to the SERMs, 'pure antioestrogens' like ZM 182780 (Wakeling *et al.* 1991, Parker 1993) are being developed. This compound is now undergoing clinical trials (Howell 1997) and has no identified ability to activate transcription through the ERα or the ERβ. The mechanism of action of pure antioestrogens is thought to be due in part to their ability to enhance proteolytic degradation of the ER as well as their ability to interfere with the transactivation function of the receptor (Gibson *et al.* 1991, Beckman *et al.* 1993). It can be considered, therefore, that pure antioestrogens give as near a functional inactivation of ER transcriptional activity as can be obtained by the genetic 'knock-out' of ER expression.

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Our studies on the role played by the ER in cell growth and function have focused on the effects of ZM 182780 in GH_3 pituitary tumour cells. These ER containing cells have been shown to be variably responsive to oestogens for growth (Riss & Sirbasku 1989, Zhou-Li *et al.* 1992), a finding that is explained by our studies showing that the ER mediates growth induced by oestrogens, growth factors (Newton *et al.* 1994*a*,*b*) and cytokine pathways (Newton *et al.* 1994*c*). With reporter genes for the ER, we have shown that the transcriptional activity of the ER is increased by oestrogens and growth factors and this is completely blocked by the pure antioestrogen, ZM 182780. In contrast to these studies conducted under serum free conditions, in medium containing serum, we have observed that ZM 182780 induces death of GH_3 cells, with some of the characteristics of apoptosis (Newton 1995). Indeed, experiments on normal rat pituitary cells have demonstrated a clear ability of functional ER ablation by ZM 182780 to induce an apparent cell loss (Newton *et al.* 1996). Given the potential importance of these findings with regard to the treatment of oestrogen dependent tumours, such as those of the pituitary and indeed those of the breast, we have sought to determine the mechanism for this effect. Most recently we have provided evidence that ZM 182780 enhances sensitivity to gamma irradiation of breast tumour cells (Newton *et al.* 1998). As it is well established that reactive oxygen species (ROS) are mediators of radiation-induced cell death (Datta *et al.* 1992), we have sought to determine the role that these agents play in the death of cells due to functional ER ablation by ZM 182780. Our data presented in the current report support the idea that ZM 182780 kills GH_3 cells by increasing their sensitivity to ROS.

Materials and Methods

Reagents

All reagents for cell culture were obtained from Life Technologies (Paisley, UK). The cell death ELISA and lactate dehydrogenase assays were obtained from Boehringer Mannheim UK (Lewes, UK). Fluorescent probes for flow cytometry were obtained from Molecular Probes (Cambridge Bioscience, UK). Other flow cytometry reagents were obtained from Beckton Dickinson UK Ltd. All other reagents were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). The pure antioestrogen was obtained as a gift from Zeneca Pharmaceuticals Ltd (Macclesfield, UK). Oestradiol, ZM 182780 and fluorescent probes were dissolved in ethanol to give ethanol concentrations within the culture medium of less than 0·1%.

Cell culture

Pituitary GH_3 cells as previously described (Newton *et al.* 1994*a*) were maintained in Dulbecco's modified Eagle's medium (DMEM) with phenol red, penicillin (50 U/ml), streptomycin (50 μg/ml), amphoteracin (2 μg/ml) and 10% foetal calf serum (GIBCO, Myoclone). For the majority of studies reported here, experiments were conducted on cells seeded into 48 well culture plates at densities over the range 4000 to 10 000 cells per cm^2. Cells were kept at 37 °C in an atmosphere of 5% CO_2 in air or for some studies in a relative hypoxia incubator where O_2 concentrations were reduced (by the addition of nitrogen) from 21% to 5%. Prior to treatments, cells were allowed to attach for 24 h.

For flow cytometry, cells were harvested from 75 cm^2 culture flasks by the addition of trypsin/EDTA and resuspended in culture medium. These cells were treated in suspension at 37 °C in a gas atmosphere of 5% CO_2 in air.

Fluorescence microscopy

To confirm the integrity of cell membranes, a mixture of propidium iodide (PI) and Hoechst 33342 was added to the cell culture medium to give final concentrations of 10 μg/ml and 1 μg/ml respectively and, after 5 min at 37 °C, cells were placed under a fluorescence microscope (Nikon Optiphot) with a filter block giving excitation at 380 nm and 480 nm. Cells with disrupted membranes preferentially gave strong red nuclear fluorescence due to the uptake of PI. Cells with intact cell membranes gave blue nuclear fluorescence, due to the uptake of the cell permeable Hoechst 33342 fluorochrome.

Determination of mitochondrial metabolism

As mitochondria have been implicated in the initiation of the death process, an estimate of mitochondrial metabolism was made by the addition of the diazo dye, 3-[4,5-dimethylthiazol-2-z-yl]-2,5-diphenyltetrazolium bromide (MTT), a compound that is metabolised at an early point in the electron transport chain (Slater *et al.* 1963) to give a blue insoluble formazan crystals. Except where stated, MTT dissolved in PBS was added to cells for 4 h to give a final concentration of 0·1 mg/ml. The blue crystals were solubilized by the addition of SDS in HCl to give final concentrations of 10% and 10 mM, respectively. Absorbance values were determined at 550 nM. This method is often used to give an estimate of viable cell numbers, as we have indicated in a previous study (Newton 1995). Due to the fact that experimental treatment periods were relatively short (24 to 48 h), with the exception of data presented in Fig. 2, results presented here reflect differences in metabolic activity, as opposed to gross differences in cell numbers.

Determination of cell death by DNA fragmentation

Previous work (Newton 1995) has suggested that ZM 182780-induced death of GH_3 cells has more of the

characteristics of apoptosis than of necrosis (Schwartzman & Cidlowski 1993). Indeed, in our previous study we have shown that antioestrogen exposure provokes cytosolic and nuclear shrinkage together with DNA fragmentation as detected by TUNEL assays (TdT-mediated dUTP nick and labelling). To quantitate cell death in the current study, we have used an ELISA that measures DNA fragmentation. According to the manufacturer of the ELISA^{PLUS} (Boehringer Mannheim), the assay is optimised to measure DNA fragmentation that occurs during apoptosis as opposed to that which occurs during necrosis. To provide some degree of independent validation of the ELISA, we have used an endothelial cell line, EA.hy 926 (Claise *et al.* 1997), that responds to low doses of hydrogen peroxide (H_2O_2) by undergoing classical apoptosis, as shown in Fig. 1b, and to higher doses of H_2O_2, by undergoing classical necrosis (Fig. 1c). Over a dose range of 300 to 900 μM H_2O_2, Fig. 1d shows that the amount of DNA fragmentation detected increases to reach a maximum over the range 500 to 700 μM and falls to that of the control, untreated cells at 900 μM where, as shown in (c), classical necrosis occurs. Accompanying the signs of necrosis, Fig. 1d also shows that the cytosolic enzyme, lactate dehydrogenase, measured according to the instructions provided by the manufacturer of the assay kit, Boehringer Mannheim, is released into the cell culture media, indicating the loss of cytosolic membrane integrity. The ELISA^{PLUS} assay is therefore specific for DNA breakdown that occurs during classical apoptosis and fails to detect DNA fragmentation in classical necrosis.

Flow cytometry for intracellular peroxides and changes in $\Delta\Psi_m$

To test for the production of intracellular ROS in response to antioestrogen treatment, GH_3 cells were removed from 75 cm^2 culture flasks and resuspended in culture medium containing 10 μM dichlorodihydrofluorescein diacetate (H_2DCF). In the presence of intracellular peroxides, this ester is cleaved to yield a fluorescent product (Behl *et al.* 1997*a*). Following a 30 min exposure to H_2DCF, cells were sampled by a Beckton Dickinson FACS^{Calibur} flow cytometer with an argon laser tuned to 480 nM and detection in the green fluorescence, FLT-1 channel (x-axis).

Mitochondrial membrane potential ($\Delta\Psi_m$) was determined by a previously published method (Decaudin *et al.* 1997). GH_3 cells pre-treated with ZM 182780 for 24 h were harvested from 75 cm^2 flasks by the addition of trypsin/EDTA and treated in suspension with 100 μM H_2O_2. After 1 h and thereafter at approximately 30 min intervals, 300 μl of cell suspension was taken to which was added 30 μl of PBS containing the cationic dye, $DiOC_6$ (final concentration 80 μM) and PI (1 μg/ml). Labelled cells were then incubated for 10 min at 37 °C prior to sampling by the FACS^{Calibur} using the same parameters as for the above assay. Loss of $\Delta\Psi_m$ was detected as a decrease in fluorescence intensity (x-axis of figures).

Transfection

GH_3 cells were transfected with the Bcl-2 expression vector, RcCMV-Bcl-2 (Grimm *et al.* 1996). Following transfection of adherant cells in 25 cm^2 culture flasks with 1 μg of the plasmid construct, using the protocol described for the FUGENE transfection reagent (Boehringer Mannheim), cells were left undisturbed for 18 h. These were then scraped from the culture surface and resuspended in 3 ml of tissue culture medium to which was added H_2O_2, as descibed above for the flow cytometry studies with $DiOC_6$.

Statistics

The statistical analysis presented here was performed on a minimum of 3 replicates using Student's *t*-test. All values presented in the figures are expressed as mean ± S.D.

Results

Characteristics of ZM 182780-induced cell death and response to antioxidant, NAC

In response to 10 nM ZM 182780, a concentration that we have previously shown to induce the complete functional inactivation of the ER (Newton *et al.* 1994*a*), pituitary GH_3 cells show signs of cell death 3 to 6 days after first treatment. For measurements made after the addition of MTT to cells on each day of a 6 day treatment period, Fig. 2a shows that the number of metabolically viable cells began to fall after 3 days treatment with 10 nM ZM 182780 and, as indicated in Fig. 2b, there was a parallel increase in DNA fragmentation. As indicated on this figure, the fall in metabolic activity with time and the increase in DNA fragmentation, were completely blocked by the inclusion of an excess of oestradiol in the culture medium. Indeed, although not shown, for all the following studies the effect of ZM 182780 was completely reversed by a 10-fold excess of oestradiol. As indicated in a previous study (Newton *et al.* 1995), cells with low metabolic activity demonstrated marked cytoplasmic and nuclear shrinkage (3- to 5-fold versus control cells), but they failed to exclude PI, as determined by fluorescence microscopy. Indeed for 5 similar experiments, where cells were treated with ZM 182780, fluorescence microscopy revealed that between 29 and 78% of the cell population had taken up PI. These observations are consistent with neither classical apoptosis nor necrosis (Schwartzman & Cidlowski 1993). In our experience of a number of cell models, including the endothelial cells shown in Fig. 1, classical necrosis is characterised by little change in cell and nuclear volume,

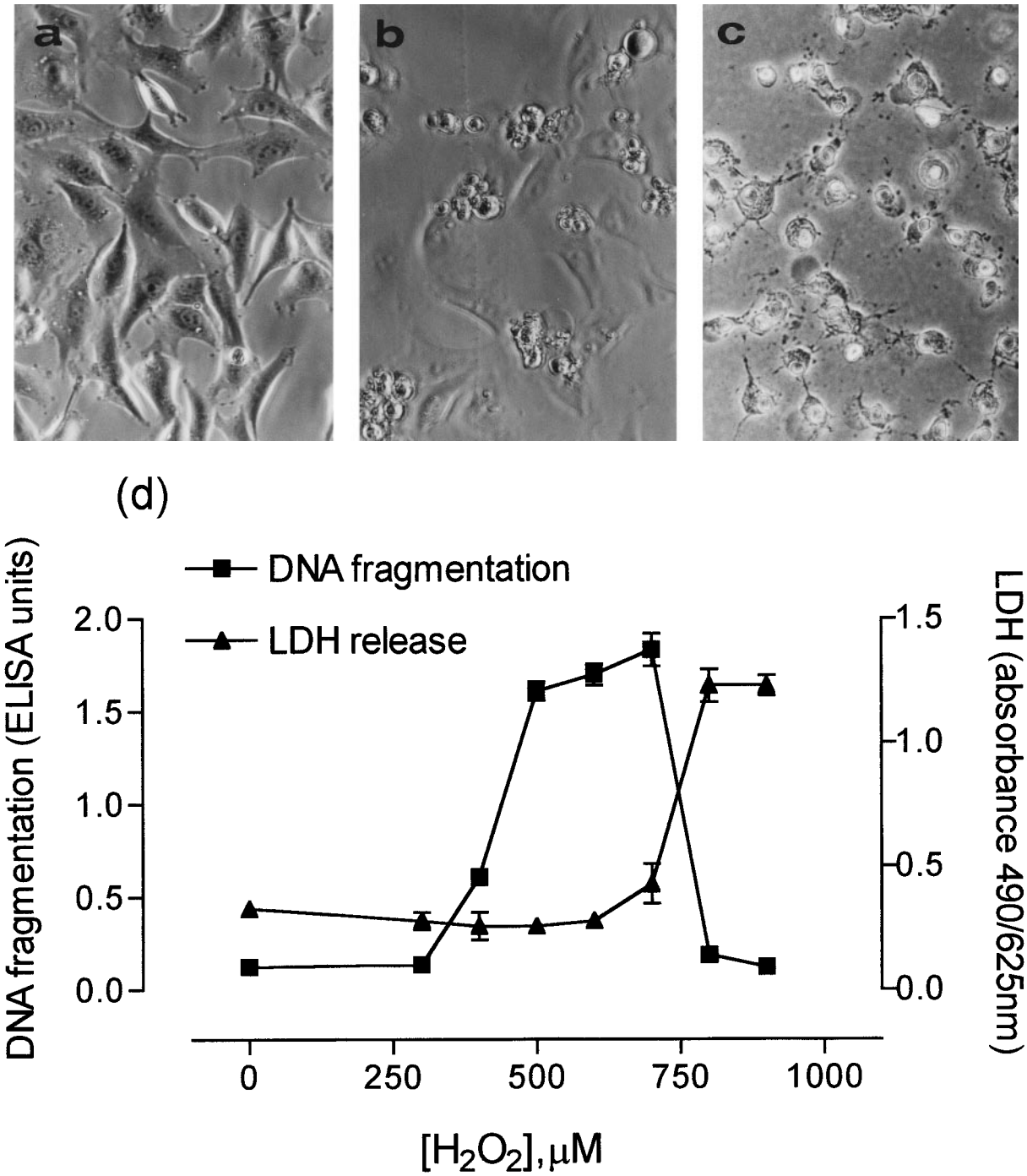


Figure 1 Normal light microscope appearance of endothelial EA.hy 926 cells treated with 500 μM H_2O_2 (b) and 800 μM (c) in comparison to control untreated cells (a). The graphic (d) of this Figure shows DNA fragmentation measured with an ELISA and in parallel, the release of lactate dehydrogenase into the culture medium 24 h after the addition of H_2O_2. For this and subsequent Figures, values are mean ± S.D. for at least 3 replicates.

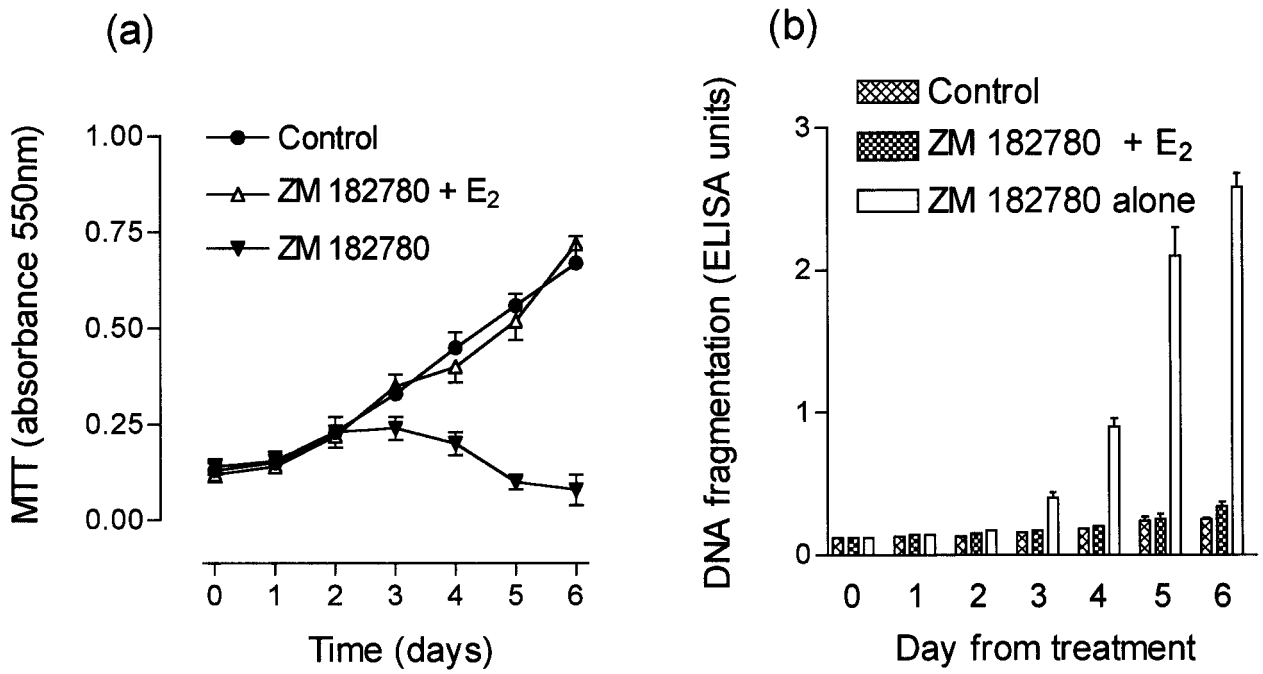


Figure 2 The response of GH_3 cells to ZM 182780 (10 nM) alone and ZM in combination with oestradiol (100 nM) over a time course of 6 days. In (a) viable cell numbers are quantitated by the addition of MTT and in (b) DNA fragmentation is measured by ELISA.

or an increase in both. For all the cell types demonstrating the necrotic morphology defined above, DNA fragmentation was not detected by the DNA fragmentation ELISA or following agarose gel electrophoresis of isolated cellular DNA (data not shown). Based on these observations, it is likely that our difficulty in characterising death of GH_3 cells induced by ZM 182780 is due to the fact necrosis intervenes before the full apoptotic programme can be completed. This idea will be addressed again later in this report.

To determine whether intracellular oxidants played a role in ZM 182780-induced GH_3 cell death, the experimental studies as indicated above were repeated in the presence of the thiol antioxidant and glutathione precursor, *N*-acetyl cysteine (NAC) (Johnson *et al.* 1996). For measurements of DNA fragmentation made with the ELISA^{PLUS} assay, Fig. 3 shows that in the absence of other additions, ZM 182780 resulted in a significant increase in DNA breakdown from day 3 onwards. In the presence of 250 μM NAC, this increase was completely blocked.

The effect of ZM 182780 on antioxidant defences, and on response to pharmacological modulation of ROS concentrations

The above study strongly indicated that ROS were involved in the cell death response of GH_3 cells to ZM 182780. As ROS are always present within cells, even under apparently anaerobic conditions (Esposti & McLennan 1998) we determined whether antioxidant defences were depressed by antioestrogen exposure. Experiments were performed where intracellular oxidant formation was measured by incubation of ZM 182780 pretreated cells with H_2DCF, followed by flow cytometry as described in Methods. Figure 4 shows there is no marked difference in the fluorescence intensity profiles after 48 h ZM 182780 exposure. Over 5 experiments, the $22 \pm 7\%$ of cells showing an increase in peroxide-induced H_2DCF cleavage was not significantly different from control untreated cells, $25 \pm 8\%$. After a further 24 h exposure, the fluorescence profile of Fig. 4 shows a shift to higher intensities, indicating an increase in intracellular oxidant concentrations ($45 \pm 9\%$ vs control, $19 \pm 7\%$, $P<0{\cdot}01$).

Further experiments to address the effects of intracellular oxidant generation were performed using the compound L-buthionine-[S,R]-sulphoxamine (BSO), an agent that blocks the endogenous synthesis of the glutathione peroxidase cofactor, glutathione (Griffith 1979), leading to hightened intracellular H_2O_2 exposure. Experiments were performed on cells under normal *in vitro* cell culture conditions containing 21% O_2 and under the relative hypoxic condition of 5% O_2. Cells treated with ZM 182780 for 24 h were then exposed to BSO over the concentration range 50 μM to 300 μM. Figure 5a shows that, at the end of a 24 h period of exposure to BSO alone, DNA breakdown could not be detected, despite the increase in intracellular oxidants, as indicated by the insert, for measurements made by flow cytometry with H_2DCF.

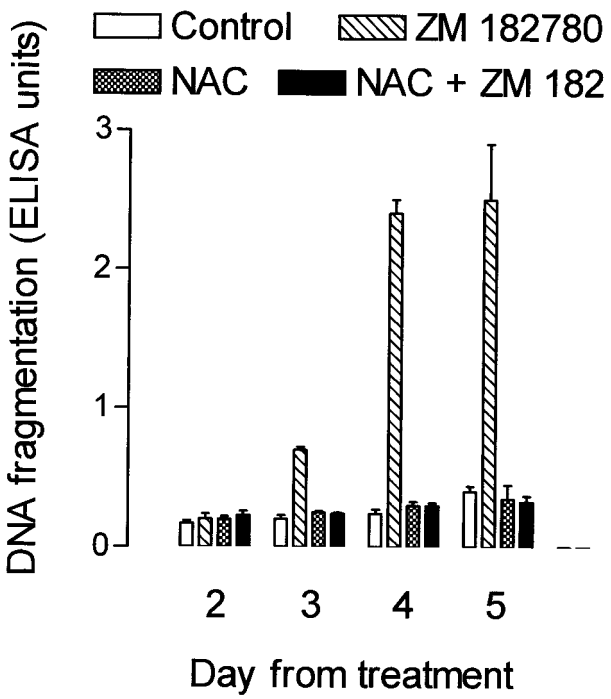


Figure 3 DNA fragmentation within GH_3 cells in response to ZM 182780 (10 nm) over a 5 day period in the absence and presence of the antioxidant, NAC (500 μM).

In contrast, cells pre-treated with ZM 182780 for 24 h under 'normoxic' conditions showed significant DNA fragmentation after 24 h exposure to BSO at concentrations from 25 μM and higher. This was completely blocked by the antioxidant, NAC, confirming that cell death was due to ROS accumulation. In parallel with the DNA fragmentation detected by ELISA, there was a large decrease in metabolic activity (Fig. 5b). Under the relative hypoxic condition of 5% O_2, no marked BSO-induced DNA fragmentation could be observed for the ZM 182780 pre-treated cells (data not shown) and metabolic activity was not affected by BSO as shown in Fig. 5c. These results strongly suggest that loss of ER function sensitises cells to H_2O_2 accumulation due to the inadequacy of the glutathione peroxidase reaction (Halliwell & Gutteridge 1990). Clearly, reducing the environmental oxygen concentrations blocks this effect.

Response of cells to H_2O_2

H_2O_2 freely diffuses across cell membranes and it has been used to induce oxidative stress in numerous previous studies (Schmidt *et al.* 1986, Meyer *et al.* 1993, Behl *et al.* 1997*a*). Preliminary experiments, where pituitary GH_3 cells were exposed to H_2O_2 alone over the concentration range 50 μM to 1 mM, revealed that cell morphology changed within 2 h, at doses of peroxide over the range 50 to 400 μM, to that observed for the long-term antioestrogen treated cells. Paradoxically, at the high H_2O_2 doses (1 mM), cell morphology remained relatively unaltered for the first 2 to 3 h with no evidence of DNA fragmentation. For one of these experiments, Fig. 6a shows that DNA fragmentation increased with time, at the higher H_2O_2 doses. Again, contrary to expectations, in contrast to the low doses, where cytosolic membrane integrity, as evident by PI uptake, was lost within 2 h of H_2O_2 treatment, for a large proportion of the cell population (range, 39–78%, for 7 experiments), the swelling, high dose treated cells excluded PI for several hours. Finally, after around 8 h post 1 mM H_2O_2, all cells showed PI uptake as observed by fluorescence microscopy.

This experiment was repeated for cells pre-treated with ZM 182780 for 24 h. Figure 6b shows these data for measurements made with the ELISA^{PLUS} assay, 2 h after the addition of H_2O_2. Antioestrogen pre-treatment clearly enhanced the ability of H_2O_2 at concentrations below 400 μM to induce DNA fragmentation and, as shown in Fig. 6c, to induce loss of mitochondrial activity, determined by the addition of MTT. Paradoxically, mitochondrial activity was maintained at the higher peroxide doses in the absence of ZM 182780 and was maintained to a lesser extent in the presence of ZM 182780.

It is more usual for low doses of H_2O_2 to induce apoptosis and high doses to induce necrosis (see Fig. 1). The GH_3 cell response to H_2O_2 is clearly unusual in that delayed DNA fragmentation is induced at the high doses whilst at the lower doses, cell death is observed within 2 h. However, given that H_2O_2 doses less than 100 μM are likely to be encountered under physiological conditions (Esposti & McLennan 1998), the rapidity of the response to these lower doses, in comparison to death induced by ZM 182780 treatment alone (see Fig. 2), begs the question as to whether the same mechanism is being addressed. Experiments were therefore performed to determine whether low dose oxidant sensitivity was enhanced as a function of the time these cells were exposed to ZM 182780. Figure 6d shows that, at least for the 72 h ZM 182780 pre-exposure, a significant increase in DNA fragmentation over and above the untreated control cells was now noted at 25 μM H_2O_2. This effect was not apparent for the cells pre-treated with the antioestrogen for 24 or 48 h. These data would further substantiate the claim that ZM 182780 enhances sensitivity to oxidants, and provide the additional information that the degree of sensitisation is a function of antioestrogen exposure time.

$\Delta\Psi_m$ disruption in response to H_2O_2 and ZM 182780 pre-treatment

Our experiments have strongly suggested that loss of ER function enhances sensitivity of GH_3 cell to ROS. Recent studies suggest that the cell death and, in particular, apoptotic cell death, is initiated by events that bring about

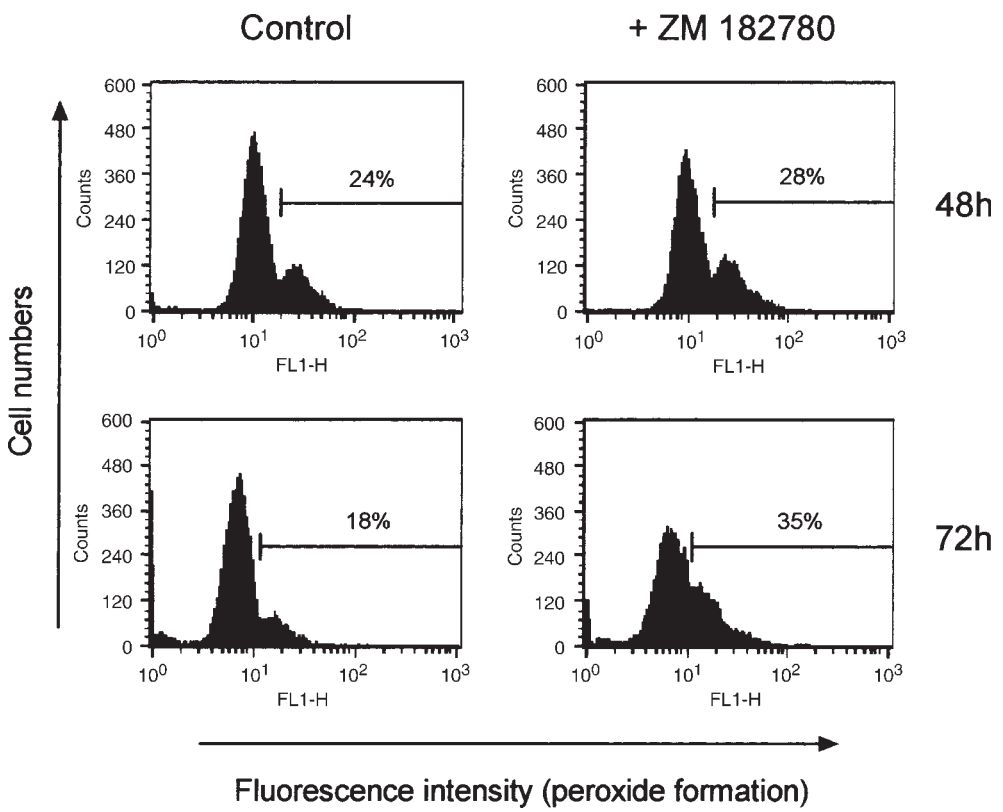


Figure 4 Accumulation of intracellular peroxides, measured by the addition of H_2DCF, following 48 h or 72 h treatment with ZM 182780 (10 nM). The area to the right of the bar indicates the percentage of the cell population showing an increased intracellular peroxide concentration.

the loss of mitochondrial membrane potential ($\Delta\Psi_m$). The $\Delta\Psi_m$ is generated by active oxidative phosphorylation, where the electron flow through the inner mitochondrial membrane is used to pump protons out of the intramitochondrial space. The resulting electrochemical gradient drives ATP synthesis. Apoptotic and necrotic cell death is thought to be initiated when mitochondria undergo a permeability transition (Zoratti & Szabò 1995). In the case of apoptosis, the loss of mitochondrial cytochrome c and as yet, uncharacterised apoptosis-inducing factors (AIFs) from the mitochondrial matrix, is thought to activate the caspase cascade (Yang *et al.* 1997, Zhivotovsky *et al.* 1998). For the current study, a direct assessment of the $\Delta\Psi_m$ was made by labelling cells with the cationic dye $DiOC_6$, as indicated in Methods. As a positively charged substance, $DiOC_6$ accumulates within the inner membrane space as a function of the $\Delta\Psi_m$ and emits green fluorescence, in proportion to its concentration. The graphic of Fig. 7 shows these data for $DiOC_6$ fluorescence at two time points following H_2O_2 addition. For all time points tested, the associated table indicates the percentage of the cell population that have low $\Delta\Psi_m$ and have taken up PI. From the figure it can be seen that little increase in the low intensity fluorescence corresponding to that induced by the mitochondrial uncoupling agent, carbonyl cyanide *m*-chlorophenylhydrazone (Fig. 7A, open profile corresponding to region M1 shown in I), was induced by short term pre-exposure (24 h) to ZM 182780 alone. However, for these ZM 182780 pre-treated cells, the majority have lost $\Delta\Psi_m$ in response to H_2O_2 treatment for 135 min. The associated table shows that the effect of ZM 182780 on H_2O_2-induced loss of $\Delta\Psi_m$ becomes statistically significant after 105 min of exposure. For the 135 min time point following H_2O_2 treatment, Fig. 7 also shows that over-expression of the mitochondrial protein, Bcl-2, partially prevents the ZM 182780 enhanced loss of $\Delta\Psi_m$. Although not shown in Fig. 7, it must be pointed out that the effects of ZM 182780 were completely blocked by co-incubation with oestradiol at a concentration of 100 nM.

Discussion

These data presented in this paper have firmly established a role for ROS in the cytotoxic response of pituitary GH_3 cells to the antioestrogen, ZM 182780. A major premise on which these studies were conducted was that ROS have been shown to have a role in several human diseases,

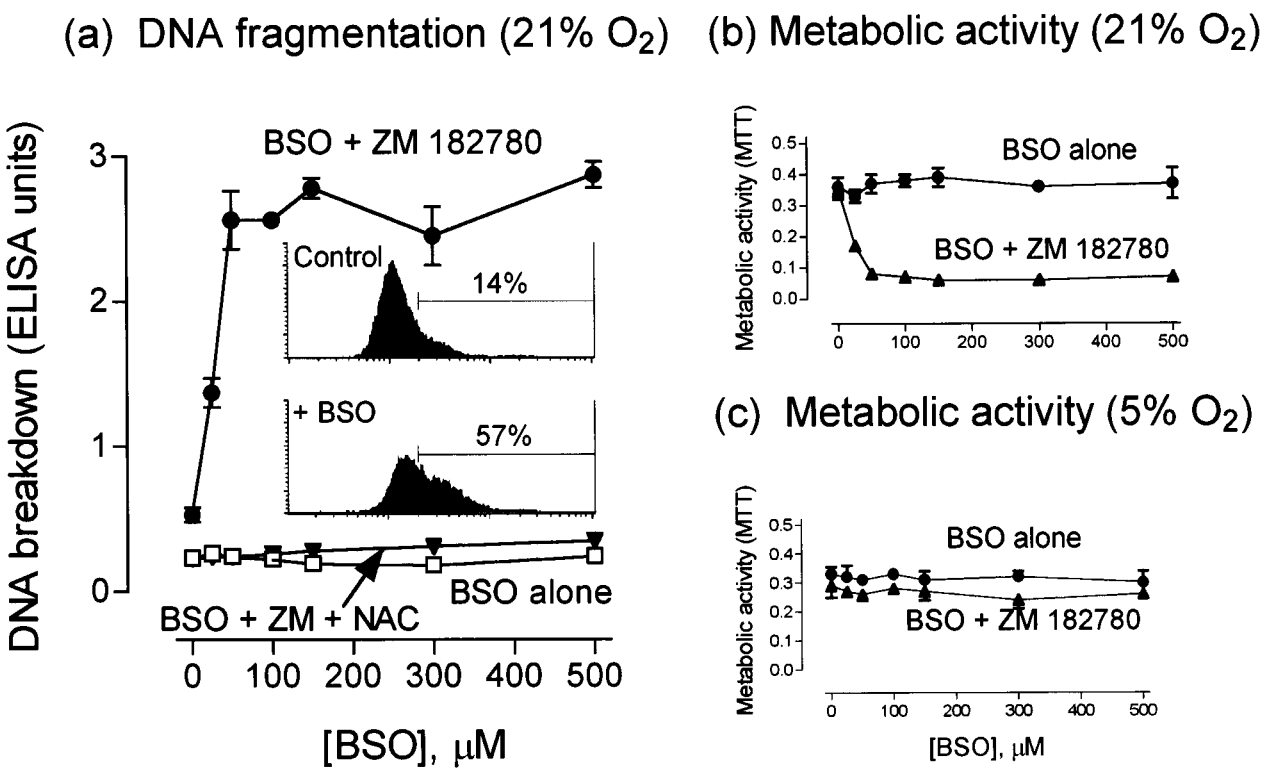


Figure 5 The effect of blocking glutathione synthesis with BSO in the absence and presence of 10 nM ZM 182780 alone or with the antioxidant NAC, under normoxic (a and b) and relative hypoxic culture conditions (c). In (a), DNA fragmentation was measured by ELISA and in (b) and (c), metabolic activity was measured by the addition of MTT 24 h after BSO exposure. The inset in (a) shows the accumulation of H_2O_2 as measured by the addition of H_2DCF, 24 h after the addition of 300 μM BSO alone. Although not shown, H_2O_2 accumulation could not be detected under the relative hypoxic condition of 5% O_2.

amongst which notable examples are Alzheimer's (Coyle & Puttfarcken 1993) and atherosclerosis (Diaz *et al.* 1997). This was reinforced by our recent demonstration that ZM 182780 enhances sensitivity to gamma irradiation (Newton *et al.* 1998), a treatment modality known to induce ROS (Datta *et al.* 1992). ROS are formed within cells as a fundamental consequence of an aerobic environment. Amongst other sites for their production (Ramasara 1982), mitochondria are one of the prinicipal sites for H_2O_2 production (Boveris & Chance 1973). Although H_2O_2 is relatively unreactive and cannot be described as a free radical, its synthesis can damage cellular structures due to the fact that, in the presence of transition metals, such as iron (Fe^{2+}), it is converted to the potent oxidizing agent, the OH radical.

Whilst our experiments with the antioxidant, NAC, have provided firm evidence that GH_3 cell death induced by ZM 182780 involves oxidants, our studies with the compound H_2DCF would suggest that intracellular oxidant concentrations are not altered by ZM 182780. Although some increase is observed 72 h after antioestrogen treatment, by this stage, DNA fragmentation has increased. It is already known that the cellular changes occurring during apoptosis are associated with heightened ROS production (Kroemer *et al.* 1997), so our observation may reflect the outcome and not the initiating event of cell death. This infers that the enzymes, glutathione peroxidase and catalase, both of which are capable of disposing of H_2O_2 generated as a result of metabolic events (Akman *et al.* 1989), are not under the control of the ER response pathway. Based on this, and our studies with NAC, a likely explanation for the effects of ZM 182780 exposure is that ROS trigger cell death, when the threshold for initiating such a response is lowered by loss of ER function. This idea finds support from studies presented in Figs 5 and 6 where the effect of endogenous manipulation of oxidant levels with BSO and exogenous administration of H_2O_2 are explored. Here it is shown that ZM 182780 exposure markedly sensitises cells to elevated ROS concentrations. The experiments presented in Fig. 5 clearly show that the outcome of manipulating glutathione concentrations by the glutathione synthase inhibitor, BSO, is absolutely dependent on the ambient O_2 concentrations. Under relative hypoxia, ZM 182780 fails to facilitate death due to glutathione depletion, strongly suggesting that cellular oxidant formation is depressed.

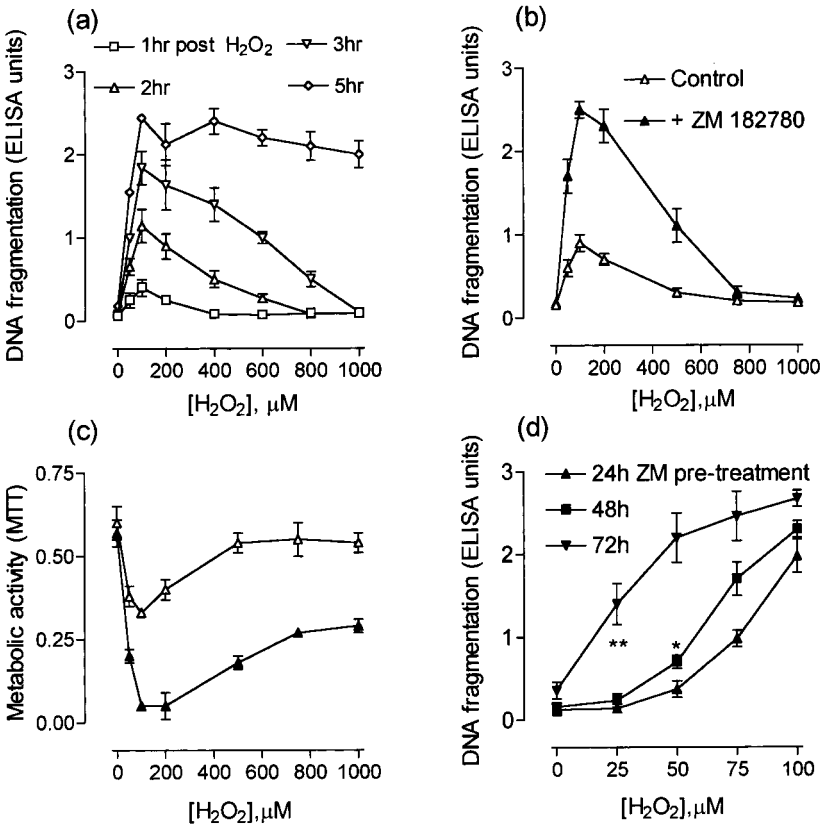
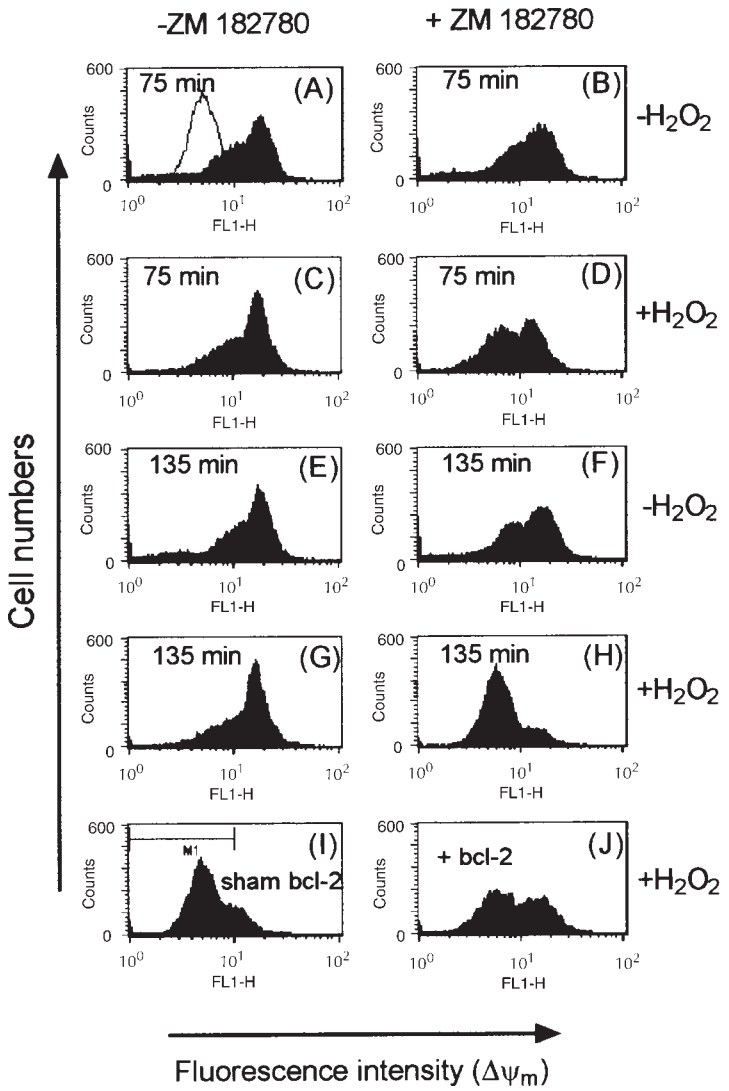


Figure 6 The effect of a concentration range of H_2O_2 on DNA fragmentation and metabolic activity within GH_3. In (a) the time course for DNA fragmentation is presented; for a separate study, (b) displays the effect of a 2 h exposure to H_2O_2 on untreated cells and cells pre-incubated with 10 nM ZM 182780 (24 h) measured as DNA fragmentation and metabolic activity after the addition of MTT (c). In (d), an experiment is shown where the effect of duration of antioestrogen exposure on DNA fragmentation in response to low H_2O_2 concentrations, is tested. vs no addition of H_2O_2: $^*P<0{\cdot}01$, $^{**}P<0{\cdot}001$.

Given that oxidants appear to be involved in the survival/death equation of these GH_3 cells and other cell types (Behl *et al.* 1997*b*), it may be no accident of nature that, as a source of ROS, mitochondria are becoming firm candidates as the main integration site for events that lead to apoptosis and necrosis (Zamzami *et al.* 1997). Mitochondria in their normal oxidative capacity generate a transmembrane potential that allows the generation of ATP. This transmembrane potential ($\Delta\Psi_m$) can collapse by the opening of permeability transition (PT) pores within the inner mitochondrial membrane (Zoratti & Szabò 1995), thus allowing mitochondria to release low molecular weight proteins and cytochrome c that together activate the cascade of the apoptotic pathway (Kluck *et al.* 1997, Petit *et al.* 1996). PT is sensitive to many factors, prominent amongst which are ROS, and mitochondrial electron transport chain disruptors (Zoratti & Szabò 1995, Reed 1997). It is currently thought that PT is regulated by a complex of proteins including the apoptosis regulator, Bcl-2 (Zamzami *et al.* 1997, Reed 1997). Studies by other groups have suggested that Bcl-2 is regulated by oestrogens (Wang & Phang 1995). Our observations on the ability of ZM 182780 to enhance the ability of H_2O_2 to collapse $\Delta\Psi_m$ and the ability of Bcl-2 over-expression to partially block this response are consistent with this, but do not exclude the possibility that the ER directly controls the expression of components of the electron transport chain. Indeed, only relatively recently was it appreciated that the transmembrane potential held the PT pore from opening and that loss of $\Delta\Psi_m$ could be the cause as well as the consequence of PT (Petronilli *et al.* 1993). In support of this, for a related cell line, GH_4C_1, others have indicated that oestrogens alter the concentration of mitochondrially encoded cytochrome c oxidase subunit II (VanItallie & Dannies 1988). Evidence also exists for the presence of DNA binding sites in the mitochondrial genome for other members of the steroid receptor superfamily (Demonacos *et al.* 1995). Therefore, the ER might directly control mitochondrial energy flux, thereby influencing the ability of ROS to induce PT and, as a consequence, cell death.



Time/min	↓$\Delta\Psi_m$ (1)		PI uptake (2)	
	-ZM	+ZM	-ZM	+ZM
45	27.7	33.0	1.0	1.9
75	26.7	41.7	2.2	30.2
105	36.6	74.5*	7.9	68.1*
135	25.1	73.9*	13.9	77.0*
205	39.7	82.2*	32.9	87.4*
310	45.1	81.2*	39.5	91.3*

Percentage of cells having lost $\Delta\Psi_m$ (1), and having taken-up PI (2) as a function of time post H_2O_2 treatment. Values are means from four experiments with SD omitted for clarity.
For cells completely untreated, 25±7% had low $\Delta\Psi_m$ equivalent to treatment with mClCCP. Statistical comparison is made in comparison to -ZM, *P<0.01.

Figure 7 The effect of ZM 182780 on $\Delta\Psi_m$ and cytosolic membrane integrity after H_2O_2 treatment of GH_3 cells. Control and antioestrogen pre-treated (24 h) cells were suspended in culture medium and treated with PBS without and with H_2O_2 (final concentration 100 μM). Aliquots of cells were then taken to which $DiOC_6$ and PI were added. Panels (A) to (H) show the fluorescence profiles with $DiOC_6$ at the two selected time points (A–D, 75 min; E–H, 135 min.), whilst the associated table shows, for all time points, the proportion of cells that had lost $\Delta\Psi_m$ and taken up PI. For cells treated with ZM 182780 for 24 h prior to H_2O_2 exposure, panel (J) shows $DiOC_6$ fluorescence in comparison with sham bcl-2 transfected cells (I). mClCCP, carbonyl cyanide m-chlorophenylhydrazone.

Before a final comment as to the significance of our findings, this discussion would be incomplete without referring to the anomalous nature of GH_3 cell death induced by ZM 182780 alone and in combination with elevated oxidant exposure. Firstly, it is not possible to clearly define the mode of cell death in response to ZM 182780 or oxidants. Cell morphology is not consistent with either apoptosis alone or necrosis alone. There is a large reduction in cell and nuclear volume with corresponding DNA fragmentation; however, this is accompanied by low mitochondrial metabolic activity and loss of cell membrane integrity. We can only propose that part of the apoptotic programme is initiated, leading to endonuclease activation, and that this is rapidy overwhelmed by a progressive loss in overall cellular energy state and an inability to maintain membrane integrity. This cell line may provide an example of what has been termed 'necrosis supervened over apoptosis' (Raffray & Cohen 1997). A second anomaly is the dose response to H_2O_2. It is generally considered that low doses of oxidants induce apoptosis and higher doses, necrosis. Indeed, this is our experience with the endothelial cells described in

Methods and also for cancer and other cell lines (CJ Newton, unpublished observation). Figure 6c clearly shows that metabolic activity is maintained at the high peroxide doses in the absence of ZM 182780, an effect that is blunted by the presence of ZM 182780. These observations suggest that the high peroxide doses trigger a strong protective mechanism such that mitochondrial activity is maintained for several hours. If one is to make this proposal, then one must also propose that at the lower oxidant doses, the cells are unable to mount a sufficient 'stress reponse' to prevent the collapse of mitochondrial function and cell death. Although not shown here, we have evidence that metabolic activity in the presence of 1 mM H_2O_2 is maintained for greater than 90% of the cell population for at least 12 h, by the inclusion of inhibitors of the enzyme poly (ADP-ribose) polymerase (PARP). During this period, DNA fragmentation was also prevented by these PARP inhibitors (CJ Newton, unpublished observations). PARP is an enzyme that is highly activated following DNA damage and results in a large fall of cellular ATP concentrations (Sims *et al.* 1983). These observations strongly suggest that it is the fall in the cellular energy state induced by the oxidant exposure, and not oxidant exposure *per se*, that is responsible for cell death.

Finally, our observation that oxidant sensitivity of this pituitary tumour cell line is increased by antioestrogen exposure should be considered in the light of therapeutic strategies where it is known that ROS are involved. As we have suggested earlier, our observations might provide the explanation for our recent findings that ZM 182780 increased the sensitivity of breast tumour cells to gamma radiation exposure (Newton *et al.* 1998). The current work also raises the question as to whether pure antioestrogens enhance the effect of ROS generating agents in other ER containing tumour cell types, such as the breast. From the experiments reported here on the role of ambient oxygen concentrations, it is quite likely that an *in vivo* role for oxidants in antioestrogen-induced cell death would depend on tissue oxygen saturation. For this reason it will be important to determine the effect that antioestrogens have on the growth and survival of ER containing cells maintained under different ambient oxygen concentrations.

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