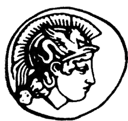
PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 73 (2000) 29–38

The Journal of
Steroid Biochemistry
&
Molecular Biology

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Response of estrogen receptor containing tumour cells to pure antiestrogens and the calmodulin inhibitor, calmidzolium chloride

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Received 27 July 1999; accepted 7 February 2000

Abstract

Cell survival is dependent on both external and internally generated signalling processes and current strategies for medical intervention in neoplastic disease are directed towards signal transduction blockade. Redundancy in signalling pathways may mean, however, that a combination of agents is required for the maximal therapeutic benefit. We have explored this idea with regard to the antiestrogen sensitivity of estrogen dependent tumours. Using estrogen receptor (ER) containing tumour cell lines, we have determined whether antiestrogens increase the cytotoxicity of the potent calmodulin inhibitior, calmidzolium chloride (CCl). For the pituitary tumour cell line GH_3, CCl induces a form of apoptotic cell death and co-treatment with the pure antiestrogen, ZM 182780, enhances sensitivity to the calmodulin inhibitor, by at least two fold. In contrast to the pure steroidal antiestrogens, the triphenylethylenes, tamoxifen and 4-hydroxytamoxifen give no enhancing effect on CCl induced cell death. Although CCl induces apoptosis of several ER containing breast cancer cell lines, unlike the pituitary tumour cells, ZM 182780 is unable to increase their sensitivity to calmodulin inhibition. Further studies strongly suggest that cell death in response to calmodulin inhibition is the result of metabolic disruption and that for GH_3 cells, this is enhanced by antiestrogen treatment. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Although great efforts are underway to develop new antitumour agents based on an ability to block signal transduction pathways, the complexity and redundancy in intracellular signalling may mean that a combination of agents will be required for fully effective therapy.

One of the most clinically successful classes of antitumour compounds has been the antiestrogens. Therefore for a number of cell types containing the estrogen receptor (ER), antiestrogens like tamoxifen have been shown to reduce cellular growth rates [1]. Tamoxifen is now one of the most widely used antiendocrine agents for the treatment of breast cancer. More recently a number of new antiestrogens have been developed and several are currently undergoing clinical trials [2]. Unlike tamoxifen, these new antiestrogens typified by the steroidal compound, ZM 182780 [3], offer the advantage that they more fully block the transcriptional activity of the ER. In contrast, tamoxifen is considered to be a selective estrogen receptor modulator (SERM), as in some cell and tissue types, it can activate gene transcription depending on the promotor context [4]. This phenomenon may, in part, be due to the presence of a second form of the ER in tumour cells, the ERβ [5,6]. Via interaction with the ERβ isoform, estradiol inhibits transcription of genes by interfering with the transcriptional activating activity of the Fos and Jun proteins that comprise the activator protein-1 (AP-1) complex [5]. In contrast, the interaction of tamoxifen with the ERβ induces transcription of genes normally responsive to the AP-1 transcription factor. Tamoxifen can therefore signal through what are normally con-

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PII: S0960-0760(00)00047-9

sidered to be serum and growth factor activated pathways [5]. In contrast to tamoxifen, the pure antiestrogens like ZM 182780, have been shown to block both estrogen and growth factor activated gene expression [7].

Although concern has been expressed with regard to the potential of tamoxifen to induce endometrial cancer [8], pure antiestrogens may result in a loss of bone mineral density with long term therapy [9]. Pure antiestrogens might, however, be particularly useful as agents to augment other therapies, like chemotherapy and radiotherapy. Recently, our studies have suggested that for a breast cancer cell line under in vitro conditions, ZM 182780 enhances the cytostatic and cytotoxic effects of gamma irradiation [10]. Recent work on the effect of ZM 182780 in a pituitary tumour cell line has also indicated that the loss of functional ER activity induced by the antiestrogen increases sensitivity to reactive oxygen species [11]. These studies have provided evidence to indicate that this is due to a role that the ER plays in supporting mitochondrial function. Indeed, evidence has been accumulating over the past three to four years that apoptotic cell death involves changes in mitochondrial function [12]. In view of this and our assertion that single agents will be less clinically efficacious than a combination, we have tested the antitumour effect of combining a potent and specific calmodulin inhibitor with the pure antiestrogen, ZM 182780.

Calmodulin inhibition was chosen as a target for combination with ZM 182780 as, (1), the protein is required in numerous metabolic pathways [13] and metabolic status is a trigger mechanism for apoptosis [14], (2), previous studies have demonstrated an interaction between the ER and calmodulin [15] and (3), analogues of the antiestrogen, tamoxifen, inhibit calmodulin's activity [16]. For this work we have used the well characterised ER containing pituitary tumour cell line, GH_3, where we have shown previously that the ER mediates both growth factor and estrogenic signals [17]. As models of a tumour type that would provide the clinical target for such a combined therapy as described here, studies are reported for the breast cancer cell lines, MCF-7, T47D and ZR 75-1.

2. Materials and methods

2.1. Reagents

Reagents for cell culture were obtained form Life Technologies (Paisley, UK). The cell death ELISA was obtained from Boehringer Mannhein UK (Lewes, England). All other reagents were obtained from Sigma-Aldrich Company (Pool, England). The pure antiestrogens ZM 182780 and RU 58668 were obtained as gifts from Zeneca Pharmaceutucals Ltd. (Macclesfield, England) and Roussel (Romainville, France), respectively, whilst 4-hydroxytamoxifen (4-OHT) was obtained from Besins Iscovesco (Paris, France). Calmidzolium chloride (CCl) was obtained from Calbiochem-Novabiochem (UK) (Nottingham, England). Estradiol and the antiestrogens were dissolved in ethanol to give ethanol concentrations within the culture medium of less than 0.1%.

2.2. Cell culture

Pituitary GH_3 cells as previously described [17] 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). Breast cancer MCF-7, T47D and ZR 75-1 were maintained in RPMI with the same additions as for the pituitary tumour cells. For the majority of studies reported here, experiments were conducted on cells seeded into 48 well culture plates at densities over the range 5000 to 50,000 cells/cm^2. Cells were kept at 37°C in an atmosphere of 5% CO_2 in air. Prior to treatments, cells were allowed to attach for 24 h. Cell numbers were determined with a Coulter Counter (Coulter Elecronics, Luton, England).

2.3. Fluorescence microscopy

To confirm the integrity of cell membranes, a mixture of ethidium bromide and acridine orange was added to the cell culture medium to give final concentrations of 1μM and 25 μM, 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 and 480 nm. Cells with disrupted membranes preferentially gave strong red nuclear fluorescence due to the uptake of ethidium bromide. Cells with intact cell membranes gave green fluorescence, due to the uptake of the membrane permeable acridine orange fluorocrome.

2.4. Determination of viable cell numbers

An estimate of the number of viable cells was made by the addition of the diazo dye 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT), a compound that is metabolised by both cytosolic and mitochondrial NAD(P)H-linked dehydrogenases [18] to give 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 dissolved by the addition of SDS in HCl to give final concentrations of 10% and 10 mM, respectively. Absorbance values were determined at

550 nm. When added to cells that have been treated for several days with cytostatic/cytotoxic agents, this method can be used to give an estimate of viable cell numbers, as we have indicated in a previous study [19].

2.5. Determination of cell death by DNA breakdown

DNA breakdown occurs during most forms of cell death. To quantitate cell death we have used an ELISA (Boehringer Mannheim cell death $ELISA^{PLUS}$) that detects DNA fragmentation. This assay employs both anti-histone and anti-DNA antibodies. In brief, cells showing a cytotoxic response are lysed with the solution provided in the assay kit and the lysate is centrifuged to sediment the chromosomal DNA. A sample of the supernatant is then added to strepavadin-coated wells in microtitre plates, and a mixture of biotinylated anti-histone and peroxidase labelled anti-DNA antibodies is added to wells. These plates are then placed on shaker for 2 h, after which wells are washed and a peroxidase substrate is added. The resulting colour is measured in a multi-well plate reader. Our previous studies with this assay have shown that it gives a high degree of specificity for DNA breakdown that occurs during classical apoptosis, as opposed to that which occurs during necrosis [11].

2.6. ATP determinations

ATP was determined in cell lysates with a luminescent assay. Medium was removed from treated cells in 48 well plates and 0.5 ml of distilled water was added. After a period of 1–2 min the cell lysate was aspirated and boiled for 5 min. This was then diluted with a further 1 ml of distilled water and 100 μl aliquots were mixed with a 100 μl aliquot of a luciferase/luciferin mixture provided by Sigma (FL-AA). Values were recorded as relative light units (RLU) and were expressed as a percentage of those obtained for untreated control cells. All values were corrected for cell numbers determined with a Coulter counter. Values obtained from experiments fell within the wide linear range of the assay.

2.7. Statistics

The statistical analysis presented here was performed on a minimum of three replicates using the student's t-test. All values presented in the figures are expressed as mean $\pm$ SD.

3. Results

3.1. Dose response of pituitary tumour cells to CCl in the absence and presence of ZM 182780

To test the response of pituitary GH_3 cells to calmodulin antagonism alone or with concomitant loss of ER function, cells were exposed for 5 days to a dose range of CCl (1–5 μM) alone or the same range with 100 nM ZM 182780. We have previously shown that the antiestrogen used at this concentration completely blocks the transcriptional function of the ER [20]. Fig. 1(a) shows the result of one of a number of experiments where a statistically significant decrease in cellular metabolic activity was observed from CCl concentrations of 3 μM and above. In the additional presence of ZM 182780, all cells were dead with 3 μM CCl. As indicated in Section 2, although strictly speaking, the addition of MTT measures cellular NAD(P)H concentrations and hence metabolic activity, for long-term treated cells, as in the case of the experiment presented in Fig. 1. MTT colour formation may also be used as an assay for live cell numbers [19]. For five experiments conducted in this way, the amount of CCl needed to reduce viable cell numbers by 50% (IC_{50} value) is decreased from 3.3 ± 0.5 to 1.6 ± 0.3 by the presence of ZM 182780. Fig. 1(b) shows that the loss of metabolic activity is matched by the large increase in DNA fragmentation, measured with the cell death ELISA, an assay that we have shown to be a good indicator of DNA fragmentation that occurs during apoptosis [11]. Fig. 2(a) shows the light microscope appearance of MTT stained cells 72 h after the addition of 2.5 μM CCl and 100 nM ZM 182780. Fig. 2(b) shows the appearance of these cells under UV light, after addition of a mixture of ethidium bromide and acridine orange. Both early nuclear fragmentation (green granules), where the cell membrane is

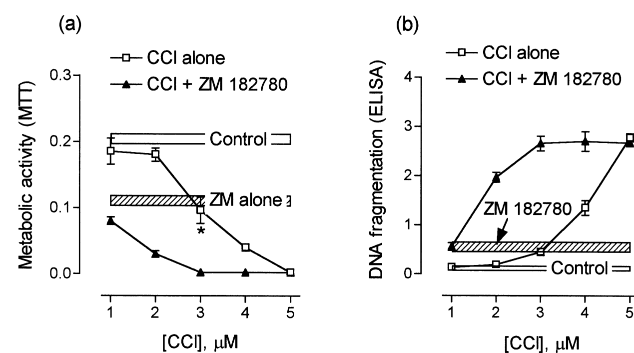

Fig. 1. The effect of a 5 day treatment of cells with a dose range of CCl in the absence and presence of 100 nM ZM measured in (a) as metabolically viable cells following the addition of MTT (a) and in (b), as DNA fragmentation measured with an ELISA. $p < 0.01$ vs. control.

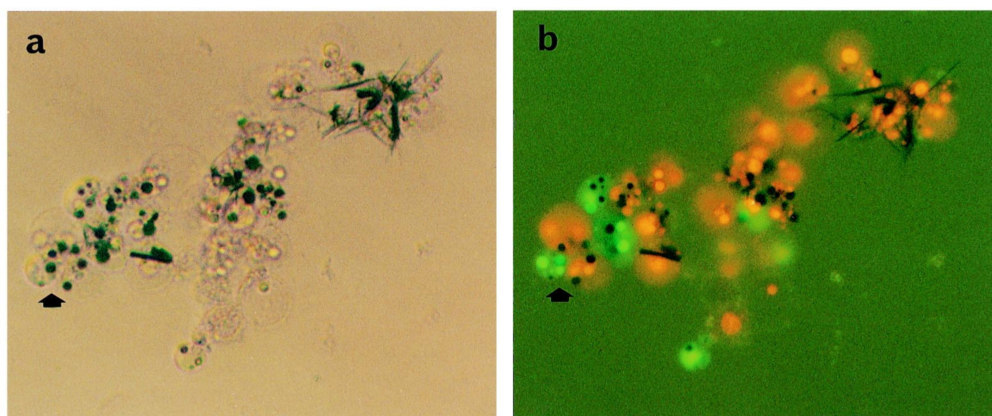

Fig. 2. The appearance of cells following a 72 h treatment period of GH_3 cells with 2.5 μM CCl and 100 nM ZM under normal light conditions (a) and under UV light (b) following the addition of ethidium bromide and acridine orange.

still intact, and late red fragmentation, where ethidium bromide uptake has occurred (membrane integrity lost), is apparent. As indicated by the arrows on Fig. 2(a), some of the MTT staining granules can be observed to correspond to the green fluorescent granules in Fig. 2(b) (arrows). Given that apoptosis involves the break-up of cells into metabolically active fragments containing nuclear and cytoplasmic components [21], these observations in combination with the ELISA data, strongly indicate that a form of apoptotic cell death was being induced by CCl.

Althought not shown in Fig. 1, it must be stressed that the inclusion of 100 nM estradiol in combination with ZM 182780, completely prevented the enhancing effect of the antiestrogen on CCl induced cell death. This finding was consistent for all subsequent studies.

3.2. Antiestrogen pre-treatment and CCl-induced cell death

A particular observation made throughout the above studies was that death at 1–3 μM CCl was apparent after 48–72 h treatment in the presence of 100 nM ZM 182780. To explore the apparent synergism between these two agents, we tested whether pre-treatment of cells with ZM 182780 would pre-dispose to cell death following a brief exposure to CCl. In order to discount the possibility that an enhancing effect of ZM 182780 was due to the ability of ZM 182780 to reduce cell numbers, a protocol was adopted where control cells were seeded at lower density than for those cells treated with ZM 182780. Both 48 h pre-treated and control cells were then exposed to 1–3 μM CCl and 24h later, MTT was added. For the exper-

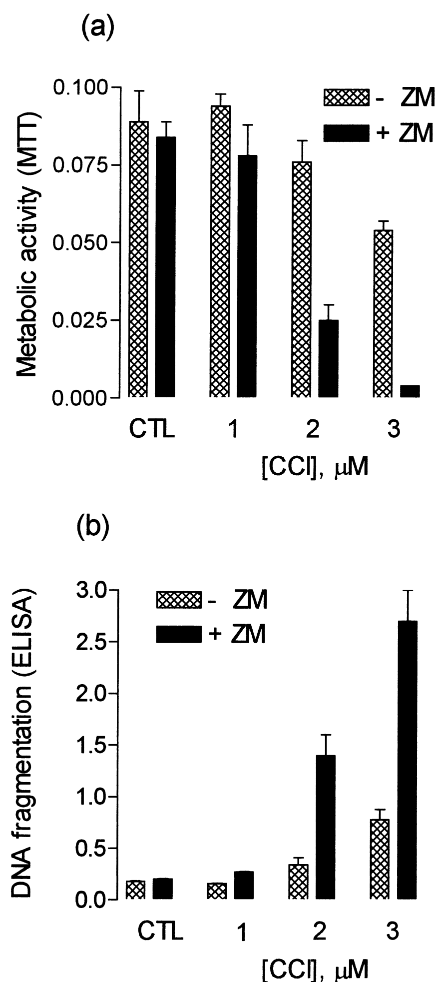

Fig. 3. The effect of pre-treatment of GH_3 cells for 48 h with ZM on the response to a 24 h exposure to CCl, measured as metabolic activity (a) and DNA fragmentation (b).

iment shown in Fig. 3, cell numbers, estimated by Coulter counter for ZM treated cells were not significantly different from control cell numbers 67,000 ± 6000 vs. 74000 ± 5000 respectively ($n = 4$), yet ZM 182780 pre-treatment markedly enhanced the CCl induced loss of metabolic activity.

3.3. Apoptotic morphology is induced by MTT for the CCl and ZM treated cells

A further observation made during the above studies, where cells were pre-treated with ZM 182780, was that at doses of CCl around 1–2 μM, apoptotic morphology, as indicated in Fig. 2(b), became apparent for a proportion of the cell population within 1–3 h after the addition of MTT. The same phenomenon was also observed in some experiments where cells were treated with 3–4 μM CCl alone and then exposed to MTT. For the first hour after MTT addition, cells stained evenly throughout the cytoplasm with the metabolically formed, blue formazan dye. Over the next 1–3 h the dye became concentrated into several 'pockets', some of which co-stained with acridine orange as indicated in Fig. 2(b).

To explore these observations further, experiments were performed where (1), DNA fragmentation was determined as a function of time after the addition of CCl (1.5 μM) for 48 h, ZM 182780 (100 nM) pre-treated cells, (2) where DNA fragmentation for 48 h ZM 182780 and 20 h, CCl-treated cells was determined as

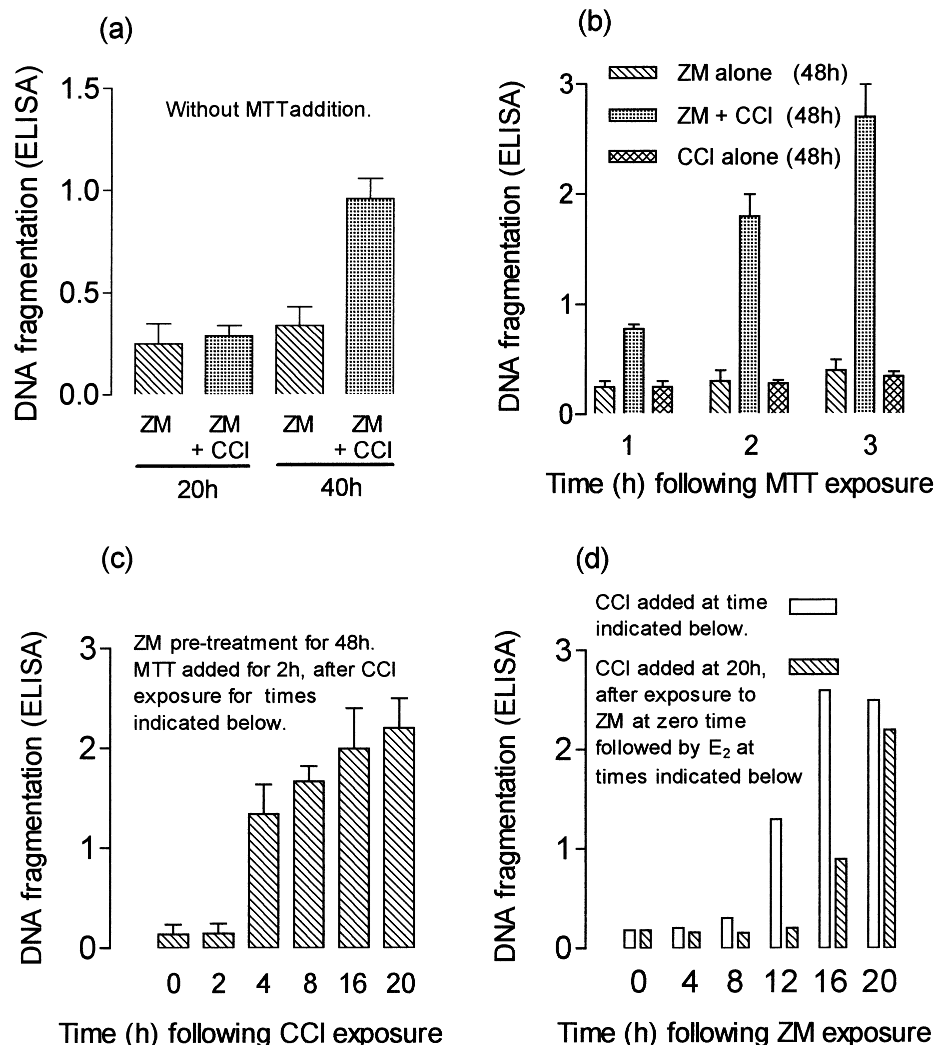

Fig. 4. Apoptotic cell death is induced by MTT exposure for the CCl and ZM exposed cells. In (a) DNA fragmentation is not observed for 48 h ZM pre-treated until cells were exposed to CCl for 40 h. In contrast, (b) shows that MTT induces DNA fragmentation for cells pre-treated with ZM for 48 h and then exposed to CCl for only 20 h. In (c) DNA fragmentation in response to MTT was measured for cells pre-treated with ZM for 48 h and then exposed to CCl for the times indicated. In (d) the effect of continuous exposure to ZM for the times indicated (open bars) prior to immediate CCl exposure is determined, and compared to the effect of CCl added 20 h after first adding ZM and after reversal of the antiestrogen effect by the addition of estradiol (hatched bars) at times indicated.

a function of MTT exposure time, (3) where DNA fragmentation in response to MTT (2 h) was determined as a function of CCl exposure time (ZM, 48 h pre-treatment), and finally (4), where MTT (2 h)-induced DNA fragmentation was determined as a function of ZM 182780 exposure time (CCl, 48 h pre-treatment). For the experiment shown in Fig. 4(a), DNA breakdown in response to CCl for the 48 h antiestrogen pre-treated cells, failed to occur until the cells had been exposed to the calmodulin antagonist for 40 h. In contrast, for cells exposed to ZM 182780 for 48 h and then to CCl for 20 h, Fig. 4(b) shows that a 1h exposure to MTT was sufficient to induce some DNA fragmentation. No effect of MTT was noted for cells only pre-treated with ZM 182780 and not exposed to CCl and also for cell only exposed to CCl. For cells pre-exposed to ZM 182780 for 48 h and then to CCl for varying time periods, Fig. 4(c) shows that maximal DNA fragmentation was induced by MTT for cells given CCl for only 4 h. Finally, Fig. 4(d) shows that continuous exposure to ZM 182780 for at least 12 h prior to the addition of CCl for 4 h was sufficient for DNA fragmentation in response to MTT. In contrast, the shaded bars of Fig. 4(d) show that when estradiol was given to block the effect of ZM 182780 at the time points indicated, and then 20 h after first exposure to ZM 182780, CCl was added for 4 h, DNA fragmentation was prevented. These data indicate that continuous exposure to ZM 182780 is required for a response to CCl and MTT to be observed.

MTT is metabolised within mitochondria and cytosol by NADH/NADPH dependent dehydrogenases and its metabolism will alter the redox state ($NAD(P)H/NAD(P)^+$ ratio). As a consequence of this, the maximum rate of ATP synthesis should be reduced. To test this, ATP measurements were made for cells exposed to ZM 182780 alone (48 h) and for cells exposed to CCl and MTT. Table 1 shows these data for two experiments. For experiment 1, in comparison to untreated cells, ZM and CCl treatment alone or the combination of both agents had little effect on ATP concentrations following 48 h exposure. In contrast, exposure to MTT for 30 min induced a highly significant reduction in ATP concentrations for cells exposed to both CCl and ZM. For experiment 2, CCl exposure alone significantly reduced ATP concentrations but this was not enhanced by a 15 min exposure to MTT. Also, for this experiment, ZM failed to enhance the CCl-induced fall in cellular ATP but an effect of ZM became apparent following exposure to MTT for 15 min.

Table 1
ATP concentrations within ZM and CCl treated GH_3 cells in response to MTT exposure expressed as a percentage of ATP concentrations in untreated controls

Treatment	Zero time	+MTT (15 min)	+MTT (30 min)
Experiment 1			
Control	100	–	93 ± 5
ZM	92 ± 6	–	83 ± 7
CCl (1 μM)	108 ± 7	–	93 ± 5
CCl (2 μM)	102 ± 3	–	78 ± 8^a
CCl (1 μM)+ZM	88 ± 7	–	67 ± 6^b
CCl (2 μM)+ZM	94 ± 2	–	51 ± 4^b
Experiment 2			
Control	100	73 ± 5^a	–
ZM	98 ± 3	56 ± 6^b	–
CCl (1 μM)	60 ± 6^a	57 ± 4^b	–
CCl (2 μM)	62 ± 4^b	65 ± 7^b	–
CCl (1 μM) +ZM	62 ± 5^b	35 ± 4^c	–
CCl (2 μM) +ZM	63 ± 6^b	33 ± 4^c	–

[a] $p < 0.05$ vs. control.
[b] $p < 0.01$ vs. control.
[c] $p < 0.001$ vs. control ($n = 5$ replicates).

To determine whether apoptosis was triggered by a reduction in ATP concentrations per se, we tested whether reducing ATP generation with the F_0F_1 ATPase inhibitor, Oligomycin [22], would have the same effect as MTT. Fig. 5 shows the time-course for the response to Oligomycin (5 μg/ml) in comparison to MTT, for 48 h, ZM 182780 and CCl pre-treated cells. As previously shown in Fig. 4(b), exposure to MTT induced DNA fragmentation within 1 h of addition. In comparison, the effect of oligomycin was more protracted, requiring at least an 8 h exposure before DNA fragmentation occurred.

3.4. Comparison of the effect of ZM 182780 with other antiestrogens

Transfection experiments with GH_3 cells using ER responsive reporter plasmids, together with other studies to determine ER responsive endpoints such as progesterone receptor expression, prolactin synthesis and growth, have shown that the pure antiestrogen, ZM 182780, is at least two orders of magnitude more

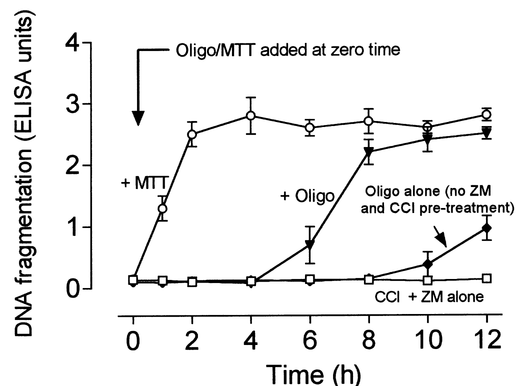

Fig. 5. Response of 48 h CCl (1.5 μM) and ZM (100 nM) treated GH_3 cells to the F_1F_0 ATPase inhibitor, oligomycin.

potent than tamoxifen [10]. At a maximally effective dose, ZM 182780 is also able to give a greater growth suppression than a maximally effective dose of tamoxifen. A comparative study of the ability of a number of antiestrogens to enhance the cytotoxic effect of CCl on GH_3 cells was therefore made. Fig. 6 shows the response of GH_3 cells to CCl over the range 1 to 3 μM in combination with, ZM 182780 (100 nM), with the pure antiestrogen, RU 58668 (100 nM) [23], with 4-hydroxytamoxifen (4-OHT; 1 μM) and with tamoxifen (1 μM). To minimise the growth inhibitory response to antiestrogens alone, cells were treated 24 h after seeding at high density (50,000 cm^{-2}). Values are given as metabolic activity 72 h treatment, transformed as a function of respective controls (no-addition or ZM 182780 only). It is only the pure steroidal antiestrogens ZM 182780 and RU 58668 that are able to enhance the response to CCl.

3.5. Response of breast cancer cells to CCl and ZM 182780

To determine the applicability of our findings to clinically relevant targets for a combination of a calmodulin antagonist and an antiestrogen, experiments were conducted with MCF-7, T47D and ZR75-1 breast cancer cells. These cells were treated for a 5 day period with a combination of CCl (2 to 5 μM) and ZM 182780 (100 nM), prior to the addition of MTT to determine metabolic activity. Fig. 7 shows that whilst ZR 75-1 and MCF-7 cells were somewhat more sensitive to CCl than T47D cells, no enhancing effect of ZM 182780 was observed for any of the three cell types. These data for MCF-7 cell sensitivity to CCl are exactly comparable to those obtained in a previous study [24].

In parallel with an observation made with the GH_3 cell line, classical apoptotic morphology became apparent for the T47D cell line treated with 4 μM CCl, 3–4 h after the addition of MTT as shown in the photograph associated with Fig. 7. Unlike GH_3 cells, the effect of MTT was independent of the presence of ZM 182780.

4. Discussion

Using the pituitary tumour cell line GH_3 as a model to study the role of the ER in the growth and survival of tumour cells, we have previously shown that the antiestrogen, ZM 182780 induces growth arrest [17] and under some conditions, cell death [19]. Our recent studies have indicated that loss of ER function, induced by pure antiestrogens, increases the sensitivity to reactive oxygen species [11]. The studies presented in the current report would suggest that sensitivity in general to cytotoxic agents is enhanced by functional ER ablation by pure antiestrogens. In contrast for three ER containing breast cancer cell lines, no enhancement of the cytotoxic response to the calmodulin antagonist CCl was noted. These data on breast cancer cells contrast with the one previous study where it was demonstrated that camlodulin antagonists synergised with tamoxifen to induce apoptotic death of ER positive and negative breast cancer cells [25]. That the effect was apparent for ER negative cells suggests that the effect of tamoxifen was not ER mediated. Indeed, tamoxifen has been shown to interfere with several intracellular pathways, in addition to binding to the ER [26]. It is conceivable that calmodulin inhibition could synergise with one of these non-ER, tamoxifen inhibited cellular pathways. Although data are not presented in the current study for each experiment, it

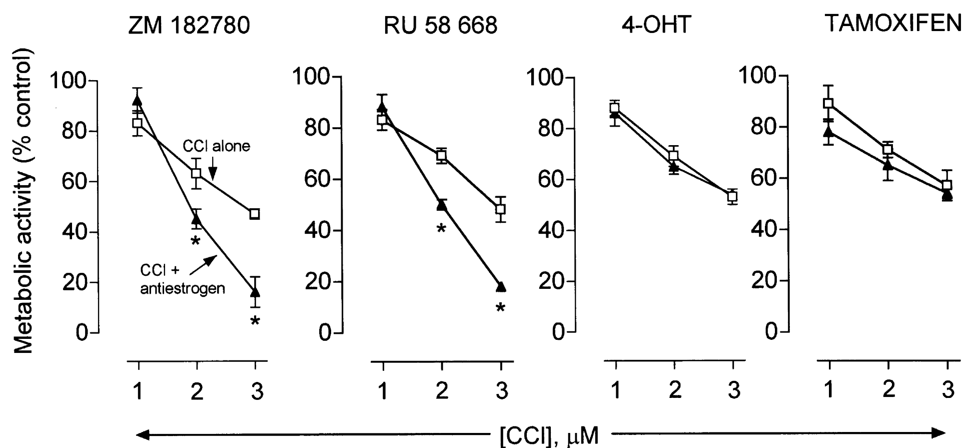

Fig. 6. Comparitive response of GH_3 cells to the antestrogens, tamoxifen (1 μM), 4-OHT (100 nM), RU58668 (100 nM) and ZM (100 nM) in combination with a dose range of CCl (▲) in comparison with CCl alone (□). Values for metabolic activity, measured with MTT, are given as a percentage of those obtained for respective controls — untreated or antiestrogen treatment alone. $p < 0.01$ vs CCl alone.

must be re-stated that 100 nM estradiol blocked the ability of ZM 182780 to enhance CCl-induced cell death, indicating an ER mediated effect. For our study presented here, in contrast to 4-OHT and tamoxifen, the ability of the pure antiestrogens, ZM 182780 and RU 58668 to enhance the response to CCl must presumably reside in their ability to completely block the transcriptional activity of the ER at the estrogen response element and perhaps also from the AP-1 response element. [5].

For our previous studies on GH_3 cells, death in response to long-term treatment with ZM 182780 alone, has been difficult to characterise, as in parallel with an increase in DNA fragmentation, cells lose the ability to exclude propidium iodide and ethidium bromide. During these earlier studies we have not been able to disassociate DNA fragmentation, from metabolic collapse and membrane disruption. This is not the case in the current study. Fig. 2 provides evidence of nuclear fragmentation when membrane integrity is still intact (green fluorescent cells).

With regard to the potential mechanism by which functional ER ablation by ZM 182780 enhances the cytotoxic effect of CCl, data presented in Fig. 4 provide significant clues. Here we have shown that the diazo dye MTT is able to induce DNA fragmentation for GH_3 cells treated with low doses of CCl and pre-treated with ZM 182780. MTT is reduced within mitochondria and cytoplasm and its metabolism will alter the redox ratio in favour of a more oxidized state. In turn, this will decrease ATP synthesis. Table 1 confirms that exposure of ZM and CCl treated GH_3 cells to MTT for just 15–30 min, reduces ATP concentrations. Currently, apoptosis is thought to be initiated by the opening of the mitochondrial permeability transition pore (PTP) and the loss of mitochondrial membrane potential ($\Delta\Psi_m$). The $\Delta\Psi_m$ is maintained by electron flow through the respiratory chain and it is

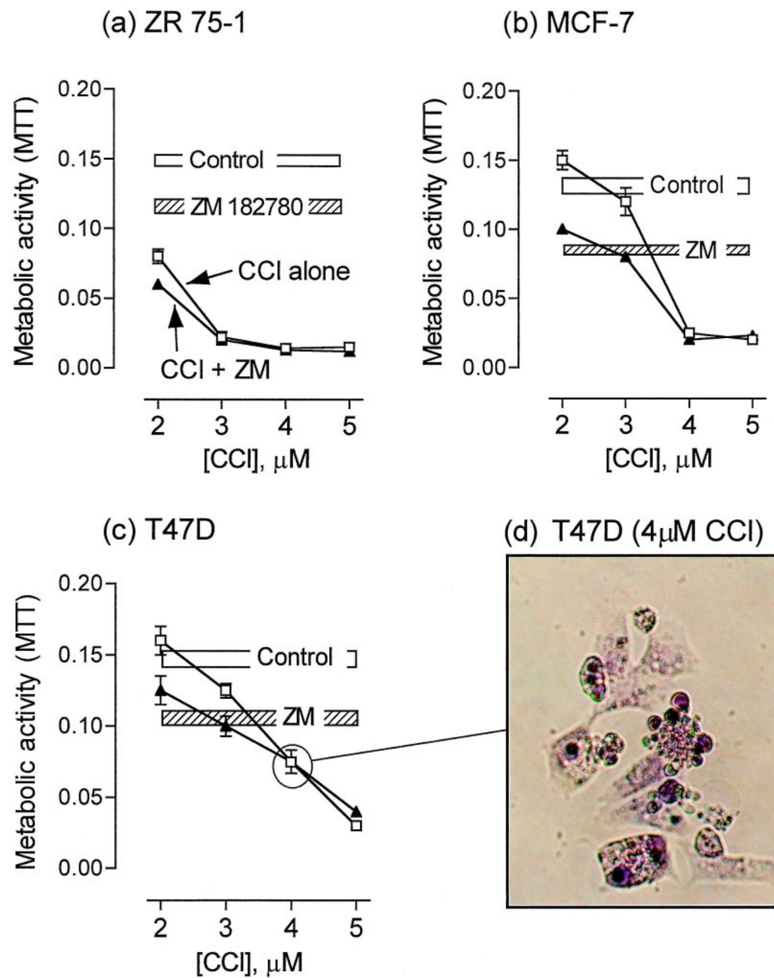

Fig. 7. The response of the breast cancer cells MCF-7 (a), ZR 75 (b) and T47D (c) to a CCl and ZM measured as metabolic activity following the addition of MTT. The light microscope picture of (d) shows the morphology of T47D cells exposed to ZM, and CCl (4 μM) and then exposed to MTT for 4 h.

used to activate ATP synthase. Several studies have shown that respiratory chain 'poisons' induce a permeability transition (PT) and apoptotic cell death [27]. Therefore, MTT-induced ATP depletion may trigger PT opening in a sufficient number of mitochondria to induce apoptotic cell death. Fig. 7, shows that classical apoptotic morphology was induced in T47D breast cancer cells treated with 4 μM CCl alone following MTT exposure. A number of reports show that key enzymes of the glycolytic pathway are regulated by calmodulin [13,28]. Recently we have demonstrated that ZM 182780 pre-treatment of GH_3 cells increases the ease with which oxidants induce a loss of $\Delta\Psi_m$. Our data presented here for GH_3 cells are consistent with these observations and suggest that CCl and ZM 182780 synergise to alter the energetic status of the cell: a condition that predisposes mitochondria to undergo a PT and to initiate the apoptotic process.

At present it is regarded that PTP opening results in the release of pro-apoptotic mitochondrial factors that in turn activate the 'executioners' of apoptosis, the cytosolic caspase [29]. It has been shown for the MCF-7 breast cancer cell line, that estrogens induce the expression of the antiapoptotic protein Bcl-2 [30]. This protein is thought to be one of a complex controlling PTP opening [31]. Whilst this mechanism may also operate in the GH_3 cells, some evidence exists for the related cell line, GH_4C_1, that estrogens control the expression of one of the mitochondrial genome encoded subunits of the terminal electron acceptor, cytochrome c oxidase [32]. For these cells it is possible therefore that a loss of electron flow, due to a reduced cytochrome c oxidase activity, could be one of the mechanisms responsible for the enhancing effect of 'pure' antiestrogens on CCl induced death. It is also possible that the ER has a role in controlling glycolytic flux. Studies have shown that the expression of one of the rate limiting enzymes of glycolysis, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is strongly induced by estrogens [33].

The current report has outlined the evidence to support the initial premise for this study, that a drug combination is more effective than individual agents used alone. However, the work on the breast cancer cell lines has shown that the presence of the ER does not guarantee that antiestrogens will enhance the cytotoxic response to calmodulin antagonism. Therefore, in contrast to the pituitary tumour cells, for the breast cancer cell lines, it would appear that other pathways activated by serum factors or those constituitively expressed, may override the 'metabolic support' provided by the ER. More recently we have demonstrated (Newton, unpublished observation) that blockade of glycolysis enhances CCl-induced apoptosis of breast MCF-7 cells. Together these data highlight the importance of metabolic events as triggers for the apoptotic process. In future studies, it may well prove instructive to determine the extent to which the ER controls the expression of key enzymes of the glycolytic and respiratory pathways in a range of ER containing tumour cells.

Acknowledgements

The authors wish to thank Yorkshire Cancer Research and The Hull and East Riding Charitable Trust for their support during this study.

References

[1] B.S. Katzenellenbogen, H.S. Bahkoo, E.R. Ferguson, N.C. Lan, T. Tatee, T.L.S. Tsai, J.A. Katzenellenbogen, Estrogen and antiestrogen action in reproductive tissues and tumours, Recent. Prog. Horm. Res. 35 (1979) 259–274.

[2] A. Howell, Antiestrogens: future prospects, Oncology 11 (1995) 59–64.

[3] A.E. Wakeling, M. Dukes, J. Bowler, A potent specific pure antiestrogen with clinical potential, Cancer Res. 51 (1991) 3867–3873.

[4] D.P. McDonnell, B.A. Lieberman, J. Norris, Development of tissue-selective estrogen receptor modulators, in: Baird Schutz (Ed.), Organ-selective actions of steroid hormones, Springer-Verlag, Krattenmacher, 1995.

[5] K. Paech, P. Webb, G.J.M. Kuiper, S. Nilsson, J.A. Gustafsson, P.J. Kushner, T.S. Scanlan, Differential ligand activation of estrogen receptors ERα and ERβ at AP-1 sites, Science 277 (1997) 1508–1510.

[6] G.J.M. Kuiper, J.A. Gustafsson, The novel estrogen receptor-β subtype: potential role in the cell- and promotor-specific actions of estrogens and anti-estrogens, FEBS Lett. 410 (1997) 87–90.

[7] C.J. Newton, R. Buric, T. Trapp, S. Brockmeier, U. Pagotto, G.K. Stalla, The unliganded estrogen receptor (ER) transduces growth factor signals, J. Steroid Biochem. Molec. Biol. 48 (1994) 481–486.

[8] A. Decensi, V. Fontana, S. Bruno, C. Gustavino, B. Gatteschi, A. Costa, Effect of tamoxifen on endometrial proliferation, J. Clin. Oncol. 14 (1996) 434–440.

[9] M. Sato, T.A. Grese, J.A. Dodge, H.U. Bryant, C.H. Turner, Emerging therapies for the prevention of postmenopausal osteoporosis, J. Med. Chem. 42 (1999) 1–24.

[10] C.J. Newton, K. Schlatterer, G.K. Stalla, E. von Angerer, B. Wowra, Pharmacological enhancement of radiosurgery response: studies on an in vitro model system, J. Radiosurgery. 1 (1998) 51–56.

[11] C.J. Newton, N. Drummond, C.H. Burgoyne, V. Speirs, G.K. Stalla, S.L. Atkin, Functional inactivation of the oestrogen receptor by the antioestrogen, ZM 182780, sensitises tumour cells to reactive oxygen species, J. Endocrinol. 161 (1999) 199–210.

[12] N. Zamzami, T. Hirsch, B. Dallaporta, P.X. Petit, G. Kroemer, Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis, J. Bioenergetic. Biomemb. 29 (1997) 185–193.

[13] L. Glass-Marmor, R. Beitner, Detachment of glycolytic enzymes from cytoskeleton of melanoma cells induced by calmodulin antagonists, Eur. J. Pharmacol. 328 (1997) 241–248.

[14] S. Martin, G.K. Schwartz, Chemotherapeutically induced

DNA damage, ATP depletion, and the apoptotic cascade, Oncology Res. 9 (1997) 1–5.

[15] A. Bouhoute, G. Leclercq, Modulation of estradiol and DNA binding to estrogen receptor upon association with calmodulin, Biochem. Biophys. Res. Commum. 208 (1995) 748–755.

[16] I.R. Hardcastle, M.G. Rowlands, R.M. Grimshaw, J. Houghton, M. Jarman, Homologues of idoxifine: variation of estrogen receptor binding and calmodulin antagonism with chain length, J. Med. Chem. 39 (1996) 999–1004.

[17] C.J. Newton, R. Buric, T. Trapp, S. Brockmeier, U. Pagotto, G.K. Stalla, The unliganded estrogen receptor (ER) transduces growth factor signals, J. Steroid Biochem. Molec. Biol. 48 (1994) 481–486.

[18] T.F. Slater, B. Sawyer, U. Sträuli, Studies on succinate-tetrazolium reductase systems III. Points of coupling of four different tetrazolium salts, Biochem. et Biophys. Acta. 77 (1963) 383–393.

[19] C.J. Newton, Estrogen receptor blockade by the pure antiestrogen, ZM 182780, induces death of pituitary tumour cells, J. Steroid Biochem. Molec. Biol. 55 (1995) 327–336.

[20] C.J. Newton, E. Arzt, G.K. Stalla, Involvement of the estrogen receptor in the growth response of pituitary tumour cells to interleukin-2, Biochem. Biophys. Res. Comm. 205 (1994) 1930–1937.

[21] R.A. Schwartzman, J.A. Cidlowski, Apoptosis: The biochemistry and molecular biology of programmed cell death, Endocr. Rev. 14 (1993) 133–151.

[22] S. Sweet, G. Singh, Accumulation of humal promyelocytic leukemic (HL-60) cells at two energetic cell cycle checkpoints, Cancer Res. 55 (1995) 5164–5167.

[23] P. Van de Velde, F. Nique, F. Bouchoux, J. Bremaud, M.C. Hameau, D. Lucas, C. Moratille, S. Viet, D. Philibert, G. Teutsch, RU 58668 a new pure antiestrogen inducing a regression of human mammary carcinoma implanted in nude mice, J Steroid Biochem Molec Biol. 48 (1994) 187–196.

[24] J.S. Strobel, V.A. Peterson, K.A. Woodfork, A survey of human breast cancer sensitivity to growth inhibition by calmodulin antagonists in tissue culture, Biochem. Pharmacol. 47 (1994) 2157–2161.

[25] O.S. Frankfurt, E.V. Sugarbaker, J.A. Robb, L. Villa, Synergistic induction of apoptosis in breast cancer cells by tamoxifen and calmodulin inhibitors, Cancer Lett. 97 (1995) 149–154.

[26] V.C. Jordan, C.S. Murphy, Endocrine pharmacology of antiestrogens as antitumour agents, Endocrine Rev. 11 (1990) 578–610.

[27] E.J. Wolvetang, K.L. Johnson, K. Krauer, S.J. Ralph, A.W. Linnane, Mitochondrial respiratory chain inhibitors induce apoptosis, FEBS Lett. 339 (1994) 40–44.

[28] T. Livnat, M. Chen-Zion, R. Beitner, Stimulatory effect of epidermal growth factor on binding of glycolytic enzymes to muscle cytoskeleton and the antagonistic action of calmodulin inhibitors, Biochem. Med. Metab. Biol. 50 (1992) 24–34.

[29] M.O. Hengartner, Death cycle and Swiss army knives, Nature 391 (1998) 441–442.

[30] T.T.Y. Wang, J.M. Phang, Effects of estrogens on apoptotic pathways in human breast cancer cell line MCF-7, Cancer Res. 55 (1995) 2487–2489.

[31] G. Kroemer, N. Zamzami, S.A. Susin, Mitochondrial control of apoptosis, Immunol. Today 18 (1997) 44–51.

[32] C.M. Van Itallie, P.S. Dannies, Estrogen induces accumulation of the mitochondrial ribonucleic acid for subunit II of cytochrome oxidase in pituitary tumour cells, Molec. Endocrinol. 2 (1988) 332–337.

[33] L. Sahlin, Dexamethasone attenuates the estradiol-induced increase of IGF-I mRNA in the rat uterus, J. Steroid. Biochem. Mol. Biol. 55 (1995) 9–15.