Tamoxifen Suppresses the Growth of Malignant Pleural Mesothelioma Cells

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Abstract. *Introduction: Malignant pleural mesothelioma (MPM) is a rare but highly aggressive malignancy most often associated with exposure to asbestos. Recent evidence points to oestrogen receptor (ER)-β having a tumour-suppressor role in MPM progression, and this raises the question of whether selective modulators of ERs could play a role in augmenting MPM therapy. Materials and Methods: We investigated the action of tamoxifen in inhibiting the growth and modulating the cisplatin sensitivity of four MPM cell lines. Results: Tamoxifen inhibited the growth of MPM cells and also modulated their sensitivity to cisplatin. The MPM cell lines expressed ERβ, but the actions of tamoxifen were not blocked by antagonism of nuclear ERs. Tamoxifen treatment repressed the expression of cyclins by MPM cells, resulting in cell-cycle arrest and caspase-3-coupled apoptosis signaling. Conclusion: The ER-independent actions of tamoxifen on MPM cell proliferation and cell-cycle progression may have clinical benefits for a subset of patients with MPM.*

Malignant pleural mesothelioma (MPM) is a rare neoplasm arising from the mesothelial cells lining the thoracic cavity, and is highly associated with exposure to asbestos (1). MPM has a long latent period, with the malignancy developing more than 30 years after exposure to the carcinogen, and an extremely poor post-diagnosis median survival time of 9 to 12 months (2, 3). The preferred therapy for MPM is determined by a number of factors, including age and associated co-morbidities, but intervention is essentially limited to surgery

(extra-pleural pneumonectomy) and chemotherapy (4). The chemotherapeutic drug of choice is cisplatin but survival is enhanced when used in combination with pemetrexed. The capacity for these interventions to prolong life is highly variable and is influenced by factors including the histological type and tumour stage at diagnosis (5).

There is a gender dichotomy in MPM survival that is, at least in part, the result of circulating oestrogens acting through the beta isoform of the oestrogen receptor (ERβ) to attenuate MPM cell growth (6), indicating MPM as an endocrine-responsive malignancy. Selective ER modulators, including tamoxifen and fulvestrant (ICI 182,780), are important therapeutic agents in the treatment of oestrogen-sensitive cancer. The suppression of breast carcinoma cell growth through ERα antagonism is an important facet of first-line treatment for this malignancy. The question remains as to whether ER modulation could play a role in novel interventions for MPM. Two small phase II and phase III clinical trials were performed to investigate whether post-surgical adjuvant therapy using cisplatin in combination with interferon-α and tamoxifen (CIT) had a patient survival benefit (7, 8). The results of these trials were inconclusive and the aim of the present study was to establish whether tamoxifen affects MPM cell growth and sensitivity to cisplatin.

Materials and Methods

Cell culture. The REN, MPP-89, Msto-211H (Instituto Scientifico Tumouri, Genoa, Italy), H2052 MCF-7 and MET5A (American Type Culture Collection, Teddington, UK) cell lines were routinely maintained as described previously (9). For experimental purposes, cells were maintained in oestrogen-free medium for 24 h prior to treatment (10). Cells were treated with vehicle control, tamoxifen (100 nM to 10 μM) or cisplatin (100 nM to 50 μM) over a time over a period of 72 h. MPM cell viability was measured after treatment using the Cell-Titer 96 Assay (Promega, Southampton, UK). Synergistic effects of tamoxifen with cisplatin were investigated by

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Table I. *Primer pairs used in reverse transcription-polymerase chain reaction analysis.*

Gene	Forward primer	Reverse primer
18S/*RNA18S1*	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT
ERα/*ESR1*	CACATGAGTAACAAAGGCATGG	ATGAAGTAGAGC CCGCAGTG
ERβ/*ESR2*	GTCAGGCATGCGAGTAACAA	GGGAGCCCTCTTTGCTTTTA
p14/*CDKN2A*	CCCTCGTGCTGATGCTACTG	CATCATGACCTGGTCTTCTAGGAA
p15/*CDKN2B*	GGGAAAGAAGGGAAGAGTGTCGTT	GCATGCCCTTGTTCTCCTCG
p16/*CDKN2A*	GGGGGCACCAGAGGCAGT	GGTTGTGGCGGGGGCAGT T
p21/*CDKN1A*	GCATGACAGATTTCTACCACTCC	AAGATGTAGAGCGGGCCTTT
p27/*CDKN1B*	CGCCATATTGGGCCACTAA	CGCAGAGCCGTGAGCAA
Cyclin A2/*CCNA2*	CTGCTGCTATGCTGTTAGCC	TGTTGGAGCAGCTAAGTCAAAA
Cyclin B1/*CCNB1*	ATAAGGCGAAGATCAACATGGC	TTTGTTACCAATGTCCCCAAGAG
Cyclin D1/*CCND1*	CTGGAGGTCTGCGAGGAA	GGGGATGGTCTCCTTCATCT
Cyclin D3/*CCND3*	GAGCTGCTGTGTTGCGAAG	GCATGTGCGGCTTGATCTC

treating cells with tamoxifen (5 μM) before the addition of cisplatin (0.1 μM to 50 μM) and then measuring viability. The involvement of ERs in transducing the effects of tamoxifen were investigated by pre-incubation with ICI182,780 (1 μM) or MPP (100 nM) before tamoxifen treatment.

Western blotting. MPM cells were incubated in oestrogen-free medium for 24 h before the treatments indicated. Lysates were prepared and subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis and western blotting using standard techniques. Following incubation with primary antibodies. All primary antibodies were purchased from Cell Signaling Technology (Leiden, The Netherlands) except for ERα and ERβ antibodies, which were obtained from Leica Microsystems (Ashbourne, Ireland). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Sigma, Arklow, Ireland) and visualized using the ECL+ detection system (GE Healthcare, Little Chalfont, UK).

Quantitative polymerase chain reaction. Total RNA was extracted from treated cells using the RNeasy extraction kit (Qiagen, Crawley, UK). RNA was quantified and its purity measured using a NanoDrop 8000 spectrophotometer (Eppendorf, Stevenage, UK). Reverse transcription of isolated mRNA to synthesize cDNA was performed using ImProm-II kit (Promega). Real-time (RT) PCR using a 7500 Fast System and SYBR Green reagents (Applied Biosystems, Warrington, UK) was used to quantify the abundance of chosen mRNA species (Table I). The abundance of each of the mRNA species was normalised against 18S rRNA and expressed as a fold change relative to untreated cells.

Immunohistochemistry. Eighty-nine cases of MPM were identified from the archive of the Pathology Unit, Regional Hospital of Mestre-Venice, Italy. Informed patient consent for access to tissue was obtained according to local ethical regulations. The tissue samples were taken following videothoracoscopy biopsy or from resected surgical specimens, fixed in neutral formalin and embedded in paraffin. Primary antibodies specific for ERα and ERβ were used in this study and the immunohistochemistry parameters for each antibody were initially optimized using a breast carcinoma tissue microarray as described previously (11). Slides were counterstained with haematoxylin.

Apoptosis assays. Caspase-3 plays an important role in apoptosis by cleaving key cellular proteins such as DNA-fragmentation factor and poly (ADP-ribose) polymerase (PARP). Caspase-3 is initially present as a 32 kDa precursor protein, which is recruited into active heterodimeric complexes when cells undergo apoptosis (12). Activated caspase-3 is composed of two subunits, p20 and p11, which result from proteolytic processing of the 32-kDa precursor during apoptosis. A second processing step involving the removal of a pro-domain from the p20 subunit generates a p17 subunit. Caspase-3 cleavage was evaluated by western blotting as described above.

For lactose dehydrogenase (LDH) release assays, $3×10^3$ cells per well were plated in 96-well plates in phenol red-free RPMI-1640 containing 1% charcoal-stripped serum. Following overnight incubation, cells were treated with different concentrations of tamoxifen or with ethanol vehicle. Cell cytotoxicity was assessed 24 h and 48 h after treatment using the LDH Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions.

Results

Cisplatin and tamoxifen effects on MPM cell growth. The MPM cell lines were treated with tamoxifen over a concentration range of 0 to 10 μM for up to 72 h. Cell viability assays were performed at 24 h, 48 h and 72 h (Figure 1A). The four MPM cell lines had different sensitivities to tamoxifen and only H2052 cells had a 50% inhibitory concentration (IC_{50}) <10 μM after 72 h. Phase-contrast microscopy revealed that after 48 h treatment with 1 μM tamoxifen (Figure 1B), the fibroblastic Msto-211H and H2052 cells showed a marked change in morphology including shrinkage, irregular shape and condensed chromatin in the nuclei. A higher tamoxifen concentration (5 μM) induced elongated lamellipodia in both cell lines and cell detachment.

The cisplatin sensitivity of the MPM cell lines was also evaluated (Figure 2A). The Msto-211H cell line was the most sensitive to cisplatin (IC_{50}=3 μM); the MPP-89 cells were the next highest in sensitivity (IC_{50}=7.5 μM); while the REN and H2052 cells were relatively insensitive to cisplatin (IC_{50} >50 μM and IC_{50}=45 μM respectively).
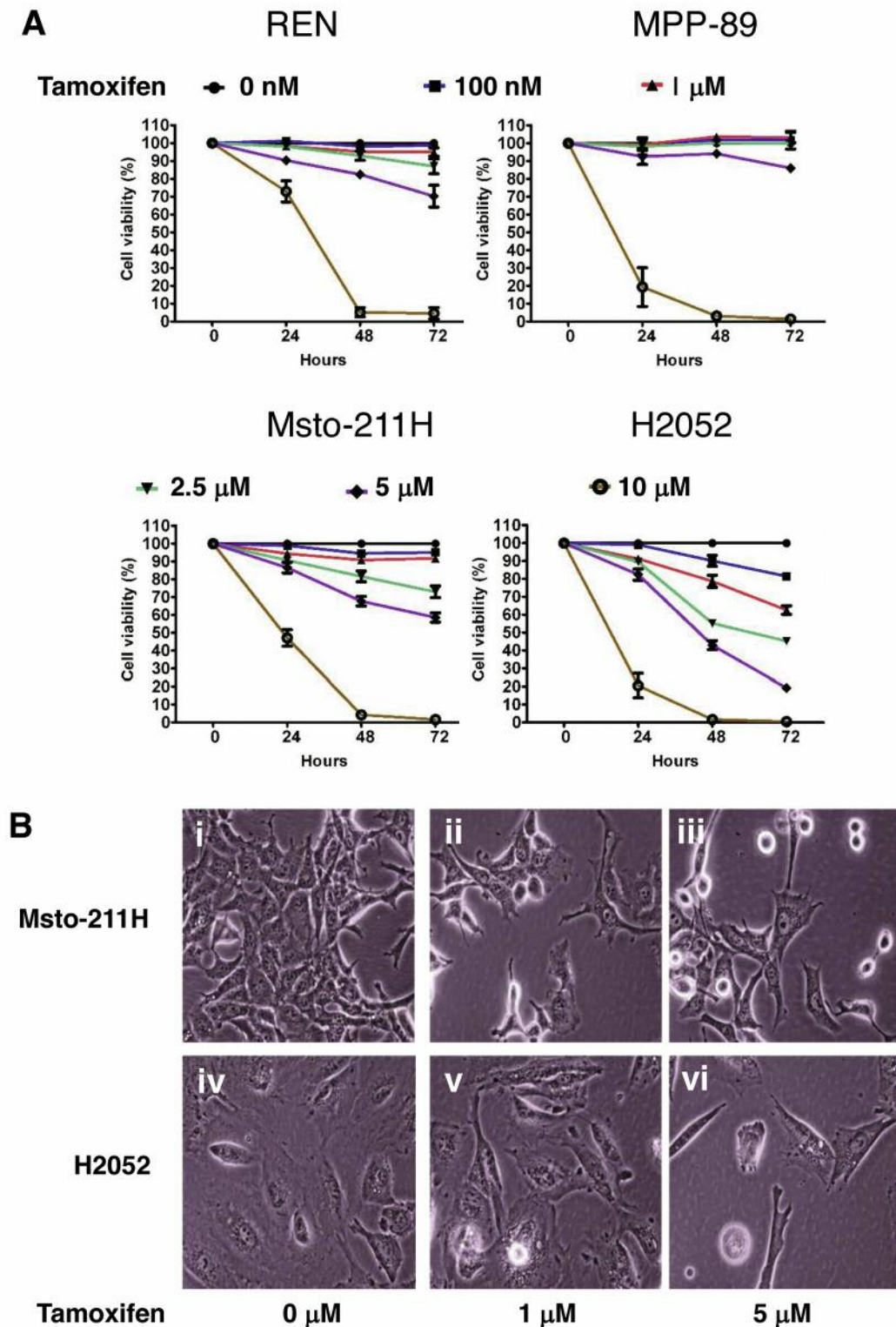
Figure 1. *A: Cell-Titer 96 cell viability assays for REN, MPP-89, Msto-211H and H2052 malignant pleural mesothelioma (MPM) cells treated with 0 to 10 μM tamoxifen over a time course of 0 to 72 h. Data are the mean±SE of three independent experiments. B: Phase-contrast microscopy images of MPM cells treated with tamoxifen over a concentration range of 0 to 5 μM for 48 h. Msto-211H (i-iii) and H2052 (iv-vi) cells were treated with tamoxifen at 1 μM (ii and v), 5 μM (iii and vi) or vehicle (ethanol diluted in medium to the same concentration as the ethanol used to initially dissolve tamoxifen); (i and iv). Representative images are shown at ×40 magnification.*
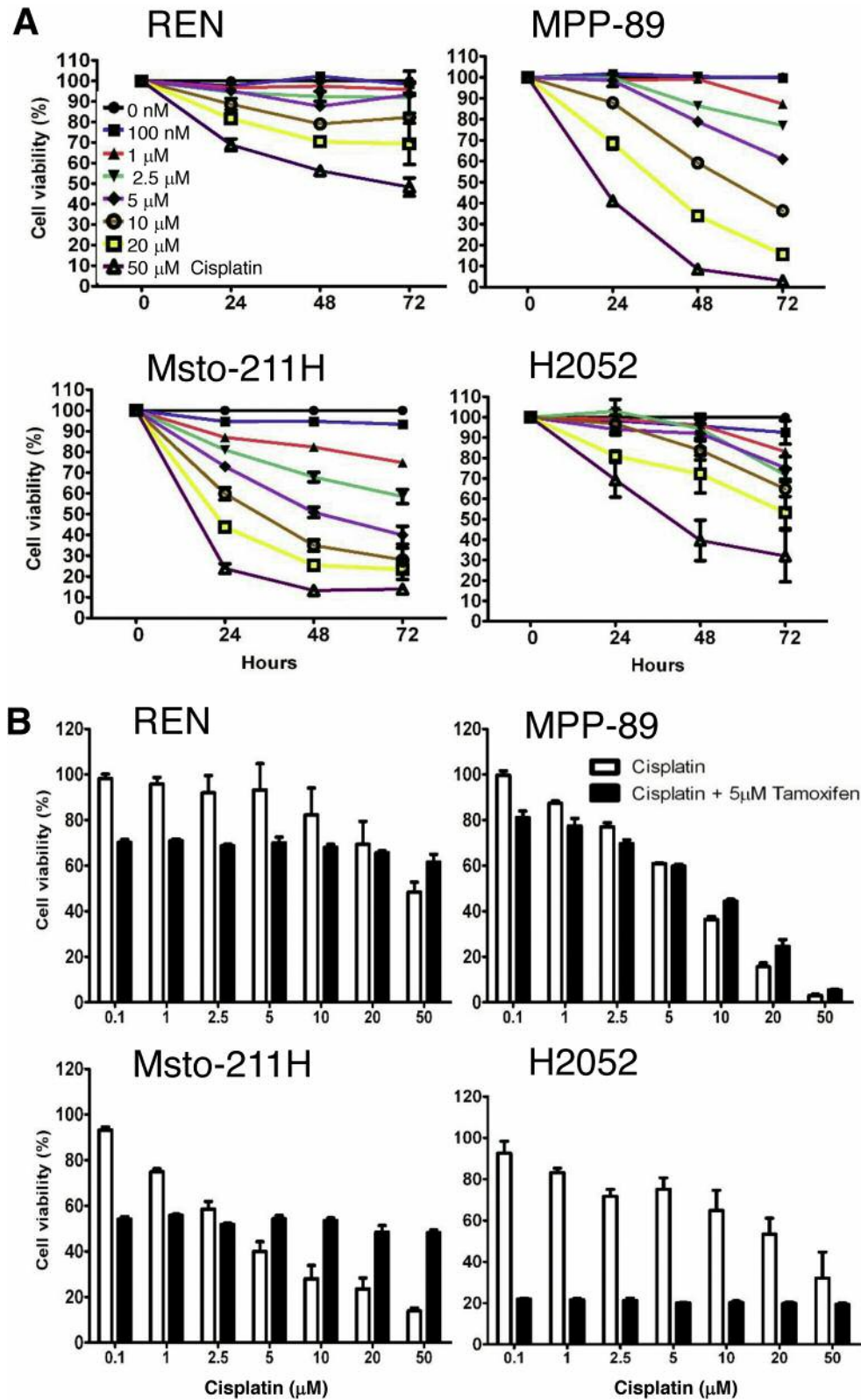
Figure 2. *Cell-Titer 96 cell viability assays for REN, MPP-89, Msto-211H and H2052 malignant pleural mesothelioma (MPM) cells treated with 0 to 50 μM tamoxifen over a time course of 0 to 72 h (A) or with cisplatin over a concentration range of 0 to 50 μM for 48 h in the presence or absence of 5 μM tamoxifen (B). Data are the mean±SE of three independent experiments.*
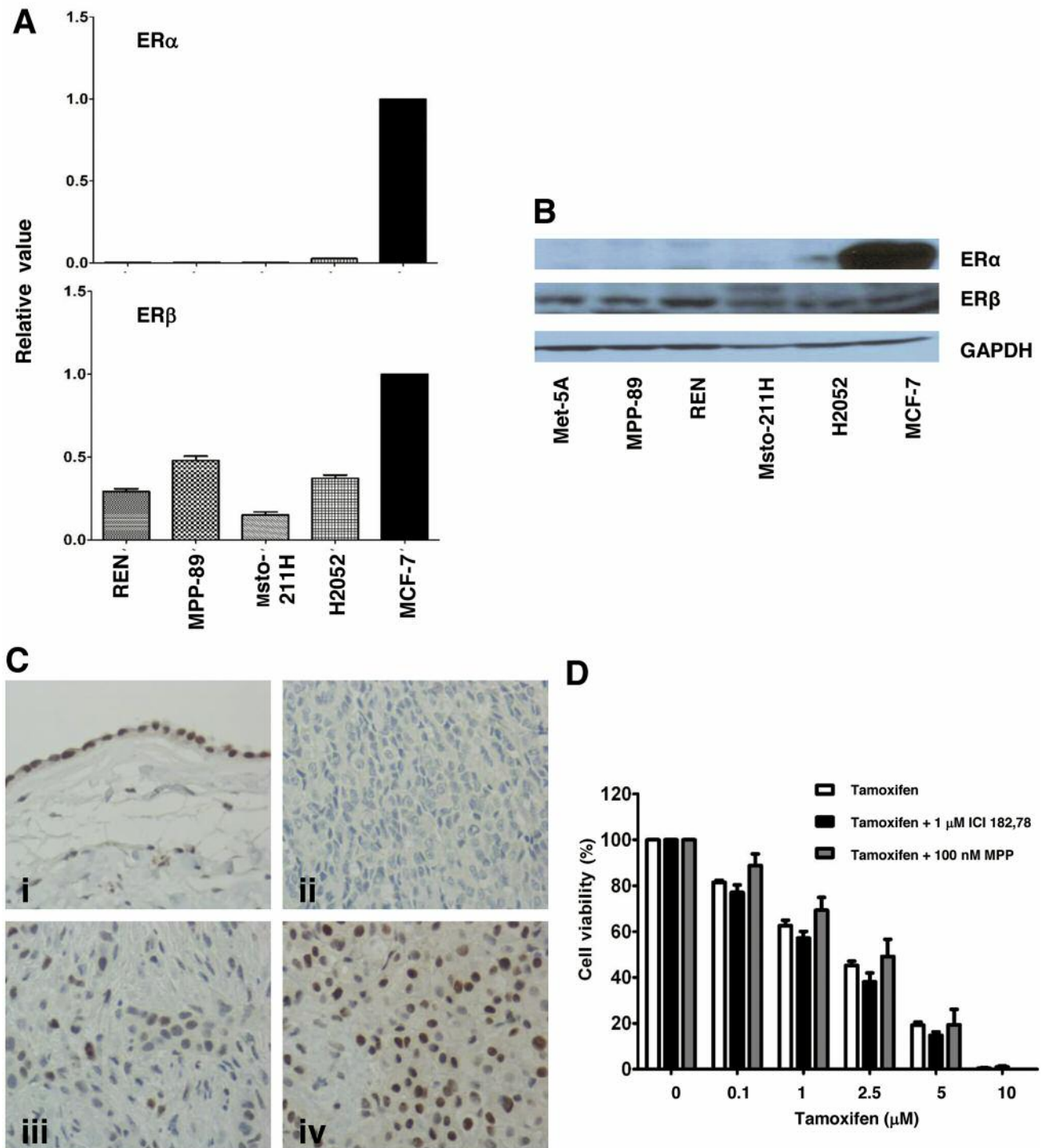
Figure 3. *A: Quantitative polymerase chain reaction analysis of oestrogen receptor (ER)-α and ERβ mRNA abundance in REN, MPP-89, Msto-211H and H2052 malignant pleural mesothelioma (MPM) cells. Data presented are the mean of three independent experiments calculated as fold difference relative to expression in MCF-7 breast cancer cells. B: Western blot analysis of ERα and ERβ expression by REN, MPP-89, Msto-211H, H2052 MPM cells, MET-5A non-malignant mesothelial cells and MCF-7 breast carcinoma cells. Data are representative of two independent experiments and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) expression was used as an equal loading control. C: Detection of ERβ expression by immunoperoxidase staining in normal pleural membrane (i) and in MPM tumour biopsies (ii-iv). The mages are representative of 89 tumour specimens examined. D: Cell viability assays for H2052 MPM cells treated with 0 to 10 μM tamoxifen in the presence or absence of MPP (100 nM) or ICI 182,780 (1 μM) for 48 h. Data are the mean±SE of three independent experiments.*
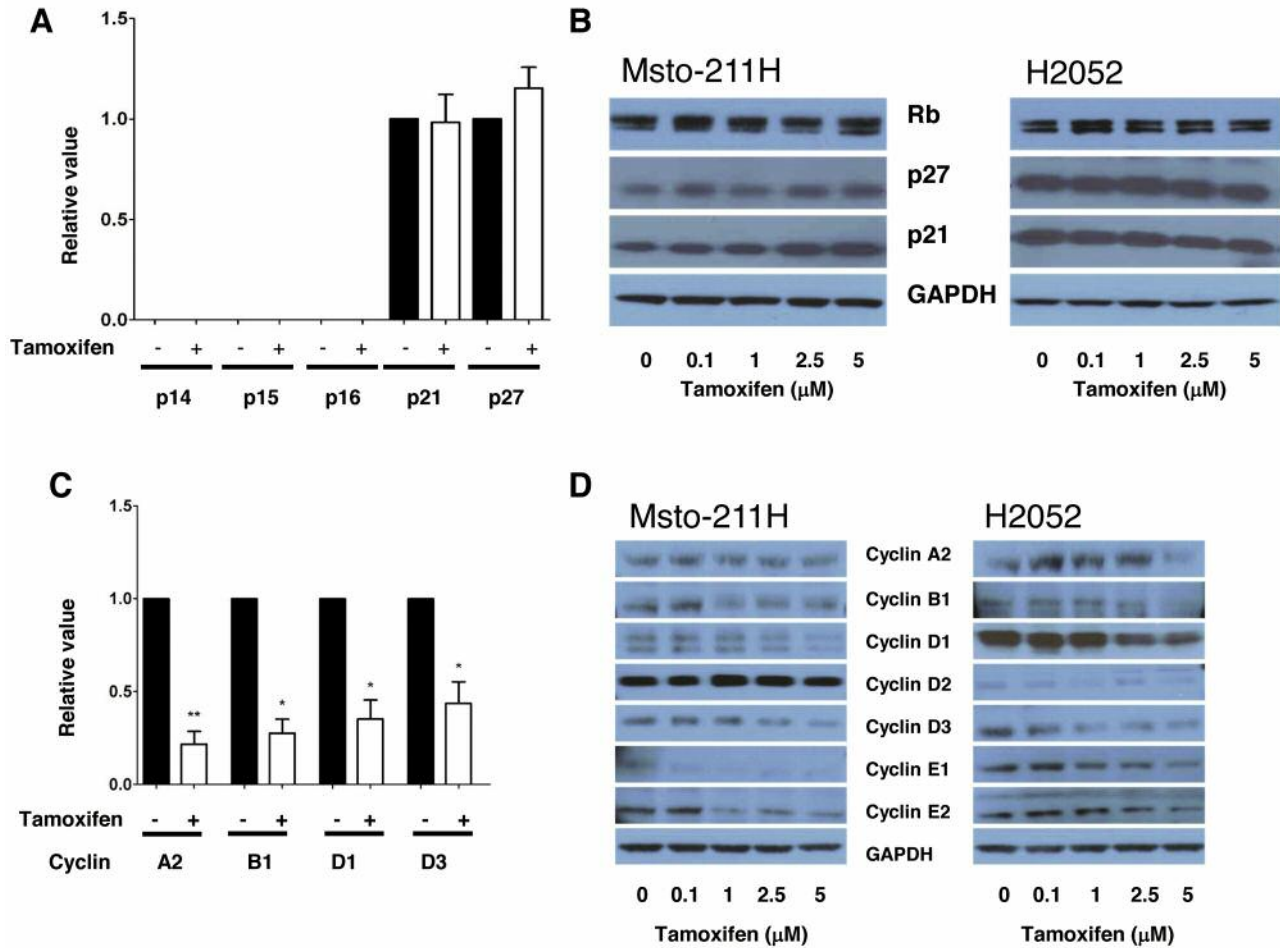
Figure 4. *A: Quantitative polymerase chain reaction (PCR) analysis of mRNA expression of p14, p15, p16, p21 and p27 cyclin-dependent kinase inhibitors by H2052 malignant pleural mesothelioma (MPM) cells treated with tamoxifen (5 µM) or vehicle for 48 h. Data are the mean±SE of three independent experiments. B: Western blot analysis of retinoblastoma protein (Rb), p27 and p21 expression by Msto-211H and H2052 cells following treatment with 0 to 5 µM tamoxifen for 48 h. Data are representative of two independent experiments and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) expression was used as an equal loading control. C: Quantitative PCR analysis of cyclin A2, cyclin B1, cyclin D1 and cyclin D3 mRNA expression by H2052 MPM cells treated with tamoxifen (5 µM) or vehicle for 48 h. Data are the mean±SE of three independent experiments calculated as fold change relative to vehicle-treated cells. D: Western blot analysis of cyclin A2, cyclin B1, cyclin D1, cyclin D2, cyclin D3, cyclin E1 and cyclin E2 expression by Msto-211H and H2052 MPM cells following treatment with 0 to 5 µM tamoxifen for 48 h. Data presented are representative of two independent experiments and GAPDH expression was used as an equal loading control. Significantly different at *p<0.05, and **p<0.01.*

We performed cell viability assays to establish whether co-treatment with tamoxifen (5 µM) affected the sensitivity of MPM cells to cisplatin treatment (Figure 2B). Each of the cell lines displayed different responses. There was no effect of tamoxifen co-treatment on the cisplatin sensitivity of MPP-89 cells which is in agreement with this cell line having the lowest sensitivity to tamoxifen (IC$_{50}$=7.5 µM with/without tamoxifen). Tamoxifen co-treatment was more cytotoxic for REN cells at doses of cisplatin below 20 µM, but tamoxifen suppressed the sensitivity to cisplatin at concentrations of >20 µM. The effect was more pronounced for Msto-211H cells, where co-treatment with 5 µM tamoxifen enhanced cytotoxicity at low doses of cisplatin (<2.5 µM), but antagonized the action of cisplatin at higher doses, suggesting that for this cell line, tamoxifen suppressed sensitivity to high-dose cisplatin. H2052 cells were highly sensitive to tamoxifen and there was an 80% loss of viability at all cisplatin concentrations tested. Tamoxifen alone or in combination with low doses of cisplatin may be effective in reducing tumour growth in a subset of patients, but the antagonistic action of tamoxifen at higher cisplatin concentrations would be detrimental.
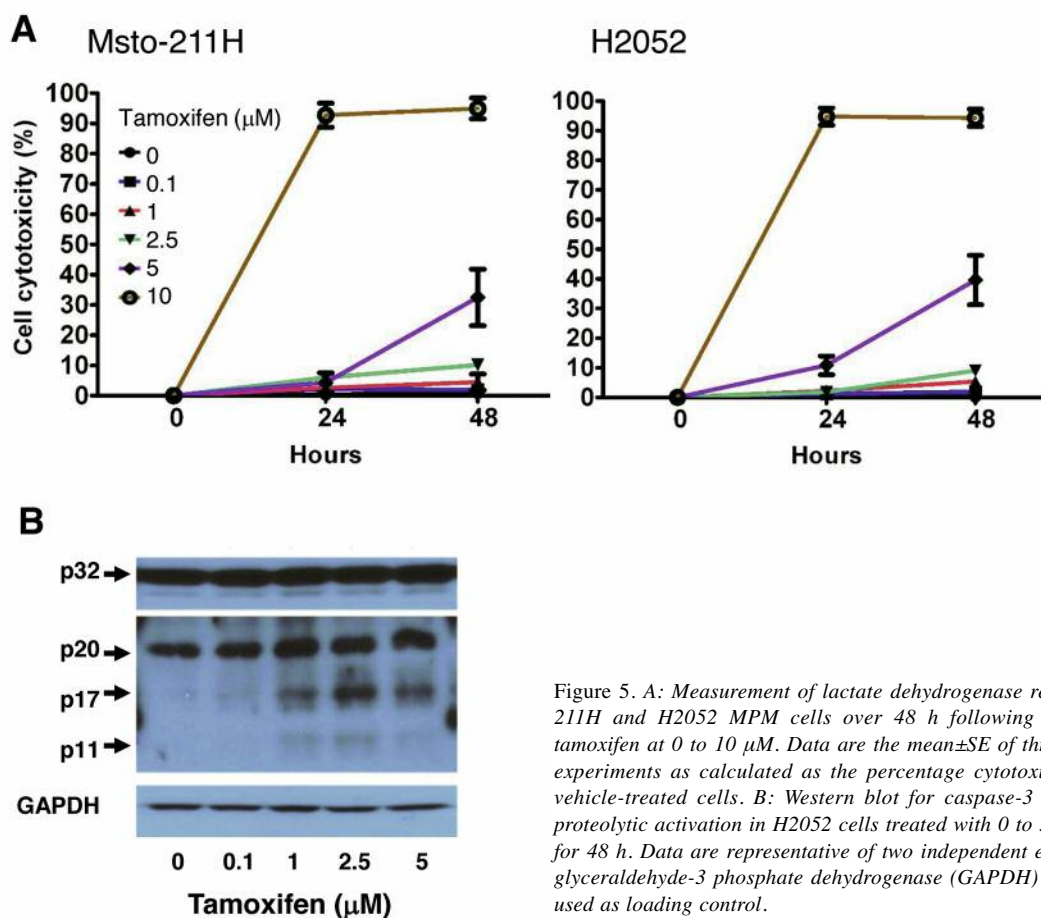
Figure 5. *A: Measurement of lactate dehydrogenase release by Msto-211H and H2052 MPM cells over 48 h following treatment with tamoxifen at 0 to 10 μM. Data are the mean±SE of three independent experiments as calculated as the percentage cytotoxicity relative to vehicle-treated cells. B: Western blot for caspase-3 expression and proteolytic activation in H2052 cells treated with 0 to 5 μM tamoxifen for 48 h. Data are representative of two independent experiments and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) expression was used as loading control.*

The action of tamoxifen on MPM cell growth is ER-independent. ERα mRNA was not expressed in REN, MPP-89 or Msto-211H cell lines but was detectable in H2052 cells (Figure 3A). ERβ mRNA was variably expressed by all four of the cell lines at a comparable abundance with that of the MCF-7 cells. ERα protein was detectable in H2502 cells but only after overexposure of the western blot, and was absent from the other three MPM cell lines (Figure 3B). ERβ protein was variably expressed in all cell lines, consistent with the quantitative PCR data. ERβ abundance varied between the 89 individual tumour specimens, with high levels of expression observed in normal mesothelial cells (Figure 3Ci), and very high to negative expression in tumour samples (Figure 3Cii-iv). ERα expression was not detected in any of the specimens (data not shown). ER involvement in MPM growth inhibition by tamoxifen was investigated using specific ER antagonists. ERα antagonism using MPP (100 nM) had no effect on H2052 MPM cell-growth inhibition by tamoxifen (Figure 3D). ICI182,780 is a highly specific antagonist for both ERα and ERβ, with no agonist activity. ICI182,780 (1 μM) co-treatment did not attenuate the effects of tamoxifen on H2052 MPM cell growth.

The effect of tamoxifen on cyclin-dependent kinase (CDK) inhibitor and cyclin expression. H2052 cells were most sensitive to tamoxifen and were therefore used to determine if tamoxifen affects CDK inhibitor expression in MPM cells. The cells were treated with 5 μM tamoxifen for 48 h and RT-PCR was carried out to measure *p14, p15, p16, p21* and *p27* mRNA abundance (Figure 4A). There was no detectable *p14, p15* or *p16* expression in H2052 cells and this was not affected by tamoxifen treatment. The expression of *p21* and *p27* was detected in H2052 cells but the abundance of mRNA (Figure 1A) and protein (Figure 4B) was not affected by tamoxifen treatment.

Cyclins are pivotal in regulating cell-cycle progression and are overexpressed in many malignancies. Quantitative PCR was used to measure cyclin A2, cyclin B1, cyclin D1 and cyclin D3 mRNA abundance following tamoxifen treatment (5 μM) of H2052 cells for 48 h (Figure 4C).

Tamoxifen treatment caused a significant reduction in the abundance of cyclin A2, cyclin B1, cyclin D1 and cyclin D3 mRNA in H2052 cells. These transcriptional changes following tamoxifen treatment were mirrored by changes in the abundance of the cyclin proteins as determined by western blotting (Figure 4D). Cyclin A2 was reduced on treatment at a concentration of 5 μM tamoxifen in H2052 cells, however, no significant reduction was observed in Msto-211H cells. Cyclin B1 protein expression was reduced in both Msto-211H and H2052 cells, with the greatest reduction observed at the highest concentration of tamoxifen. Cyclin D1 and cyclin D3 protein expression levels were reduced in both Msto-211H and H2052 cells following tamoxifen treatment; however, no changes in cyclin D2 protein expression levels were observed. Both cyclin E1 and cyclin E2 were also reduced following tamoxifen treatment in Msto-211H and H2052 cells.

Tamoxifen promotes apoptosis of MPM cells. Tamoxifen at concentrations of >1 μM had significant effects on the morphology of MPM cells (Figure 1B) and these morphological changes were consistent with apoptosis or necrosis. LDH assays were performed to evaluate the direct cytotoxicity of tamoxifen on MPM cells. LDH release is a marker of cytotoxicity and is associated with cell membrane disintegration. Tamoxifen treatment caused the dose-dependent release of LDH from both Msto-211H and H2052 cells (Figure 5A). H2052 cells were treated with tamoxifen over a concentration range of 0.1 to 5 μM for 48 h and the abundance and proteolytic activation of caspase-3 was evaluated by western blotting (Figure 5B). Tamoxifen treatment did not affect the abundance of pro-caspase-3, but a tamoxifen concentration as low as 1 μM did increase the abundance of the activated caspase-3 p17 and p11 subunits, indicating that tamoxifen stimulated apoptosis of H2052 cells.

Discussion

MPM cells either do not express ERα or its abundance is extremely low in the case of the H2052 cells, and the mechanisms that transduce tamoxifen action independently of ERα activation in other experimental systems are controversial (13, 14). Tamoxifen inhibits the survival of other tumour cell types *via* diverse ER-independent mechanisms, including modulation of the action of transforming growth factor (TGF)-β and c-MYC activation of Janus kinase signalling, enhanced oxidative stress, increased ceramide generation, and alterations in mitochondrial permeability transition (15). Tamoxifen inhibits cellular proliferation in a variety of tissue and cancer types through up-regulation of CDK inhibitors such as p15 (16), p16 (17), p21 (16, 17) and p27 (18). The activation of ERβ by oestrogen suppresses MPM cell growth by mechanisms

which include the transcriptional regulation of CDK inhibitors (6). Data from our present study indicate that tamoxifen acts through different mechanisms that modulate the expression of cyclins rather than CDK inhibitors, and that this suppression in cyclin expression is ERβ-independent.

Tamoxifen treatment induces apoptosis of breast cancer cells (19), cholangiocarcinoma cells (20) and rhabdomyosarcoma cells (21), at least in part, through induction of caspase-3 activity. The stimulation of apoptosis by tamoxifen has been described in a number of tissues and pathological conditions. Various mechanisms have been ascribed to these actions including calmodulin antagonism, c-MYC induction, protein kinase C activation, caspase-3 activation and TGFβ induction [reviewed in (15). In the present study, we found that tamoxifen treatment simultaneously induced apoptosis and cell-cycle arrest in MPM cells. In common with our observations for MPM, tamoxifen induced cell death in malignant peripheral nerve sheath tumours at <5 μM and inhibited mitosis at <1 μM; these effects were independent of nuclear ER, but were mimicked by direct calmodulin inhibition (22). The interaction of calmodulin with other effector proteins is modulated by the availability of intracellular Ca^{2+}, and the dysregulation of intracellular Ca^{2+} homeostasis has also been attributed to ER-independent actions of tamoxifen on breast cancer cells (23).

In conclusion, the growth-inhibitory effect of tamoxifen on MPM cells is achieved through suppression of cyclin expression and the induction of caspase-3 activation *via* ER-independent mechanisms. Tamoxifen may have potential as a therapeutic agent in the treatment of MPM; however, its use in combination therapies may be compromised by its effects on slowing cell-cycle progression, which renders MPM cells less sensitive to platinum-based chemotherapy.

Acknowledgements

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