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13. ABSTRACT (Maximum 200 Words)

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with promise for cancer chemotherapy. 2-ME can arrest mitosis and induce apoptosis in a wide variety of cancer cells, including androgen-independent prostate cancer. To better understand its anti-prostate cancer action, we have focused on events related to mitotic cell cycle arrest (G2/M) and induction of apoptosis in LNCaP, DU 145, and PC-3 human prostate cancer cell lines. Treatment with 2-ME for 24 h increased cyclin B1 protein and its associated kinase (cdk1) activity followed by later induction of apoptosis at 48 and 72 h. Cyclin-dependent kinase inhibitors alsterpaullone and purvalanol A prevented 2-ME-mediated increase in cyclin B1-dependent kinase activity and induction of apoptosis. Decrease in cyclin B1- and cyclin A-dependent kinase activity occurred at later times when apoptosis was increased. It is likely, however, that the higher levels of the anti-apoptotic proteins Bcl-xL and survivin in DU 145 and PC-3 compared to LNCaP accounts for the differential induction of apoptosis by 2-ME (LNCaP > DU 145 > PC-3). Our results suggest a common mechanism of 2-ME inhibition of the different types of prostate cancer cells.

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INTRODUCTION

One of the more promising emerging chemotherapeutic agents is 2-methoxyestradiol (2-ME), an endogenous metabolite of estradiol [1-3]. 2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer (AI-PC) [4,5] utilizing a remarkable number of diverse mechanisms that include mitotic cell cycle arrest and induction of apoptosis [1-3]. 2-ME's anti-prostate cancer activity, however, is poorly understood. A better understanding of the mechanisms of 2-ME's anti-prostate cancer effects will be helpful to better evaluate its clinical potential in managing AI-PC. 2-ME may be an example of a chemotherapeutic agent that takes advantage of the molecular and biochemical differences between cancer and normal cells. One such difference may be the requirement for cell cycle proteins like cyclins and cyclin-dependent kinases. Our hypothesis is that one of the cancer-specific mechanisms whereby 2-ME exerts its anti-prostate cancer activity is the deregulated activation of cyclin B1/cdc2 kinase during the cell cycle, which results in the induction of apoptotic cell death. The purpose and scope of this research proposal is to (1) determine the molecular mechanisms of the 2-ME-mediated G2/M cell cycle arrest in prostate cancer cell lines; (2) determine whether activation of cyclin B1/cdc2 kinase by 2-ME is required for induction of apoptosis in prostate cancer and non-transformed normal cells; and (3) identify synergisms and mechanisms of interaction between 2-ME and other clinically relevant chemotherapeutic drugs. In this annual report, we present our accomplishments in the first year of the proposal.

BODY

To better understand 2-ME's anti-prostate cancer action, we have focused on events related to mitotic cell cycle arrest (G2/M) and induction of apoptosis in LNCaP, DU 145, and PC-3 human prostate cancer cell lines. *Our results are presented in a manuscript submitted for publication and included as an* ***appendix***. Here we report results demonstrating strong similarities of 2-ME with paclitaxel in regard to an increase in cyclin B1 protein and its associated kinase activity followed by induction of apoptosis of prostate cancer cells. Inhibition of cyclin B1-dependent kinase activity blocked subsequent induction of apoptosis by both agents. We also showed that 2-ME and paclitaxel caused a later inhibition of cyclin A-dependent kinase activity at a time when apoptosis was increased. These results suggest a common mechanism of 2-ME inhibition of the different types of prostate cancer cells and this mechanism involves several steps in common with paclitaxel, a clinically relevant chemotherapeutic agent for AI-PC. Given that paclitaxel has an effect on patients with AI-PC as a single drug and in combination with other drugs [6], our results hold promise that 2-ME will have a similar efficacy in AI-PC.

The effects of 2-ME on cyclin B1- and cyclin A-dependent kinase activity cannot explain the differential induction of apoptosis in prostate cancer cells (LNCaP > DU 145 > PC-3). The levels of 2-ME-mediated increase in cyclin B1 protein and kinase activity are similar between these prostate cancer cell lines (see Fig. 2B, appended manuscript). Although the levels of caspase-3 protein are 2-fold higher in LNCaP compared to DU 145 cells, the levels in PC-3 cells are similar to the levels in LNCaP, and therefore cannot explain why caspase-3 activity is higher in 2-ME-treated LNCaP cells (see Fig. 8B, appended manuscript). A more likely explanation is that the higher levels of Bcl-xL (**Fig. 1**) and survivin proteins in DU 145 and PC-3 compared to LNCaP accounts for the differential induction of apoptosis by 2-ME. In addition, 2-ME treatment of LNCaP cells resulted in a 2-fold decrease in the XIAP protein, a member of the Inhibitor of Apoptosis Protein (IAP) family [7] (**Fig. 2**). Therefore, it is likely that greater induction of apoptosis resulting from the 2-ME-mediated increase in cyclin B1-dependent

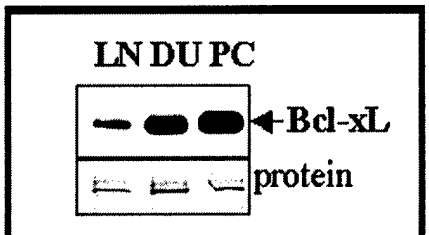

Fig. 1. Western blot analysis of Bcl-xL expression normalized to total protein.

kinase activity requires a decrease in the levels of anti-apoptosis proteins like XIAP and survivin (see Fig. 9, appended manuscript). This should shift the overall balance towards apoptosis even in the most resistant AI-PC cells like DU 145 and PC-3.

The following sections will report our findings associated with each task in the approved statement of work.

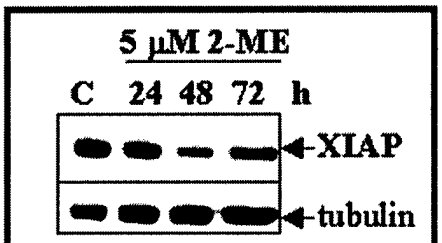

Fig. 2. Western blot analysis of XIAP expression in LNCaP cells treated with 5 μM 2-ME normalized to tubulin.

Specific Aim 1: Determine the molecular mechanisms of the 2-ME/2-EE-mediated G2/M cell cycle arrest in prostate cancer cell lines (months 1-30).

1. Determine the effect of 2-ME/2-EE on the cdc2 phosphorylation status of thr-14 and thr-161 positions in the human prostate cancer cell lines LNCaP, DU 145, and PC-3 using quantitative Western blot (months 1-4).

Dephosphorylation of thr-14 and tyr-15 positions of cdk1 (cdc2), mediated by cdc25C phosphatse, is an important mechanism for activating cdk1 and is required for cells to progress from the G2 to M stage of the cell cycle (8). Phosphorylation at the thr-161 position, mediated by cdk activating kinase (CAK) (9), results in the activation cdk1 activity during mitosis. Phosphorylation of the thr-14 and tyr-15 positions, mediated by myt1 and wee1, results in inactivation of cdk1 activity and is required for cells to exit mitosis (9).

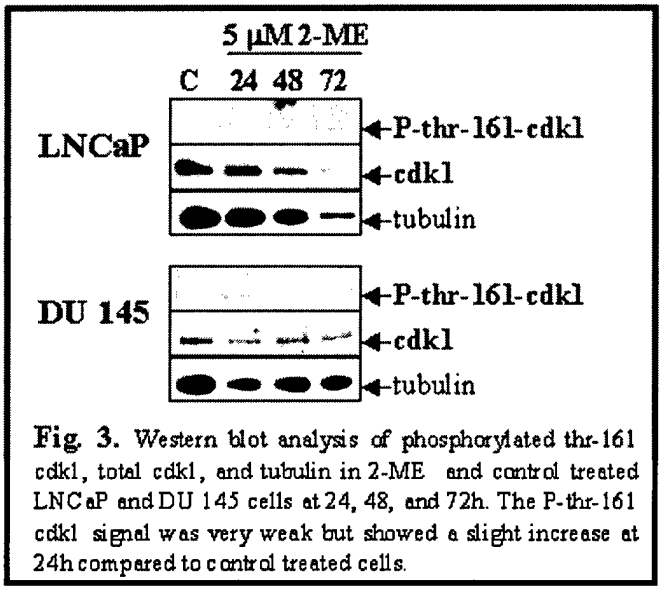

Fig. 3. Western blot analysis of phosphorylated thr-161 cdk1, total cdk1, and tubulin in 2-ME and control treated LNCaP and DU 145 cells at 24, 48, and 72h. The P-thr-161 cdk1 signal was very weak but showed a slight increase at 24h compared to control treated cells.

We used Western blot analysis to determine the effects of 2-ME on the expression of cdc25c phosphatase, wee1 kinase, and phosphorylated thr-161 cdk1. Using the phospho-cdk1 (thr-161) antibody from Cell Signaling (Beverley, MA), the results showed a slight increase after 24h treatment with 2-ME in LNCaP and DU 145 cells (**Fig. 3**). In addition, we prepared pooled LNCaP cells (n=4) treated with 5 μM 2-ME or control for 24h and the samples analyzed by the Kinetworks Phospho-Site Screen (KPSS 4.0) (Kinexus, Vancouver, Canada). The results showed a small (22%) increase in phospho-cdk1 (thr-161) and a 50% decrease in phospho-cdk1 (tyr-15) in 2-ME treated LNCaP cells compared to control. **We conclude that the changes in phosphorylation status of thr-161 (activation) and tyr-15 (inhibition) are consistent with the observation that 2-ME activates cdk1.**

2. Identify quantitative differences in the total levels of cdc25C, myt1, wee1, and CAK proteins in 2-ME/2-EE treated prostate cancer cell lines using quantitative Western blot (months 1-4).

Using the rabbit polyclonal anti-cdc25c (C-20) from Santa Cruz and Western blot analysis, results showed that the G2/M-promoting doses of 2-ME (≥5 μM; see Fig. 1, appended manuscript) shifted cdc25c to a slower migrating form in LNCaP but not in DU 145 cells (**Fig. 4**). This shift in cdc25c is likely due to increased phoshphorylation mediated by cyclin B1-cdk1 or polo kinase during mitotic block (10). Whether

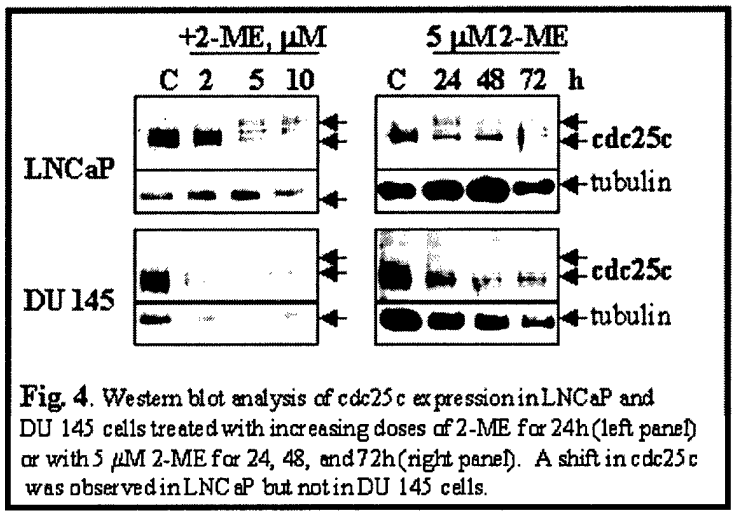

Fig. 4. Western blot analysis of cdc25c expression in LNCaP and DU 145 cells treated with increasing doses of 2-ME for 24h (left panel) or with 5 μM 2-ME for 24, 48, and 72h (right panel). A shift in cdc25c was observed in LNCaP but not in DU 145 cells.

2-ME increases phosphorylation of cdc25c can be examined using commercially available P-cdc25c (thr48) from Cell Signaling. Activation and inhibitory phosphorylations of cdc25c are required for progression through mitosis [10]. The shift/phosphorylation of cdc25c in LNCaP mediated by 2-ME peaked at 24h and decreased by 48 and 72h (**Fig 4**). There were no obvious differences in the total levels of cdc25c when normalized to tubulin loading control. If this shift represents activation of cdc25c phosphatase activity, then it correlates with the increased activity of cdk1 because it removes inhibitory phosphate at thr-14 and tyr-15. Interestingly, there was no shift in cdc25c in 2-ME treated DU 145 cells but there was activation of cyclin B1-cdk1 and block in G2/M (appended manuscript). These results are potentially interesting because 2-ME induced apoptosis in LNCaP cells to a greater extent than in DU 145 cells (see Fig. 8B, appended manuscript). There were no differences in the levels of wee1 kinase in 2-ME treated LNCaP and DU 145 cells (not shown). **We tentatively conclude that 2-ME activation of cdc25c phosphatase and not inhibition of wee1 kinase results in increase in cdk1 activity.**

3. Determine the effect of the novel anti-cancer cdc25C inhibitor MX7174 and the wee1 inhibitor PD0166285 on 2-ME/2-EE-mediated G2/M cell cycle arrest in prostate cancer cell lines using quantitative Western blot and flow cytometry (months 2-6).

We have not yet made progress in this task. Results should be informative especially in relation to the role of cdc25c phosphatase activity in mediating 2-ME increase in cyclin B1-cdk1 activity and apoptosis.

4. Determine the effect of 2-ME/2-EE on the subcellular localization of cyclin B1 and the other regulators of cdc2 kinase using Western blot and immunocytochemistry (months 2-6).

Nuclear and cytoplasmic protein lysates were prepared from DU 145 cells treated with control and 5 μM 2-ME for 24h and analyzed for cyclin B1 and cdk1 expression by Western blot analysis (**Fig. 5**). Surprisingly, cyclin B1 was found to be predominantly localized in the nuclear compartment in both control and 2-ME treated DU 145 cells, whereas cdk1 was localized in both nuclear and cytoplasmic compartments. These results need to be confirmed by immunocytochemical staining of cyclin B1 and microscopic analysis. We have not yet done this analysis in LNCaP or PC-3 cells. It would be interesting if there is a difference in cyclin B1 localization in LNCaP (greater apoptosis) compared to DU 145 and PC-3 (less apoptosis). From the previous results with cdc25c, it will also be interesting to determine if 2-ME treatment of LNCaP (but not DU 145) results in nuclear localization of cdc25c protein.

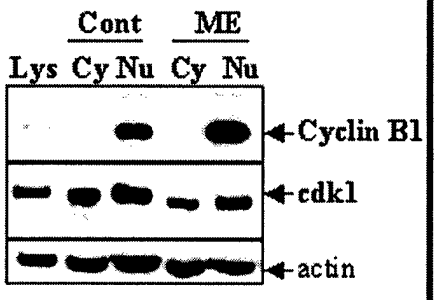

Fig. 5. Western blot analysis of cyclin B1 and cdk1 expression in nuclear (Nu) and cytoplasmic (Cy) lysates prepared from DU 145 cells treated with 5 μM 2-ME or control for 24h. Total lysate (Lys) is shown in lane 1.

5. Develop and characterize stable LNCaP, DU 145, and PC-3 Tet-Off inducible cell lines containing the dn-cdc2, cdc2-AF, and cyclin B1-AS genes regulated by the addition (off) or removal (on) of dox in the media (months 4-24).

This task is in progress. We have cloned the dominant negative cdk1 cDNA into the pTRE-Tight vector from Clontech and have co-transfected this plasmid with the pTK-Hyg hygromycin selection plasmid into the LNCaP-Tet-Off cell line (from Kerry Burnstein). The pTRE-Tight vector offers the advantage of reduced basal level expression and increased overall induction of the gene of interest. We are also cloning the cyclin B1 cDNA in the antisense orientation into the the pTRE-Tight vector.

6. Determine the effect of expressing dn-cdc2, cdc2-AF, and cyclin B1-AS on 2-ME/2-EE-mediated G2/M arrest in LNCaP, DU 145, and PC-3 Tet-Off inducible cell lines using flow cytometry (months 8-30).

Not yet started.

<u>Specific aim 2:</u> Determine whether activation of cyclin B1/cdc2 kinase by 2-ME/2-EE is required for induction of apoptosis in the Tet-Off inducible prostate cancer cell lines and in stably transfected non-transformed normal cells (months 1-30).

1. Determine whether 2-ME/2-EE treatment of the non-transformed/normal cell lines (BPH-1, NRP-152, primary prostate, CD34+ bone marrow progenitor) results in G2/M arrest and apoptosis (flow cytometry), and correlate with the expression levels of cyclin B1 protein (quantitative Western blot) (months 1-24).

The propidium/hypotonic citrate method (see methods page 7 in appended manuscript) was used to study cell cycle distribution of 2-ME treated non-transformed NRP-152 prostate cells and the primary human mesenchymal stromal cells (MSC) (**Fig. 6**, top panel). 2-ME treatment resulted in G2/M arrest at 24h, which was maintained at 48 and 72h in both NRP-152 and MSC cell lines. The DAPI staining apoptosis assay (see methods page 10 in appended manuscript) was used to measure the effect of 2-ME on induction of apoptosis in NRP-152 and MSC cells. We also compared 2-ME with docetaxel (taxotere, Txt) and flavopiridol (FP). Results showed that 2-ME and Txt induced little apoptosis in NRP (5%) and MSC (1-2%) cells but FP induces greater apoptosis

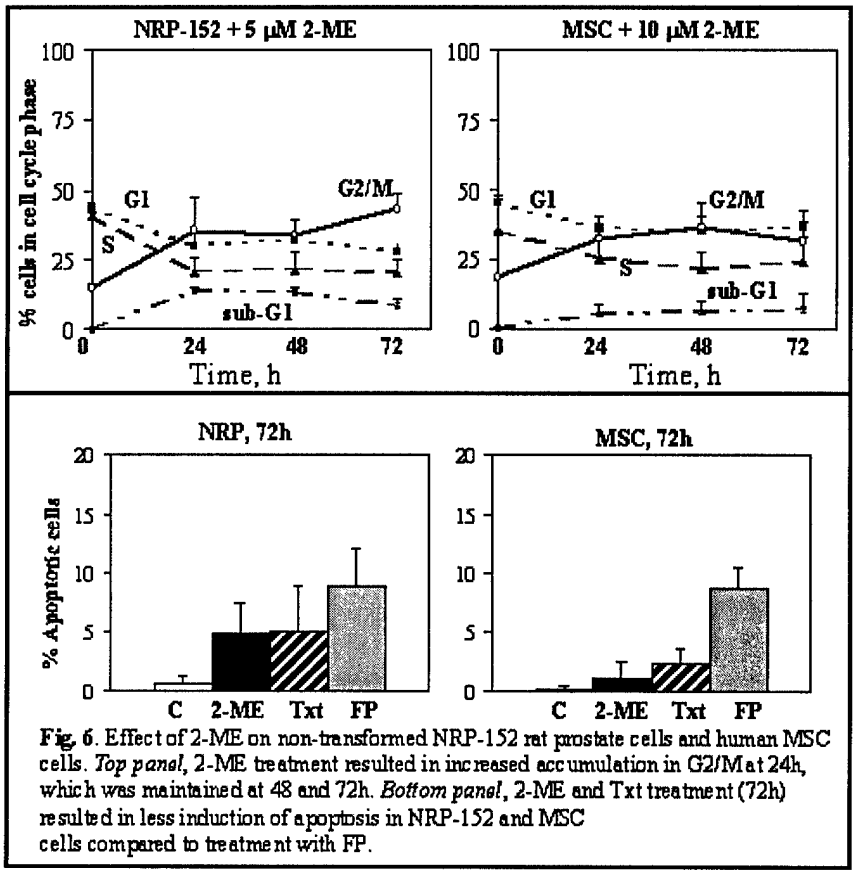

Fig. 6. Effect of 2-ME on non-transformed NRP-152 rat prostate cells and human MSC cells. *Top panel*, 2-ME treatment resulted in increased accumulation in G2/M at 24h, which was maintained at 48 and 72h. *Bottom panel*, 2-ME and Txt treatment (72h) resulted in less induction of apoptosis in NRP-152 and MSC cells compared to treatment with FP.

(9%) in these cells (**Fig. 6**, bottom panel). Unlike in prostate cancer cells, cyclin B1 protein was not detected in NRP-152 and MSC cells. We conclude that 2-ME treatment results in a G2/M cell cycle block in non-transformed (undetectable cyclin B1 protein) NRP-152 and MSC cells. Unlike treatment of LNCaP cells with 2-ME, however, NRP-152 and MSC cells are maintained in G2/M at 48 and 72h (see Fig. 4 in appended manuscript). This result is similar to results obtained with 2-ME treatment of DU 145 and PC-3 prostate cancer cells (see Fig. 4 in appended manuscript), in which there is less apoptosis at 72h (2-12%) compared to LNCaP cells (31%). **We conclude that there is a correlation with increase in apoptosis mediated by 2-ME and decrease in cells blocked in G2/M (48/72h) that is independent of cyclin B1 levels.**

2. *Use Tet-Off inducible prostate cancer cell lines to determine if inhibition of cdc2 kinase with dn-cdc2, cyclin B1-AS, and MX7174 will decrease 2-ME/2-EE-mediated apoptosis (months 8-30).*

We are waiting for the identification of Tet-Off inducible cell lines before starting this task.

3. *Use Tet-Off inducible prostate cancer cell lines to determine if further activation of cdc2 kinase with cdc2-AF and PD0166285 will increase 2-ME/2-EE-mediated apoptosis (months 8-30).*

We are waiting for the identification of Tet-Off inducible cell lines before starting this task.

4. *Determine whether stable expression of cyclin B1 in NRP-152 and MSC sensitizes them to 2-ME/2-EE-mediated apoptosis (months 4-24).*

This task is in progress. G418 resistant NRP-152 clones containing cyclin B1 expression plasmid (driven by CMV enhancer/promoter) are currently being isolated and screened for cyclin B1 expression by Western blot analysis.

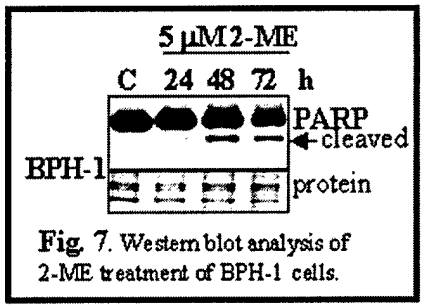

Fig. 7. Western blot analysis of 2-ME treatment of BPH-1 cells.

5. *Determine whether stable expression of cyclin B1-AS in BPH-1 will reduce cyclin B1 protein levels and decrease 2-ME/2-EE-mediated apoptosis (months 4-24).*

Not yet started. The BPH-1 cell line expresses SV40 T antigen [11] and cyclin B1 (not shown). Our data showed that the IC_{50} for 2-ME treatment of BPH-1 was the lowest (0.3 μM) compared to prostate cancer cells. Treatment of BPH-1 cells with 5 μM 2-ME resulted in the induction of apotposis as determined by DAPI analysis (not shown) and PARP cleavage (**Fig. 7**). These results indicate that the BPH-1 cell line will be a good model to test whether reduction of cyclin B1 protein levels will affect 2-ME-mediated apoptosis.

<u>Specific aim 3:</u> **Identify synergisms and mechanisms of interaction between 2-ME/2-EE and other clinically relevant chemotherapeutic drugs (months 8-36).**

1. *Identify the in vitro growth condition (multicellular spheroids using polyhema) whereby prostate cancer cells are in a non-proliferative state and determine if 2-ME/2-EE will induce G2/M arrest and apoptosis (months 8-10).*
2. *Correlate the levels of cyclin B1 protein in the non-proliferation condition with the ability of 2-ME/2-EE to increase cdc2 kinase activity and induce G2/M arrest and apoptosis (8-10).*

Our only experiment to date in these tasks was to treat LNCaP cells with control or 5 μM 2-ME in 0.1% FBS for 24, 48, and 72h (**Fig. 8**). We are assuming that these conditions result in G1 arrest and little cell proliferation. The results showed that in control treated cells, there was a decrease in cyclin B1 protein from 24 to 48/72h whereas in 2-ME treated cells, cyclin B1 was increased at 24h and decreased by 48 and 72h. These results are similar to results obtained in 5% FBS. We do not yet know if 2-ME can induce apoptosis using low cell proliferation conditions. However, we can tentatively conclude that using low cell proliferation conditions, 2-ME can still increase cyclin B1 expression in LNCaP cells.

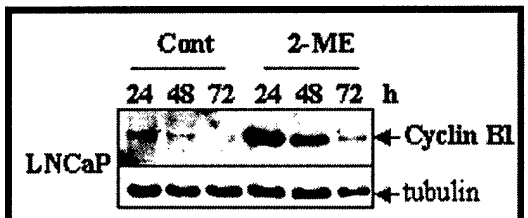

Fig. 8. Western blot analysis of cyclin B1 expression in LNCaP cells grown in low proliferation condition (0.1% FBS) and treated with control or 5 μM 2-ME for 24, 48, and 72h.

3. Determine the IC_{50} dose and the effect on the cell cycle for docetaxel (taxotere), R-roscovitine, and etoposide in prostate cancer cell lines (months 10-14).

The human prostate cancer cell line LNCaP represents a well-differentiated prostate cancer cell line that is dependent on androgens for growth (androgen-dependent), express androgen receptor (AR), PSA, and maintain a functional G1 cell cycle checkpoint control (wild type p53) [12]. Unlike LNCaP cells, the LN-AI cells are androgen-independent and can grow without the presence of androgens (LN-AI/CSS) (13). Using a six-day cell growth assay (see methods page 6 appended manuscript), we determined the IC_{50} for LNCaP, LN-AI, LN-AI/CSS, and DU 145 cells treated with docetaxel (Txt) (**Fig. 9**). The IC_{50} concentrations were as follows: LNCaP, 0.11 nM; LN-AI, 0.07 nM; LN-AI/CSS, 0.02 nM, and DU 145, 0.28 nM. **These results indicated that Txt inhibited androgen-independent LNCaP cells more effectively than androgen-dependent LNCaP cells.** Flow cytometric and Western blot analysis of Txt treated LNCaP and DU 145 cells were similar to results obtained with paclitaxel (see appended manuscript).

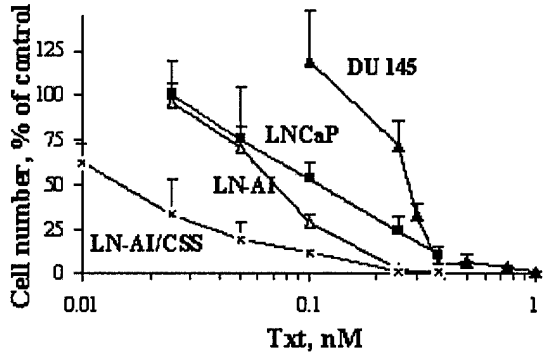

Fig. 9. Six day cell growth assay showing that Txt inhibits androgen-independent LNCaP cells (LN-AI and LN-AI/CSS) more effectively than androgen-dependent LNCaP and compared to DU 145.

KEY RESEARCH ACCOMPLISHMENTS

- Treatment of human prostate cancer cells LNCaP, DU 145, and PC-3 with 2-ME (≥5 μM) for 24 h increased cyclin B1 protein and its associated kinase (cdk1) activity followed by later induction of apoptosis at 48 and 72 h.
- Cyclin-dependent kinase inhibitors alsterpaullone and purvalanol A prevented 2-ME-mediated increase in cyclin B1-dependent kinase activity and induction of apoptosis.
- Decrease in cyclin B1- and cyclin A-dependent kinase activity occurred at later times when apoptosis was increased.
- A likely explanation for the differential induction of apoptosis by 2-ME is the higher levels of proteins that inhibit apoptosis (Bcl-xL and survivin) in DU 145 and PC-3 compared to LNCaP.

- The changes in phosphorylation status of thr-161 (activation) and tyr-15 (inhibition) are consistent with the observation that 2-ME activates cdk1.
- Treatment of LNCaP cells but not DU 145 cells with 5 μM 2-ME results in a shift in the migration of the cdc25c phosphatase protein, possibly due to phosphorylation/activation.
- There is a correlation with increase in apoptosis mediated by 2-ME and decrease in cells blocked in G2/M (48/72h) that is independent of cyclin B1 levels.
- Docetaxel inhibited androgen-independent LNCaP cells more effectively than androgen-dependent LNCaP cells.

REPORTABLE OUTCOMES

1. **Perez-Stable CM** and A De Las Pozas. 2003. 2-Methoxyestradiol and taxol inhibit prostate cancer cells by increasing cyclin B1-dependent kinase followed by induction of apoptosis. AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics, Boston, MA. Nov. 17-21, 2003. Poster Presentation.

2. **Perez-Stable CM**. 2004. 2-Methoxyestradiol and paclitaxel inhibit prostate cancer cells by increasing cyclin B1-dependent kinase activity followed by induction of apoptosis. Submitted to *Biochemical Pharmacology.*

3. Brown JW, S Cappell, **C Perez-Stable**, and LM Fishman. 2004. Extracts from two marine sponges lower cyclin B1 levels, cause a G2/M cell cycle block and trigger apoptosis in SW-13 human adrenal carcinoma cells. *Toxicon.* In Press.

CONCLUSIONS

We suggest that 2-ME-mediated induction of apoptosis in prostate cancer cells requires activation of cyclin B1-dependent kinase that arrests cells in G2/M and ultimately leads to the induction of apoptotic cell death. The cancer-specific induction of apoptosis by 2-ME may be mediated by changes (up and down) in cyclin B1 protein, predominantly expressed in cancer cells [14], and by inhibition of cyclin A-dependent kinase activity, which sensitizes cancer cells to apoptosis [15]. Our results suggest a common mechanism of 2-ME inhibition of the different types of prostate cancer cells and this mechanism involves several steps in common with paclitaxel, a clinically relevant chemotherapeutic agent for AI-PC. It is likely, however, that the higher levels of the anti-apoptotic proteins Bcl-xL and survivin in DU 145 and PC-3 compared to LNCaP accounts for the differential induction of apoptosis (LNCaP > DU 145 > PC-3). Therefore, in considering drugs that will work best in combination with 2-ME, decreasing the levels of anti-apoptosis proteins should shift the overall balance towards apoptosis even in the most resistant AI-PC cells like DU 145 and PC-3.

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2-Methoxyestradiol and paclitaxel inhibit prostate cancer cells by increasing cyclin B1-dependent kinase activity followed by induction of apoptosis

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Abbreviations: 2-ME, 2-methoxyestradiol; AI-PC, androgen-independent prostate cancer; cdk1, cyclin-dependent kinase 1; JNK, c-Jun N-terminal kinase; IAP, inhibitor of apoptosis; DMSO, dimethylsulfoxide; PI, propidium iodide; DAPI, 4'-6-diamidino-2-phenylindole

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Abstract

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with promise for cancer chemotherapy. 2-ME can arrest mitosis and induce apoptosis in a wide variety of cancer cells, including androgen-independent prostate cancer, but normal cells are resistant to this effect. To better understand its anti-prostate cancer action, we have focused on events related to mitotic cell cycle arrest (G2/M) and induction of apoptosis in LNCaP, DU 145, and PC-3 human prostate cancer cell lines. Treatment with 2-ME for 24 h increased cyclin B1 protein and its associated kinase (cdk1) activity followed by later induction of apoptosis at 48 and 72 h. Similar results were obtained with paclitaxel, a clinically relevant agent used to treat advanced prostate cancer. Cyclin-dependent kinase inhibitors alsterpaullone and purvalanol A prevented 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity and induction of apoptosis. Decrease in cyclin B1- and cyclin A-dependent kinase activity occurred at later times when apoptosis was increased. We suggest that 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells requires activation of cyclin B1-dependent kinase that arrests cells in G2/M and ultimately leads to the induction of apoptotic cell death. The cancer-specific induction of apoptosis by 2-ME and paclitaxel may be mediated by changes (up and down) in cyclin B1 protein, predominantly expressed in cancer cells, and by inhibition of cyclin A-dependent kinase activity, which sensitizes cancer cells to apoptosis.

Key words: Cyclin B1; Prostate Cancer; Mitotic Block; Apoptosis; Cyclin A

1. Introduction

One of the more promising emerging chemotherapeutic agents is 2-methoxyestradiol (2-ME), an endogenous metabolite of estradiol [1,2]. 2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer (AI-PC) [3,4] utilizing a remarkable number of diverse mechanisms that include cell cycle arrest [5-7], induction of apoptosis [8,9], disruption of microtubules [10,11], inhibition of angiogenesis [12], and increasing oxidative damage [13]. What makes 2-ME a promising chemotherapeutic is that it does not harm quiescent or proliferating normal cells and it does not exert significant estrogenic effects from binding estrogen receptors [2,14]. In fact, because of 2-ME's anti-cancer activity without toxicity to normal cells, it is currently in Phase II human trials for breast and prostate cancer [15]. 2-ME's anti-prostate cancer activity, however, is poorly understood. A better understanding of the mechanisms of 2-ME's anti-prostate cancer effects will be helpful to better evaluate its clinical potential in managing AI-PC.

One of the proposed mechanisms for 2-ME's anti-cancer effect is the disruption of microtubule function and subsequent block in the G2/M phase of the cell cycle [10,16]. We have previously shown that 2-ME inhibits both androgen-dependent LNCaP and androgen-independent DU 145 and PC-3 prostate cancer cells independent of the expression of androgen receptors and tumor suppressors p53 and Rb [3]. 2-ME blocks LNCaP, DU 145, and PC-3 prostate cancer cells in the G2/M phase of the cell cycle and induces apoptosis [3,4]. Specific mechanisms for 2-ME induced inhibition in prostate cancer cells are proposed to be mediated by the inhibition of cyclin-dependent kinase 1 (cdk1) activity [4], activation of c-Jun N-terminal

kinase (JNK) and inactivation of the anti-apoptosis proteins Bcl-2/Bcl-xL [17-19], and up-regulation of the death receptor 5 and induction of the extrinsic pathway of apoptosis [20]. There appears, however, to be no evidence for a common mechanism of action in all prostate cancer cells sensitive to 2-ME.

Paclitaxel is a well studied chemotherapeutic agent that stabilizes microtubules and has clinical efficacy in a variety of cancers, including AI-PC [21]. Paclitaxel-mediated microtubule damage activates the mitotic checkpoint and blocks the degradation of cyclin B1, leading to a prolonged activation of cyclin B1-cdk1 and mitotic arrest [22-24]. The prolonged activation of cdk1 is required for paclitaxel-mediated apoptosis in the MCF-7 breast cancer cell line, as demonstrated by the use of the chemical inhibitor of cdk1, olomoucine, and antisense oligonucleotides specific for cyclin B1 [22]. It appears, however, that the subsequent reduction of cyclin B1-cdk1 activity and exit from the paclitaxel-mediated mitotic block is important for induction of apoptosis [25]. The mechanism proposed is that increased cdk1 activity results in phosphorylation and stabilization of survivin, a member of the inhibitor of apoptosis (IAP) family and a substrate for cdk1 [26]. The subsequent decrease in cyclin B1-cdk1 activity results in a decrease in the levels of survivin and increased sensitivity to induction of apoptosis. Whether this mechanism is generally applicable to paclitaxel-mediated inhibitory effects in different types of prostate cancer cells is not clear.

To better understand how 2-ME and paclitaxel may function as anti-prostate cancer agents, we focused on the effect of these drugs on cyclin B1 protein, which is overexpressed in cancer but not in normal cells [27], and its associated kinase activity. We previously showed that LNCaP cells are more sensitive to growth inhibition by 2-ME compared to DU 145 and PC-3

cells [3]. Here we report results demonstrating strong similarities of 2-ME with paclitaxel in regard to an increase in cyclin B1 protein and its associated kinase activity followed by induction of apoptosis of prostate cancer cells. Inhibition of cyclin B1-dependent kinase activity blocked subsequent induction of apoptosis by both agents. We also showed that 2-ME and paclitaxel caused a later inhibition of cyclin A-dependent kinase activity at a time when apoptosis was increased. These results suggest a common mechanism of 2-ME inhibition of the different types of prostate cancer cells and this mechanism involves several steps in common with paclitaxel, a clinically relevant chemotherapeutic agent for AI-PC.

2. Materials and methods

2.1. Reagents

2-ME, paclitaxel, dimethylsulfoxide (DMSO), lithium chloride (LiCl), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). 2-ME was dissolved in DMSO (100 mM stock) and stored at room temperature and paclitaxel was dissolved in DMSO and stored −20° C. Histone H1 protein was purchased from Roche Applied Sciences (Indianapolis, IN, USA). 4'-6-Diamidino-2-phenylindole (DAPI), olomoucine, roscovitine, purvalanol A, allsterpaullone, PD 98059, caspase-3 substrate (colorimetric) and inhibitor were purchased from Calbiochem (San Diego, CA, USA)

2.2. Cell culture and treatment with 2-ME and paclitaxel

Human prostate carcinoma cell lines LNCaP-FGC [28], DU 145 [29], and PC-3 [30] were obtained from the American Type Culture Collection (Rockville, MD, USA). Cultures were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA USA) with 5% fetal bovine serum (Hyclone, Logan, UT USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Invitrogen). For treatment with 2-ME or paclitaxel, 7 x 10^5 LNCaP cells, 3 x 10^5 DU 145 cells, and 4 x 10^5 PC-3 cells were seeded per 6-cm dish and allowed to attach overnight. The next day, the cells were ~50% confluent and fresh media containing different doses of 2-ME (0.5 to 10 μM), paclitaxel (0.5 to 50 nM), or DMSO (0.1%) control was added and the cells cultured for varying times (4–72 h). In all the experiments, floating and trypsinized attached cells were pooled for further analysis.

2.3. *Six-day cell growth assay for paclitaxel*

LNCaP (5 x 10^4 cells/well), DU 145 (1.5 x 10^4 cells/well), and PC-3 (2 x 10^4 cells/well) were seeded in 6-well plates. The next day, fresh media containing different doses of paclitaxel (0.025-10 nM) or control (0.1% DMSO) were added; fresh media was changed after 3 days. On day 6, cells were removed by trypsin-EDTA, and viable cells counted with a hemacytometer. The cell count was normalized against the vehicle control and the data expressed as a percentage of control from three independent experiments done in duplicate.

2.4. *Flow cytometric analysis*

Propidium/hypotonic citrate method [31] was used to study cell cycle distribution of 2-ME and paclitaxel treated prostate cancer cells. After harvesting and washing cells with phosphate-buffered saline (PBS), the cell pellets were resuspended in 0.5 ml of PI staining solution (0.1% sodium citrate, 0.03% NP40, and 50 μg/ml PI), vortexed to release nuclei, and DNA distribution histograms generated by analysis of 10,000 nuclei in a Coulter XL flow cytometer. The percentage of cells in the sub-G1, G1, S, and G2/M DNA content were determined by the ModFit program (Verity Software House, Topsham, ME USA) from 6-8 samples analyzed from at least three independent experiments.

2.5. Western blot analysis

For Western blot and kinase assays, 1.8 x 10^6 LNCaP cells, 8 x 10^5 DU 145 cells, and 1 x 10^6 PC-3 cells were seeded per 10-cm dish and allowed to attach overnight. After treatment with 2-ME, paclitaxel, or DMSO control, floating and attached prostate cells were centrifuged and cell pellets stored at −80°C. Cell pellets were resuspended in NP40 cell lysis buffer (1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, protease inhibitor tablet [Roche Applied Sciences], 50 mM NaF, and 0.1 mM $NaVO_4$), lysed by vortex, left on ice for 30 min, centrifuged, and the protein concentrations of the supernatant determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA USA). After separation of 25–50 μg protein by SDS-PAGE, proteins were transferred by electrophoresis to Immobilon-P membrane (Millipore Corp, Bedford, MA USA) and incubated in 5% nonfat dry milk, PBS, and 0.25% Tween-20 for 1 h. Antibodies specific for cyclin B1 (GNS1), cyclin A (H-432), cdk1 (17), cdk2

(D-12), p53 (DO-1), p21 (C-19), p27 (C-19), Bax (N-20), survivin (FL-142), actin (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA USA) were diluted 1/1000 in 5% nonfat dry milk, PBS, and 0.25% Tween-20 and incubated overnight at 4°C. Similarly, antibodies specific for caspase-3 (CPP32), PARP (C2-10), and Bcl-xL (polyclonal) (BD Biosciences Pharmingen, San Diego, CA USA), and Bcl-2 (124) (Dako Corp, Carpinteria, CA USA) were diluted 1/2000. Membranes were washed in PBS and 0.25% Tween-20 (three times, 10 min each time) and incubated with horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG1/2a or anti-rabbit; 1/2000 dilution; Santa Cruz Biotechnology) for 1 h, washed in PBS and 0.25% Tween-20, and analyzed by exposure to X-ray film (X-Omat, Eastman Kodak Co, Rochester, NY USA) using enhanced chemiluminescence plus (ECL plus, Amersham Pharmacia Biotech, Arlington Heights, IL USA). Goat polyclonal antibodies specific for actin (C-11) and horseradish peroxidase-conjugated secondary antibody (anti-goat IgG; 1/2000 dilution; Santa Cruz Biotechnology) were used for protein loading controls. Total proteins were stained with Coomassie blue for an additional protein loading control. X-ray films were scanned using an Epson Perfection 2450 Photo scanner and the pixel intensity measured using UN-SCAN-IT digitizing software, version 5.1 (Silk Scientific Corp, Orem, UT USA). Changes in protein levels of 2-ME and paclitaxel treated cells was determined by normalizing values to actin and comparing to values of control treated cells (equals 1.0) in at least three different samples analyzed from 2-5 independent experiments. To determine the overall levels of caspase-3, Bcl-xL, and survivin in LNCaP, DU 145, and PC-3 cells, the scanned bands from the same blot were normalized to scanned total protein (n=6, two independent experiments).

2.6. Cyclin B1 and A-dependent kinase assay

Four hundred μg of total protein were incubated with 2 μg anti-cyclin B1 (GNS1 or H-433), cyclin A (H-432), or cdk2 (D-12) antibody for 3 h on ice, followed by the addition of 20 μl protein A/G-agarose (Santa Cruz Biotechnology), and incubated overnight at 4°C. with agitation. Immune-complexes were collected by centrifugation, washed 3x with NP40 cell lysis buffer, 3x with kinase buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM DTT), resuspended in kinase buffer containing 2 μg histone H1 substrate protein, 25 μM ATP, 5 μCi γ^{32}P-ATP, and incubated for 30 min at 30°C. Reactions were stopped with SDS gel loading buffer, samples electrophoresed on SDS-PAGE, electroblotted to Immobilon P membranes, and analyzed by autoradiography. Coomassie blue staining of membranes revealed similar loading of histone proteins. The histone band was cut out from the paper and ^{32}P measured by scintillation counting. Changes in kinase activity of 2-ME and paclitaxel treated cells was determined by normalizing the ^{32}P-histone values to the scanned H1 protein value and comparing to values of control treated cells (equals 1.0) in at least three different samples analyzed from 2-5 independent experiments.

2.7. p21 immunoprecipitation and cdk2 western blot

Four hundred μg of LNCaP total protein were incubated with 2 μg anti-p21 (C-19) or rabbit IgG antibody for 3 h on ice, followed by the addition of 20 μl protein A/G-agarose, and incubated overnight at 4°C. with agitation. Immune-complexes were collected by centrifugation,

washed 3x with NP40 cell lysis buffer, and analyzed by Western blot using cdk2 (D-12) antibody.

2.8. DAPI staining apoptosis assay

Floating and attached prostate cancer cells grown in 6-cm dishes were centrifuged, washed with PBS, and resuspended in 0.6 ml 4% paraformaldehyde/PBS. After fixation for 15 min, cells were centrifuged, washed with PBS, and resuspended in 0.5 ml of DAPI (1 μg/ml)/PBS. After 10 min, cells were centrifuged, washed with PBS, and 10 μl of concentrated cells were added on a microscope slide followed by placement of a coverslip. Cells containing densely stained and fragmented chromatin were identified as apoptotic using a Nikon fluorescence microscope with a DAPI filter. The number of apoptotic cells in at least 250 total cells was determined from at least four random microscope fields. Changes in apoptosis from 2-ME and paclitaxel treated prostate cancer cells was determined as percentage of apoptotic cells in at least five different samples from three independent experiments. There was minimal apoptosis detected in control treated cells (less than 0.1%). Images were captured using IP Lab, Scanalytics Inc (Fairfax VA USA).

2.9. Caspase-3 assay

Floating and attached prostate cancer cells grown in 6-cm dishes were centrifuged, washed with PBS, resuspended in 50-100 μl ice cold cell lysis buffer (50 mM HEPES, pH 7.4,

100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA), and incubated 5 min on ice. Cells were centrifuged for 10 min at 4°C and the supernatant stored at - 80 °C. Fifty μg of cell extract was added to assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol) containing caspase-3 substrate I (200 nM) and incubated at 37 °C. for 15-18 h. Absorbance at 405 nM was determined using a microtiter plate reader and the changes in caspase-3 activity from 2-ME and paclitaxel treated prostate cancer cells was determined as fold control treated cells (equals 1.0). Addition of caspase-3 inhibitor (50 nM) was used to confirm specificity.

2.10. Purvalanol A and alsterpaullone cdk inhibitors

Selective chemical inhibitors of cdk activity were used to investigate the effect on 2-ME and paclitaxel-mediated apoptosis in prostate cancer cells. We initially utilized olomoucine but a very high dose (100 μM) was required to inhibit cyclin B1 kinase activity, which by itself induced apoptosis. Subsequently, we utilized roscovitine but sub-lethal doses (10-20 μM) failed to inhibit 2-ME-mediated increase in cyclin B1-dependent kinase activity in LNCaP cells. Finally, we investigated the more potent cdk inhibitors purvalanol A and alsterpaullone [32,33]. Dose response experiments determined that 5 μM purvalanol A blocked 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity in DU 145 but not LNCaP cells. For alsterpaullone, a dose of 5 μM in LNCaP and 10 μM in PC-3 blocked 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity. The effect of these doses on 2-ME and paclitaxel-mediated apoptosis was determined using the methods described above.

2.11. Statistical analysis

Statistical differences between 2-ME or paclitaxel-treated and control cells were determined by two-tailed Student's *t*-test with $P<0.05$ considered significant.

3. Results

3.1. LNCaP cells are more sensitive to growth inhibition by 2-ME and paclitaxel

Using a six-day cell growth assay, we previously showed that androgen-dependent LNCaP cells are more sensitive to inhibition by 2-ME compared to androgen-independent DU 145 and PC-3 prostate cancer cells. The half-maximal inhibitory concentrations (IC_{50}) were as follows: LNCaP, 0.5 μM; DU 145, 1.2 μM; and PC-3, 1.8 μM [3]. Similar to inhibition by 2-ME, LNCaP cells were also more sensitive to inhibition by paclitaxel compared to DU 145 and PC-3 cells, with the IC_{50} equaling 0.2 nM, 0.5 nM, and 0.7 nM, respectively. Subsequent experiments sought to determine why LNCaP cells are more sensitive to inhibition by 2-ME and paclitaxel compared to DU 145 and PC-3 cells.

3.2. Dose response of 2-ME and paclitaxel-treated prostate cancer cells and flow cytometric analysis

To further investigate the cell cycle effects of 2-ME and paclitaxel on prostate cancer cells, we used flow cytometric analysis after treatment of cells with varying doses of 2-ME (0.5 - 10 μM) and paclitaxel (0.5 - 50 nM) for 24 h (Fig. 1). Treatment of LNCaP cells with 2 μM 2-ME and 2 nM paclitaxel resulted in an increase of cells in the G1 phase of the cell cycle. Similar doses of 2-ME and paclitaxel did not cause G1 accumulation in DU 145 and PC-3 cells, probably because their G1 cell cycle checkpoints are defective [34,35]. Treatment of all prostate cancer cells with ≥5 μM 2-ME and ≥10 nM paclitaxel resulted in an increase in G2/M with concomitant decrease in G1. These results indicated that LNCaP cells were more sensitive to growth inhibition by 2-ME and paclitaxel because lower doses blocked cells in the G1 phase of the cell cycle.

3.3. Increase of cyclin B1 protein and kinase activity in 2-ME and paclitaxel-treated prostate cancer cells

To investigate potential molecular mechanisms involved in 2-ME and paclitaxel-mediated G2/M cell cycle arrest in prostate cancer cells, we analyzed expression of cyclin B1 by Western blot (Fig. 2). The transition from the G2 to the M phase of the cell cycle requires accumulation of cyclin B1 and activation of its associated kinase, cdk1. The end of the G2/M transition and exit from mitosis requires proteolysis of cyclin B1 and reduction of cdk1 activity [36]. Treatment of LNCaP cells with the G2/M-promoting doses of 2-ME (≥5 μM) and paclitaxel (≥10 nM) for 24 h resulted in an early 3-4-fold increase of cyclin B1 protein, whereas the levels of cdk1 protein remained unchanged (Fig. 2A). The 2-ME and paclitaxel-mediated

increase in cyclin B1 protein also corresponded to a 3–4-fold increase in cyclin B1-dependent kinase activity in LNCaP cells. Similar results were obtained in 2-ME and paclitaxel treated DU 145 and PC-3 cells (Fig. 2B). These results indicated that G2/M-promoting doses of 2-ME and paclitaxel increased cyclin B1 protein and kinase activity to similar levels in the LNCaP, DU 145, and PC-3 prostate cancer cells.

3.4. Inhibition of cyclin A-dependent kinase in LNCaP cells treated with G1-promoting doses of 2-ME and paclitaxel

Cyclin A protein increases during the S and G2 phase of the cell cycle and is believed to be important for DNA replication [37]. In contrast to cyclin B1, treatment of prostate cancer cells for 24 h with varying doses of 2-ME and paclitaxel did not result in significant changes in the protein levels of cyclin A or its associated kinase cdk2 (Fig. 3). However, LNCaP cells treated with the G1-promoting doses of 2-ME and paclitaxel resulted in a significant 4–5-fold decrease in cyclin A-dependent kinase activity (Fig. 3A). The G2/M-promoting doses of 2-ME and paclitaxel, however, did not inhibit cyclin A-dependent kinase in LNCaP cells. At 24 h, there was no significant inhibition of cyclin A-dependent kinase activity by any dose of 2-ME and paclitaxel in the G1 defective DU 145 and PC-3 cells (not shown). These results indicated that the G1-promoting doses of 2-ME and paclitaxel inhibited cyclin A-dependent kinase without lowering the levels of cyclin A or cdk2 protein and may explain why LNCaP cells accumulated in G1.

3.5. *Changes in the cell cycle distribution over time in 2-ME and paclitaxel-treated prostate cancer cells*

To analyze the long-term effects (>24 h) on the cell cycle after treatment with 2-ME and paclitaxel over 4, 24, 48, and 72 h, we chose the G2/M-promoting dose of 2-ME (5 μM for LNCaP and DU 145 and 10 μM for PC-3) and paclitaxel (10 nM was used for all cell lines) (see Fig. 1). After 4 h of 2-ME treatment, there was a significant increase in cells with G2/M DNA content in DU 145 but not in LNCaP or PC-3 cells (Fig. 4). All cells treated with 2-ME and paclitaxel accumulated in G2/M after 24 h with a concomitant decrease in G1. In LNCaP, there was a significant decrease in G2/M after 48 and 72 h treatment with 2-ME and paclitaxel. In DU 145 and PC-3 cells, however, cells remained blocked in G2/M after treatment for 48 and 72 h with 2-ME but not with paclitaxel. After the initial decrease of LNCaP cells in G1 at 24 h, there was a significant increase after 48 and 72 h in 2-ME but not paclitaxel treated cells. There was a significant increase in sub-G1, often associated with apoptotic cells [38], in 2-ME and paclitaxel-treated LNCaP and DU 145 cells, and paclitaxel-treated PC-3 cells, but little increase in 2-ME-treated PC-3 cells. Although these results revealed a common G2/M block at 24 h, there were differences in the cell cycle distribution at 48 and 72 h between 2-ME and paclitaxel-treated prostate cancer cell lines.

3.6. *Changes in cyclin B1 protein and kinase activity with time in 2-ME and paclitaxel-treated prostate cancer cells*

We sought to further analyze the cyclin B1 protein levels and its associated kinase activity in prostate cancer cells after treatment with 2-ME and paclitaxel over time. Since all prostate cancer cells were blocked at G2/M after 24 h treatment with high doses of 2-ME and paclitaxel, it was not surprising that there was a marked accumulation of cyclin B1 protein (Fig. 2). However, there was a significant increase in cyclin B1 protein and its associated kinase in LNCaP cells treated with 2-ME after only 4 h, a time when there was no increase in cells with G2/M DNA content (Fig. 4,5). This indicated that the accumulation of cyclin B1 protein was not simply due to increase in the G2/M fraction. In general, the levels of cyclin B1 protein peaked at 24 h and decreased at 48 and 72 h after treatment with 2-ME and paclitaxel in all prostate cancer cells (Fig. 5B). Cyclin B1-dependent kinase activity tended to correlate with cyclin B1 protein levels with the exception of LNCaP cells treated with paclitaxel, in which activity remained significantly elevated. These results indicated that there were differences in the later (>24 h) effects of 2-ME and paclitaxel on cyclin B1-dependent kinase activity after the initial prolonged activation at 24 h.

3.7. Decrease in cyclin A protein and kinase activity after prolonged treatment (48-72h) with 2-ME and paclitaxel

When cyclin B1 protein and its associated kinase activity increased at 4 and 24 h with the G2/M-promoting doses of 2-ME and paclitaxel, there were no changes in the levels of cyclin A protein and its associated kinase activity (Fig. 5,6). However, there was a consistent 2–3-fold decrease in cyclin A protein in LNCaP and PC-3 cells (but not in DU 145) after 48 and 72 h 2-

ME (less decrease with paclitaxel) treatment, whereas the cdk2 protein levels did not change (Fig. 6). There was a consistent 2–5-fold decrease in cyclin A-dependent kinase activity in all prostate cancer cell lines at 48 and 72 h (Fig. 6B). These results indicated that 2-ME and paclitaxel decreased cyclin A-dependent kinase at a later time when apoptotic cells were increased (see Fig. 8).

3.8. Increased p53 and p21 but not p27 proteins in 2-ME and paclitaxel-treated prostate cancer cells

To further investigate molecular mechanisms involved in 2-ME and paclitaxel-mediated G1 and G2/M cell cycle arrest in LNCaP cells, we analyzed expression of p53, p21, and p27 by Western blot (Fig. 7). The G1-promoting doses of 2-ME and paclitaxel that decreased cyclin A-dependent kinase activity (see Fig. 3) resulted in a 3–4-fold increase in p53 protein (Fig. 7A); p53 is mutated in DU 145 cells and PC-3 cells and is non-functional [35]. Similarly, p53 protein levels were increased at 24 h (but not at 4 h) using the G2/M-promoting doses of 2-ME and paclitaxel and remained elevated at 48 and 72 h (Fig. 7B). Because p53 is known to increase transcription of the cdk inhibitor p21 gene [39], we also analyzed expression of p21 protein by Western blot. The G1-promoting dose of 2-ME and paclitaxel resulted in a small but significant 2–fold increase in p21 protein, whereas the G2/M-promoting doses did not increase p21 (despite high levels of p53 protein) (Fig. 7A). There was an increased association of p21 with cdk2 when the G1-promoting dose of 2-ME was utilized (Fig. 7C), indicating this may be a mechanism for inhibition of cyclin A-dependent kinase and blocking of LNCaP cells in G1. At 48 and 72 h

treatment with 2-ME and paclitaxel in LNCaP cells, there was a 2–6-fold increase in p21 protein and this correlated with increased number of cells in G1 (2-ME treated LNCaP) and decreased cyclin A-dependent kinase activity (Fig. 4,6). The levels of p21 were very low in DU 145 and PC-3 cells and did not change with 2-ME and paclitaxel treatment (not shown). There were no significant changes in the levels of the cdk inhibitor p27 using varying doses or times of treatment with 2-ME and paclitaxel.

3.9. Increased apoptosis in prostate cancer cells after 2-ME and paclitaxel-mediated mitotic block

We previously showed that DU 145 prostate cancer cells undergo caspase-dependent apoptosis when treated with 5 μM 2-ME [3]. To further analyze the time of the appearance of apoptotic cells relative to G2/M block, we performed DAPI staining and caspase-3 assays on prostate cancer cells treated with 2-ME (5–10 μM) and paclitaxel (10 nM) for 24, 48, and 72 h (Fig. 8). In LNCaP, the DAPI assay showed a significant increase of apoptotic cells from 6, 16, and >30% after 24, 48, and 72 h treatment with 2-ME and paclitaxel, which also corresponded with a significant increase in caspase-3 activity (Fig. 8B). In general, less apototic cells and caspase-3 activity were identified in 2-ME treated DU 145 and PC-3 cells compared to LNCaP cells, probably explaining the differential growth inhibition (LNCaP>DU 145> PC-3). A striking difference was observed in paclitaxel treated DU 145 cells, where there were greater number of apoptotic cells (6.5, 25, 27%) compared to 2-ME treated cells (2, 9, 13%). However, there was not a corresponding increase in caspase-3 activity in paclitaxel treated DU 145 cells. In PC-3,

the prostate cancer cell line that was least sensitive to 2-ME and paclitaxel, longer treatment (6 days) was required to obtain increased number of apoptotic cells (8%) (see Fig. 10B). Finally, there was a discrepancy in measurement of apoptotic cells identified by flow cytometry (sub-G1) and by the DAPI and caspase-3 assays (for example, DU 145 cells treated with 2-ME at 24 h showed high sub-G1 [see Fig.4] but low apoptosis by DAPI and caspase-3 assays). This high sub-G1 with low apoptosis has been attributed to the presence of hypodiploid cells resulting from aberrant mitosis (40). Overall, these results indicated that in prostate cancer cells, 2-ME and paclitaxel-mediated induction of apoptosis predominantly occurred after the G2/M cell cycle block at 24 h.

3.10. Effect of 2-ME and paclitaxel on expression of proteins important in apoptosis

In order to determine why 2-ME and paclitaxel-treated LNCaP cells undergo apoptosis to a greater degree than DU 145 and PC-3 cells, we sought to identify differences in the levels of proteins important in apoptosis by Western blot analysis. The results showed that reduction of the caspase-3 protein levels and cleavage of the PARP protein, both indicative of apoptosis, occurred to a greater extent in 2-ME and paclitaxel treated LNCaP and DU 145 compared to PC-3 cells (Fig. 9). There were no obvious differences in the levels of the pro-apoptotic protein Bax (mutated in DU 145 cells [41]) and the anti-apoptotic protein Bcl-xL in all prostate cancer cell lines treated with 2-ME and paclitaxel. The anti-apoptotic protein Bcl-2 was more highly expressed in LNCaP cells and showed no significant differences when apoptosis was increased. The levels of the anti-apoptosis protein survivin increased when cells were in the G2/M phase

and subsequently decreased to a level similar to or below that of control cells (Fig. 9). A potential reason for the differential sensitivity to apoptosis may be the 1.5–2-fold higher levels of the Bcl-xL and survivin proteins in DU 145 and PC-3 compared to LNCaP cells, which may have protected these cells from 2-ME and paclitaxel-mediated induction of apoptosis.

3.11. Inhibition of the 2-ME and paclitaxel-mediated increase of cyclin B1-dependent kinase activity blocks induction of apoptosis

To determine whether the increase in cyclin B1 protein and its associated kinase activity was required for 2-ME and paclitaxel-mediated induction of apoptosis, we utilized the potent cdk inhibitors purvalanol A and allsterpaullone [32,33]. Treatment of LNCaP cells with 5 μM allsterpaullone and DU 145 cells with 5 μM purvalanol A for 24 h resulted in an increase in G2/M (Table 1) and blocked the 2-ME and paclitaxel-mediated increase of cyclin B1-dependent kinase activity (Fig. 10A). Allsterpaullone also decreased cyclin A-dependent kinase in control and 2-ME and paclitaxel-treated LNCaP cells. In contrast, purvalanol A, which has a higher specificity for the inhibition of cyclin B1-dependent kinase, increased cyclin A-dependent kinase in control and 2-ME and paclitaxel-treated DU 145 cells (Fig. 10A). Flow cytometric analysis indicated that the pattern of the cell cycle distribution at 24 h was similar when the cdk inhibitors were added individually or simultaneously with 2-ME or paclitaxel (Table 1). At 72 h, allsterpaullone and purvalanol A blocked 2-ME and paclitaxel-mediated induction of apoptosis in LNCaP and DU 145 cells, as determined by DAPI assay, caspase-3 activity, and PARP cleavage (Fig. 10B,C). Similar results were obtained in PC-3 cells treated for 6 days with 10 μM

allsterpaullone (+/- 2-ME and paclitaxel), although inhibition of caspase-3 activity was not significant. Kinase inhibitors PD 98059 (20 μM) and LiCl (30 mM) did not significantly block the 2-ME-mediated induction of apoptosis in LNCaP and DU 145 cells (not shown). These results suggested that 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase was required for induction of apoptosis in prostate cancer cells.

4. Discussion

In this report, we suggest a common anti-prostate cancer mechanism for 2-ME and paclitaxel in the growth inhibition and induction of apoptosis utilizing several human prostate cancer cell lines. Our results showed a requirement for increased cyclin B1-dependent kinase activity at the G2/M phase of the cell cycle followed by induction of apoptosis after mitotic exit. Androgen-dependent LNCaP cells were more sensitive to growth inhibition by 2-ME and paclitaxel because they responded to lower drug doses by increasing p53 and p21 proteins and inhibiting cyclin A-dependent kinase activity, which resulted in block at the G1 phase of the cell cycle. Higher doses of 2-ME and paclitaxel blocked all prostate cancer cells, including androgen-independent DU 145 and PC-3 cells, in G2/M followed by induction of apoptosis, at which time cyclin A-dependent kinase activity was inhibited. The differential induction of apoptosis by 2-ME and paclitaxel in prostate cancer cells, however, likely depends on the levels of proteins that inhibit caspase activation. Overall, our results indicated that 2-ME has a similar mechanism as paclitaxel in the growth inhibition and induction of apoptosis of human prostate cancer cells.

Similar to paclitaxel treatment of prostate cancer cells, 2-ME increased cyclin B1 protein and blocked cells in mitosis [22-24]. The mechanism proposed for the paclitaxel-mediated increase in cyclin B1 protein is by inhibition of the proteosomal degradation system, which is a key component in the reduction of cyclin B1 protein levels required for anaphase to metaphase transition during mitosis [42]. In LNCaP cells, cyclin B1 mRNA did not increase with 2-ME treatment and the activity of the cyclin B1 promoter was inhibited by 2-ME, suggesting that the increase in cyclin B1 protein resulted from a post-transcriptional mechanism (not shown). We are currently investigating whether 2-ME can inhibit the proteosomal degradation of cyclin B1 protein and play a key role in its mechanism of action. The end result of increasing cyclin B1 protein is the increase of its associated cdk activity, which has been shown to be important in the induction of mitotic catastrophe and many forms of apoptosis [43].

Our data agrees with data obtained from paclitaxel treatment of breast and epidermal cancer cells showing that increase of cyclin B1 protein and its associated cdk is required for induction of apoptosis [22,23]. The results presented here indicated that simultaneous treatment of prostate cancer cells with 2-ME or paclitaxel with the cdk inhibitors allsterpaullone or purvalanol A resulted in drug antagonism and inhibition of apoptosis. Given that single chemotherapeutic drugs like 2-ME and paclitaxel will likely not be sufficient for the treatment of AI-PC, a better understanding of the mechanisms of action of these drugs will be critical to determine how they will interact and function with other chemotherapeutic drugs for the maximal induction of apoptosis.

An issue is whether 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells requires the increase of cyclin B1-dependent kinase and/or the subsequent reduction

of cyclin B1-dependent kinase activity. The decrease of cyclin B1-dependent kinase activity is proposed to cause apoptosis in sensitive cells by reducing the levels of survivin, a member of the IAP family of proteins [25]. Despite the similar decreased levels of cyclin B1-dependent kinase activity in 2-ME treated prostate cancer cells, however, there was a greater induction of apoptosis in LNCaP compared to DU 145 and PC-3 cells (Fig. 5,8). In addition, LNCaP cells treated with paclitaxel had elevated cyclin B1-dependent kinase activity at a time when apoptosis was increased. In contrast, DU 145 cells treated with paclitaxel resulted in a faster exit from mitotic block and lower levels of survivin at 72 h, possibly contributing to the greater induction of apoptosis compared to 2-ME treated DU 145 cells (Fig. 4,8,9). These results suggested that the mechanisms for induction of apoptosis resulting from decreased cyclin B1-dependent kinase activity and exit from mitotic block varied between 2-ME and paclitaxel treatment of prostate cancer cell types. Therefore, we favor the idea that the 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity and not the subsequent decrease in activity is more important in the induction of apoptosis. The substrates for cyclin B1-dependent kinase, in addition to survivin that may mediate this effect are yet to be identified.

Our results suggest a common mechanism of 2-ME and paclitaxel-mediated growth inhibition and induction of apoptosis in several prostate cancer cell lines involving the expression of cyclin B1 protein. In contrast, a previous report suggested that 2-ME blocks prostate cancer cells in the G2 phase of the cell cycle by inhibiting cdk1 activity [4]. In addition to our results demonstrating that 2-ME and paclitaxel increased cyclin B1-dependent kinase activity, the inhibitory phosphorylation of the tyrosine-15 position in the cdk1 protein was reduced in DU 145 cells (not shown). Other reports suggest that 2-ME and paclitaxel activates JNK, which

phosphorylates Bcl-2/Bcl-xL and inhibits its anti-apoptosis activity [17-19]. Our results showed that phosphorylated Bcl-2 (slower migrating protein in Fig. 9) in 2-ME-treated LNCaP cells occurred predominantly at 24 h (peak of G2/M block), a time when apoptosis was lower compared to 72 h. It is not clear whether phosphorylation of Bcl2 inactivates function or is a result of cells arrested in G2/M [44].

One possibility why 2-ME has anti-cancer activity but does not kill normal cells may be that cyclin B1 protein is more commonly overexpressed in cancer cells compared to normal cells [27]. We have preliminary data indicating that primary human mesenchymal stromal cells derived from the bone marrow [45] do not express cyclin B1 protein and undergo minimal apoptosis when treated with high doses of 2-ME and paclitaxel (not shown). Currently, we are investigating whether expression of cyclin B1 is required for 2-ME and paclitaxel-mediated apoptosis by overexpressing cyclin B1 protein in non-transformed cells and by inhibiting cyclin B1-dependent kinase activity in prostate cancer cells.

Another common mechanism for chemotherapeutic drug inhibition of cancer cells is the increase in p53 and p21 proteins and block in the G1 phase of the cell cycle [46]. A similar mechanism was also evident using lower doses of 2-ME and paclitaxel in LNCaP cells [47], which inhibited cyclin A-dependent kinase activity. In order to maximize induction of apoptosis, however, G2/M-promoting doses of 2-ME and paclitaxel were required. Our results showed inhibition of cyclin A-dependent kinase activity at a time when apoptosis was maximized at 48 and 72 h of treatment. An important role for inhibition of cdk2 and induction of apoptosis in cancer but not normal cells was reported, perhaps further explaining why 2-ME and paclitaxel tend to kill cancer and not normal cells [48]. However, a recent report casts doubt on the

importance of cdk2 inhibition in cancer therapy [49]. Our results showed that treatment of DU 145 cells with 2-ME or paclitaxel plus the cdk inhibitor purvalanol A resulted in the inhibition of cyclin B1- and increase of cyclin A-dependent kinase activity that completely blocked induction of apoptosis. In contrast, treatment of LNCaP cells with 2-ME or paclitaxel plus allstepaullone inhibited both cyclin B1- and cyclin A-dependent kinase activity and a difference in apoptosis was only observed after 72 h (Fig. 10). These results suggested that inhibition of cyclin A-dependent kinase activity may be an important mechanism for 2-ME and paclitaxel inhibition of prostate cancer cells.

The effects of 2-ME and paclitaxel on cyclin B1- and cyclin A-dependent kinase activity cannot explain the differential induction of apoptosis in prostate cancer cells (LNCaP > DU 145 > PC-3). Although the levels of caspase-3 protein are 2-fold higher in LNCaP compared to DU 145 cells, the levels in PC-3 cells are similar to the levels in LNCaP, and therefore can not explain why caspase-3 activity is higher in 2-ME and paclitaxel-treated LNCaP cells. A more likely explanation is that the higher levels of Bcl-xL and survivin proteins in DU 145 and PC-3 compared to LNCaP accounts for the differential induction of apoptosis by 2-ME and paclitaxel. Overexpression of Bcl-xL has a well established role as a powerful anti-apoptosis factor in prostate cancer and inhibition of Bcl-xL by antisense oligonucleotides can sensitize PC-3 cells to drug-mediated apoptosis [50,51]. A predominant role for members of the IAP family in the regulation of the induction of apoptosis in prostate cancer cells has been proposed [52]. In addition, it has been reported that expression of c-IAP-2, another member of the IAP family that can inhibit caspase activity, is highest in DU 145 and PC-3 compared to LNCaP cells and correlates with resistance to apoptosis [53]. Therefore, drugs that can decrease the levels of anti-

apoptosis proteins should shift the overall balance towards apoptosis even in the most resistant AI-PC cells.

Anti-cancer chemotherapeutic agents ideally should take advantage of the molecular differences between transformed and normal cells and induce apoptosis only in cancer cells. Two such differences may be the expression of cyclin B1 protein predominantly in cancer cells and the differential sensitivity of cancer cells to the inhibition of cyclin A-dependent kinase [27, 48]. We suggest that 2-ME and paclitaxel may take advantage of these differences to inhibit the growth of prostate cancer cells and induce apoptosis. Given that paclitaxel has an effect on patients with AI-PC as a single drug and in combination with other drugs [54], our results hold promise that 2-ME will have a similar efficacy in AI-PC.

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Table I

Flow cytometric analysis of cell cycle effects of 2-ME, paclitaxel, plus cdk inhibitors at 24 h in LNCaP and DU 145 prostate cancer cell lines.

Cells	Treatment[a]	sub-G1[b]	G1	S	G2/M
LNCaP					
	Control	0.02	67.2	23.8	8.75
	2-ME	8.19	24.8	18.5	49.1
	Pacl.	5.99	28.6	15.4	50.2
	Allst.	5.13	64.1	11.6	19.4
	2-ME/Allst.	1.78	64.1	12.6	21.5
	Pacl./Allst.	5.04	63.2	14.8	17.2
DU 145					
	Control	0.47	44.7	42.2	12.7
	2-ME	25.3	26.4	14.8	38.3
	Pacl.	16.5	4.24	12.9	67.7
	Purv.	1.24	13.3	17.9	67.5
	2-ME/Purv	3.59	11.8	21.5	63.3
	Pacl./Purv.	1.81	12.5	19.7	66.3

[a] The doses were 5 μM 2-ME, 10 nM paclitaxel, 5 μM allsterpaullone, and 5 μM purvalanol A.

[b] The percentage of cells in the sub-G1, G1, S, and G2/M DNA content.

Figure Legends

Fig. 1. Flow cytometric analysis of 2-ME and paclitaxel dose response in prostate cancer cells. Human prostate cancer cell lines LNCaP, DU 145, and PC-3 were treated with varying doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the percentage of cells in the cell cycle phases (G1, S, and G2/M) determined by flow cytometry. In LNCaP, 2 μM 2-ME and 2 nM paclitaxel increased cells in G1 (arrows; $P< 10^{-6}$). Higher doses of 2-ME (≥5 μM) and paclitaxel (≥10 nM) blocked all prostate cancer cells in G2/M with concomitant reduction in G1. Results are expressed as means ± standard deviation (error bars).

Fig. 2. G2/M-promoting doses of 2-ME and paclitaxel increased cyclin B1 protein and its associated kinase activity in prostate cancer cells. (A) LNCaP cells were treated with increasing doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the levels of cyclin B1 and cdk1 proteins determined by Western blot analysis, normalized to actin protein, and compared to control (C) treated cells. Cyclin B1 protein was immunoprecipitated and its associated kinase activity (^{32}P-histone) determined from the same lysates. Kinase activity was normalized to the amount of histone protein detected by Coomassie blue. (B) 2-ME and paclitaxel treatment of LNCaP, DU 145, and PC-3 cells resulted in similar increases in cyclin B1 protein and its

associated kinase activity compared to control treated cells (*, $P< 0.03$). Results are expressed as means (fold control=1) ± standard deviation (error bars).

Fig. 3. G1-promoting dose of 2-ME and paclitaxel inhibit cyclin A-dependent kinase activity in LNCaP cells. LNCaP cells were treated with increasing doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the levels of cyclin A and cdk2 proteins determined by Western blot analysis and compared to control (C) treated cells. Cyclin A protein was immunoprecipitated and its associated kinase activity (^{32}P-histone) determined from the same lysates. 2 μM 2-ME and 2 nM paclitaxel inhibited cyclin A-dependent kinase (arrows) without decreased cyclin A and cdk2 proteins.

Fig. 4. Changes in cell cycle distribution with time after treatment of prostate cancer cells with 2-ME and paclitaxel. LNCaP, DU 145, and PC-3 cells were treated with 2-ME (5 μM for LNCaP and DU 145; 10 μM for PC-3) and paclitaxel (10 nM) for 4, 24, 48, and 72 h and the percentage of cells in the cell cycle phases (sub-G1, G1, S, and G2/M) analyzed by flow cytometry. In 2-ME treated DU 145 cells, there was a significant increase in G2/M after 4 h (arrow; $P<0.03$). At 24 h, all cells showed increase in G2/M with a concomitant reduction in G1. At 48 and 72 h, there was a decrease in G2/M, except with 2-ME treated DU 145 and PC-3 cells. Results are expressed as means ± standard deviation (error bars).

Fig. 5 Changes in cyclin B1/cdk1 protein and cyclin B1-dependent kinase activity with time after treatment of prostate cancer cells with 2-ME and paclitaxel. (A) LNCaP cells were treated

with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the levels of cyclin B1 and cdk1 proteins analyzed by Western blot and compared to control (C) treated cells. Cyclin B1 protein was immunoprecipitated and its associated kinase activity (^{32}P-histone) determined from the same lysates. Cyclin B1-dependent kinase activity decreased in 2-ME but not paclitaxel treated LNCaP cells at 48 and 72 h. (B) 2-ME and paclitaxel treatment of LNCaP, DU 145, and PC-3 cells resulted in a peak of cyclin B1 protein and its associated kinase activity at 24 h with subsequent decline at 48 and 72 h. The exception is paclitaxel treated LNCaP cells, which maintained high cyclin B1-dependent kinase activity at 48 and 72 h, despite low cyclin B1 protein. There was a greater decrease in cyclin B1-dependent kinase activity in paclitaxel compared to 2-ME treated DU 145 cells (*, $P<0.05$). Results are expressed as means (fold control=1) ± standard deviation (error bars).

Fig. 6. 2-ME and paclitaxel decrease cyclin A protein and its associated kinase activity in prostate cancer cells after 48 and 72 h of treatment. (A) LNCaP cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the levels of cyclin A and cdk2 proteins analyzed by Western blot and compared to control (C) treated cells. Cyclin A but not cdk2 protein decreased in 2-ME treated LNCaP cells at 48 and 72 h. Cyclin A protein was immunoprecipitated and its associated kinase activity (^{32}P-histone) determined from the same lysates. Cyclin A-dependent kinase activity decreased in both 2-ME and paclitaxel treated LNCaP cells at 48 and 72 h. (B) 2-ME and paclitaxel treatment of LNCaP, DU 145, and PC-3 cells resulted in significantly decreased cyclin A-dependent kinase activity at 48 and 72 h. Cyclin A protein was significantly decreased only in 2-ME treated LNCaP and PC-3 cells (*, $P<0.03$),

whereas cyclin A-dependent kinase activity decreased in all 2-ME and paclitaxel treated prostate cancer cells (*, $P<0.04$) at 48 and 72 h. Results are expressed as means (fold control=1) ± standard deviation (error bars).

Fig. 7. 2-ME and paclitaxel increased p53 and p21 proteins in LNCaP cells. (A) LNCaP cells were treated with increasing doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the levels of p53, p21, and p27 proteins determined by Western blot and compared to control (C) treated cells. p53 increased with 2-ME (≥2 μM) and paclitaxel (≥2 nM), whereas p21 significantly increased with only 2 μM 2-ME and 2 nM paclitaxel ($P<0.05$). There were no significant differences in p27. (B) LNCaP cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the levels of p53, p21, and p27 proteins analyzed by Western blot. p53 increased at 24 h and remained elevated at 48 and 72 h, whereas p21 increased at 48 and 72 h. (C) Increased association of p21 with cdk2 protein in 2-ME treated LNCaP cells. p21 protein was immunoprecipitated (+ ip) from LNCaP cells treated with 5 μM 2-ME (M) or control (C) for 24 h and samples analyzed by Western blot for cdk2 protein. Positive control was LNCaP (LN) cell total lysate and negative control was immunoprecipitation with non-specific rabbit Ig (– ip).

Fig. 8. 2-ME and paclitaxel increased apoptosis and caspase-3 activity in prostate cancer cells at 48 and 72 h. (A) LNCaP cells were treated with 5 μM 2-ME for 24, 48, and 72 h and cells analyzed by DAPI staining of nuclei for apoptotic cells; control treated cells were at 24 h. After 24 h of 2-ME treatment, many cells were blocked in mitosis (arrowhead) and by 48 and 72 h,

there was an increase of apoptotic cells with densely stained and fragmented chromatin (arrows). (B) 2-ME and paclitaxel treatment of LNCaP, DU 145, and PC-3 cells resulted in significantly increased apoptotic cells (DAPI) and caspase-3 activity at 48 and 72 h (*, $P<0.04$). Increased apoptotic cells and caspase-3 activity was greatest in LNCaP cells. In DU 145 cells, paclitaxel increased apoptotic cells greater than 2-ME at 48 and 72 h. Results are expressed as means (fold above control=1) ± standard deviation (error bars).

Fig. 9. PARP and caspase-3 cleavage was greatest in 2-ME and paclitaxel treated LNCaP and DU 145 compared to PC-3 prostate cancer cells. LNCaP (LN), DU 145 (DU), and PC-3 (PC) cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 24, 48, and 72 h and the levels of caspase-3, PARP, Bax, Bcl-2, Bcl-xL, and survivin determined by Western blot and compared to control (C) treated cells. There was a significant decrease of pro-caspase-3 and increased PARP cleavage in LNCaP and DU 145 compared to PC-3 cells. There was greater cleavage of PARP in paclitaxel compared to 2-ME treated DU 145 cells. No significant differences were notable in Bax, Bcl-2, and Bcl-xL. The presence of a slower migrating Bcl-2 protein was greatest in LNCaP cells treated with 2-ME for 24 h. Mitotic block resulted in increased survivin, which decreased at 48 and 72 h. The overall levels of Bcl-xL and survivin was greater in DU 145 and PC-3 compared to LNCaP cells.

Fig. 10. Inhibition of the 2-ME and paclitaxel-mediated increase of cyclin B1-dependent kinase activity blocked induction of apoptosis. (A) LNCaP and DU 145 cells were treated for 24 h with 5 μM 2-ME (M), 10 nM paclitaxel (P), 5 μM allsterpaullone (A, LNCaP), 5 μM purvalanol A

(Pu, DU 145), and 2-ME or paclitaxel combined with allsterpaullone (MA, PA) and purvalanol A (MPu, PPu). Cyclin B1 and A proteins were immunoprecipitated and the associated kinase activity (B1/kinase and A/kinase) determined and compared to control (C) treated cells. In LNCaP, allsterpaullone decreased both cyclin B1- and A-dependent kinase activity, whereas in DU 145 purvalanol A decreased cyclin B1-dependent kinase activity with a concomitant increase in cyclin A-dependent kinase activity. (B) Cdk inhibitors allsterpaullone and purvalanol A significantly inhibited 2-ME and paclitaxel-mediated apoptosis in LNCaP, DU 145 (72 h), and PC-3 (6 days) as determined by DAPI staining and caspase-3 activity (*, $P<0.01$). (C) Western blot showing that allsterpaullone and purvalanol A blocked 2-ME and paclitaxel-mediated cleavage of PARP in LNCaP and DU 145 cells at 72 h. A non-specific band was noted below the cleaved PARP fragment.

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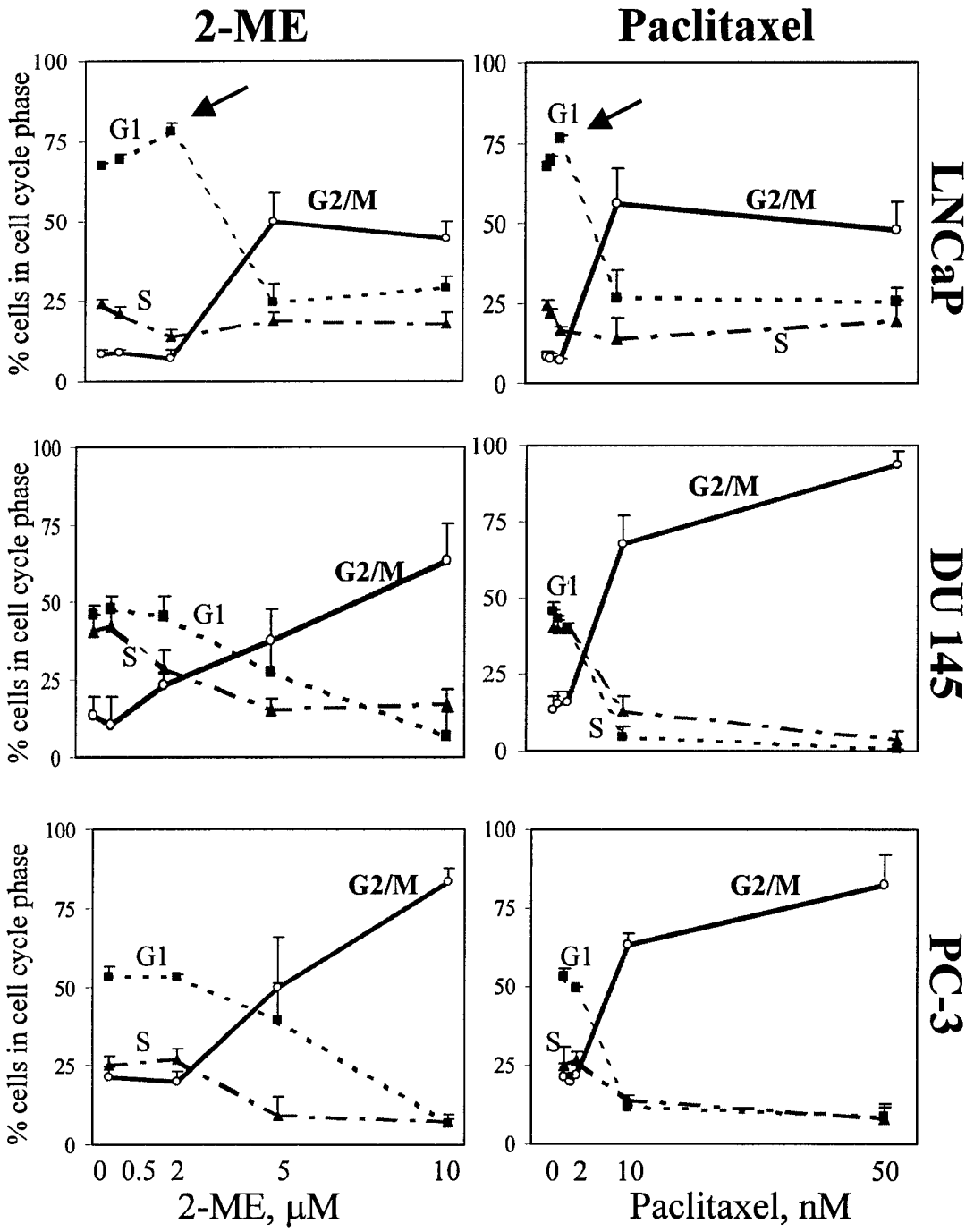
2-ME
Paclitaxel
LNCaP
DU 145
PC-3
% cells in cell cycle phase
100
75
50
25
0
G1
S
G2/M
0 0.5 2 5 10
2-ME, μM
0 2 10 50
Paclitaxel, nM

Figure 1

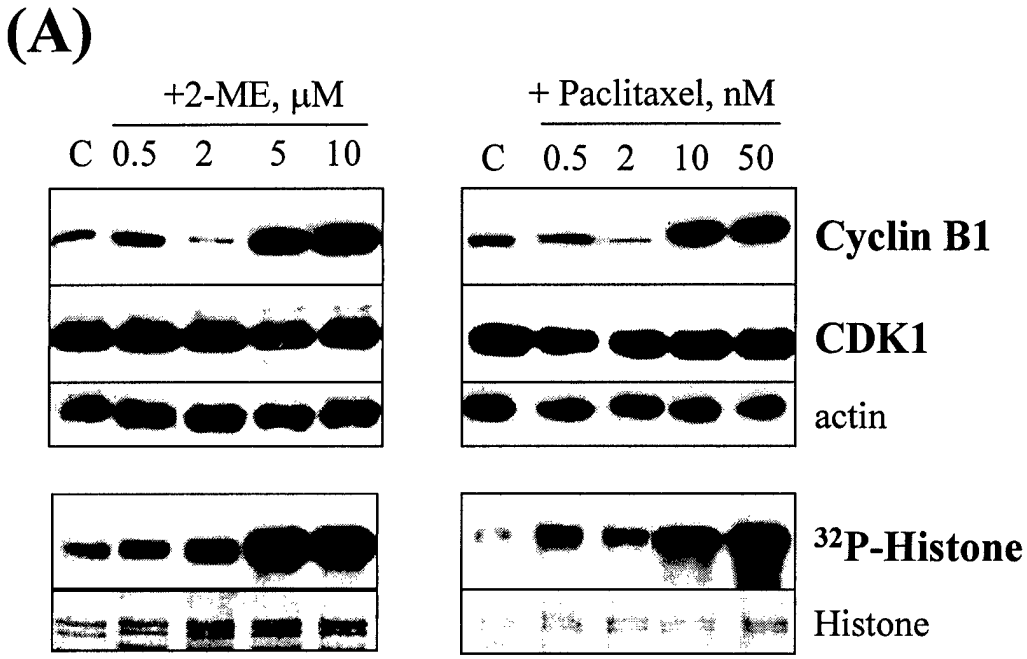
(A)
+2-ME, μM
C 0.5 2 5 10
+ Paclitaxel, nM
C 0.5 2 10 50
Cyclin B1
CDK1
actin
32P-Histone
Histone

Figure 2A

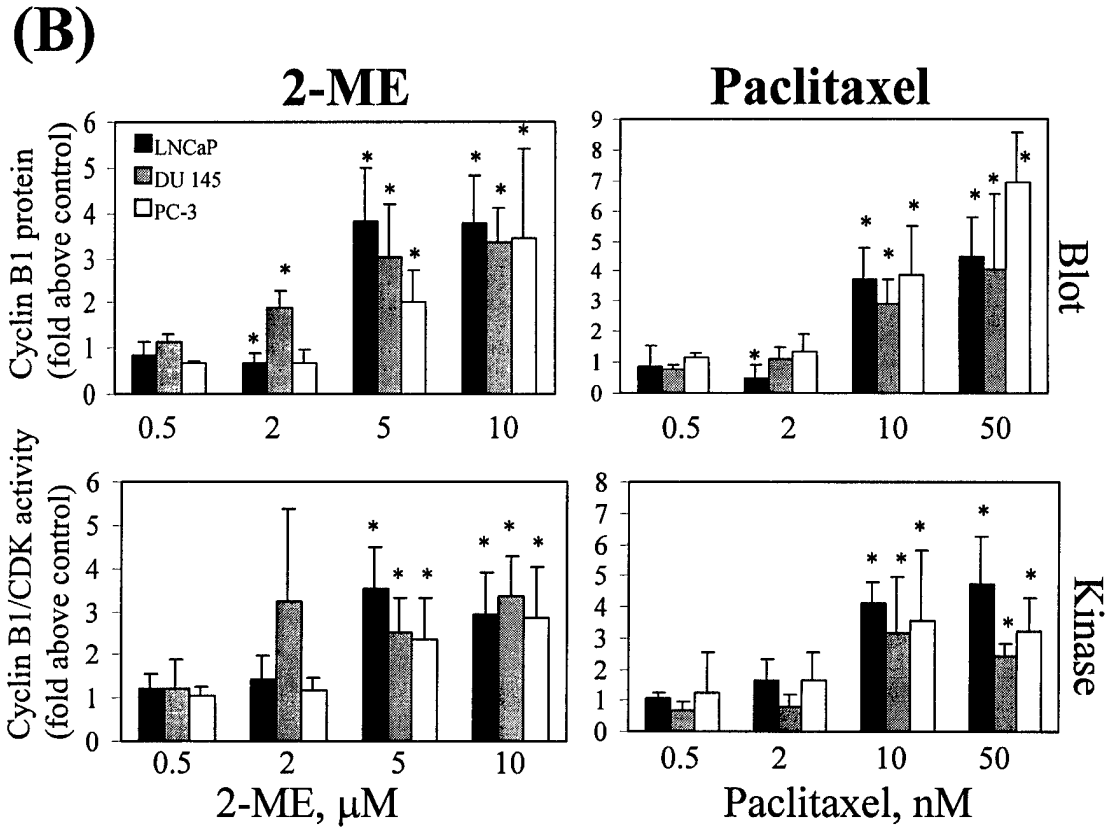
(B)
2-ME
Paclitaxel
LNCaP
DU 145
PC-3
Cyclin B1 protein (fold above control)
Cyclin B1/CDK activity (fold above control)
Blot
Kinase
0.5
2
5
10
50
2-ME, μM
Paclitaxel, nM

Figure 2B

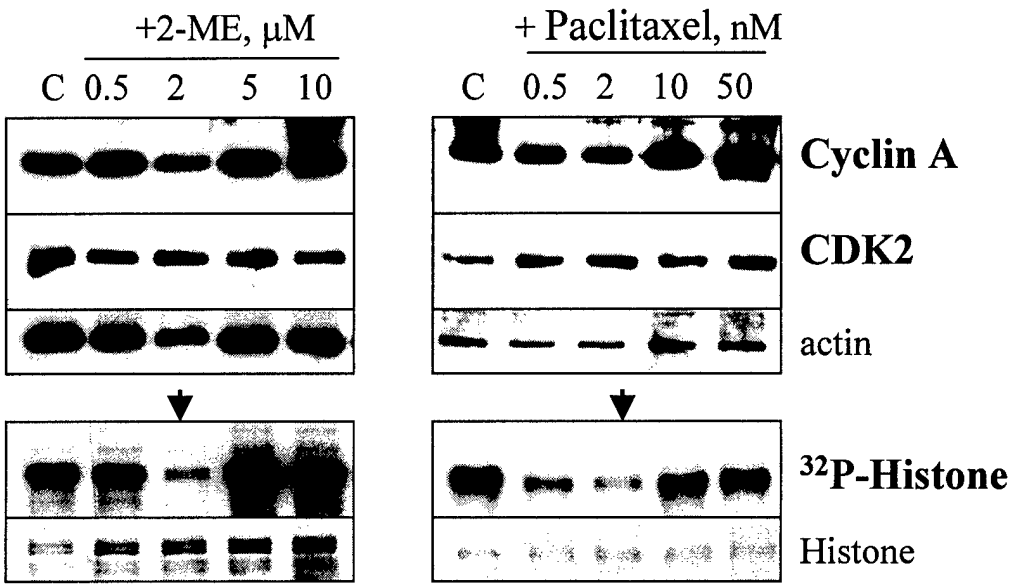
+2-ME, μM
C 0.5 2 5 10
+ Paclitaxel, nM
C 0.5 2 10 50
Cyclin A
CDK2
actin
32P-Histone
Histone

Figure 3

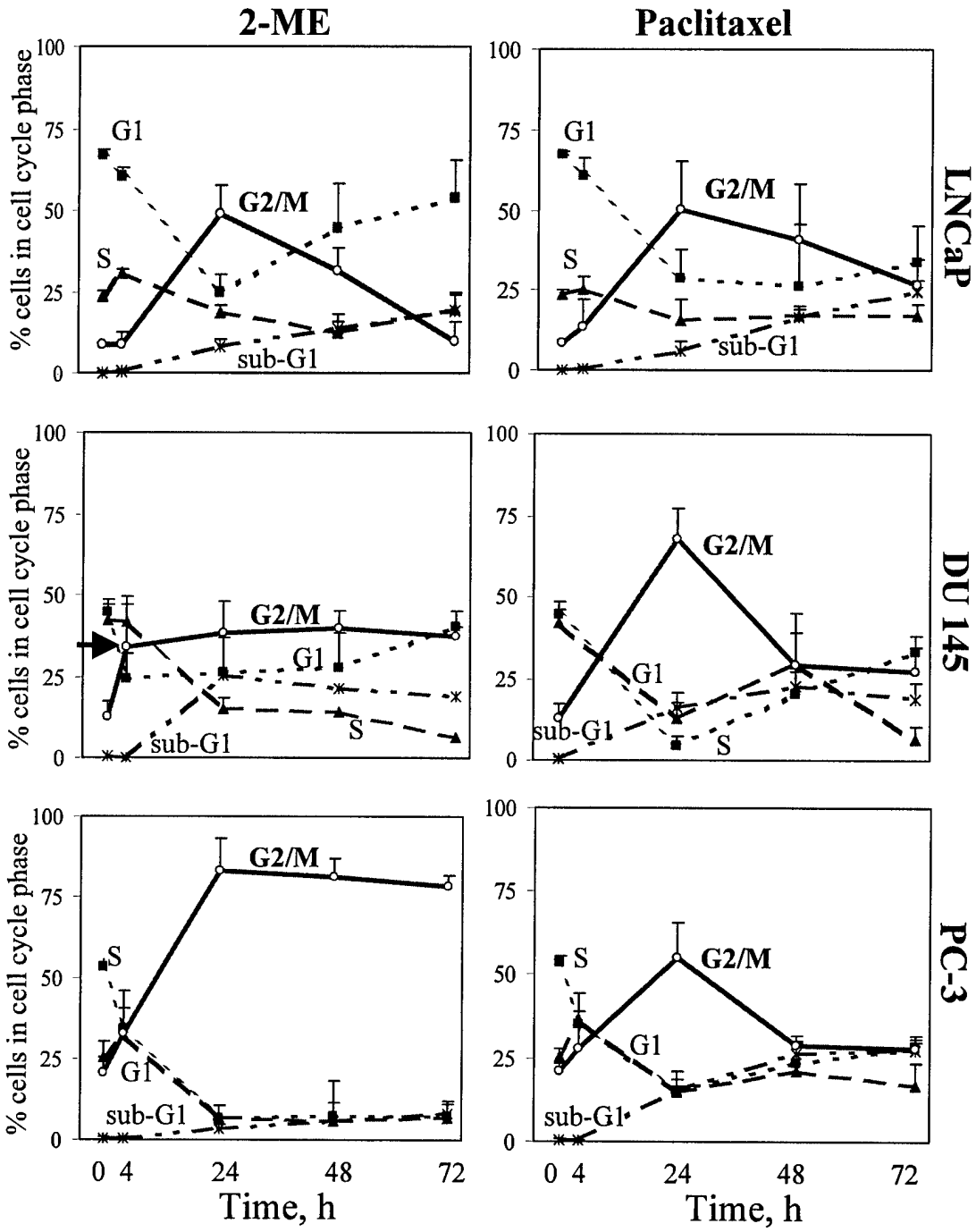
2-ME
Paclitaxel
LNCaP
DU 145
PC-3
% cells in cell cycle phase
100
75
50
25
0
G1
G2/M
S
sub-G1
0 4
24
48
72
Time, h

Figure 4

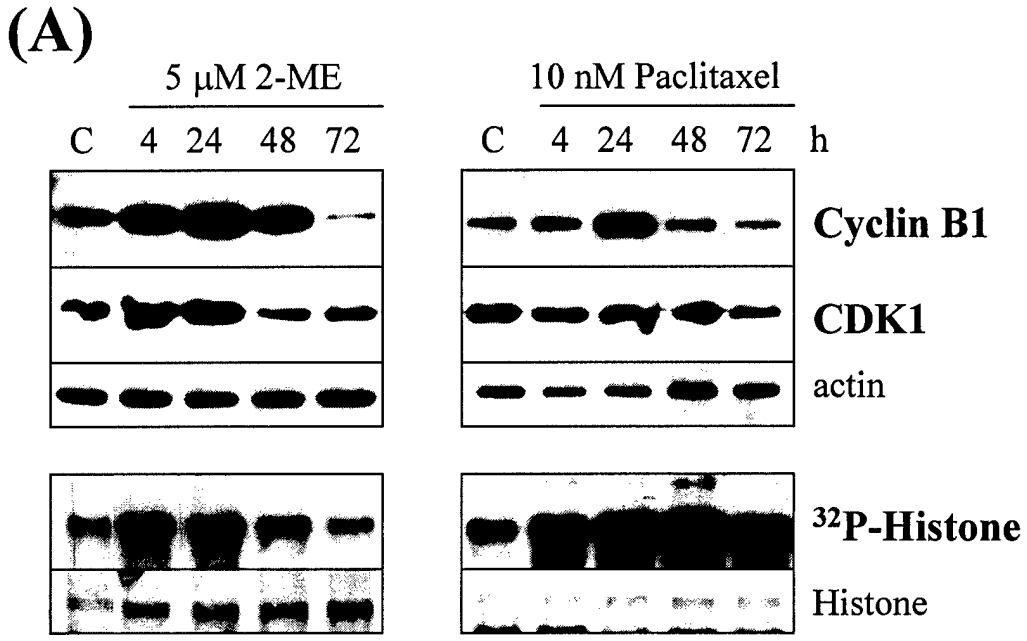
(A)
5 μM 2-ME
10 nM Paclitaxel
C 4 24 48 72
C 4 24 48 72 h
Cyclin B1
CDK1
actin
32P-Histone
Histone

Figure 5A

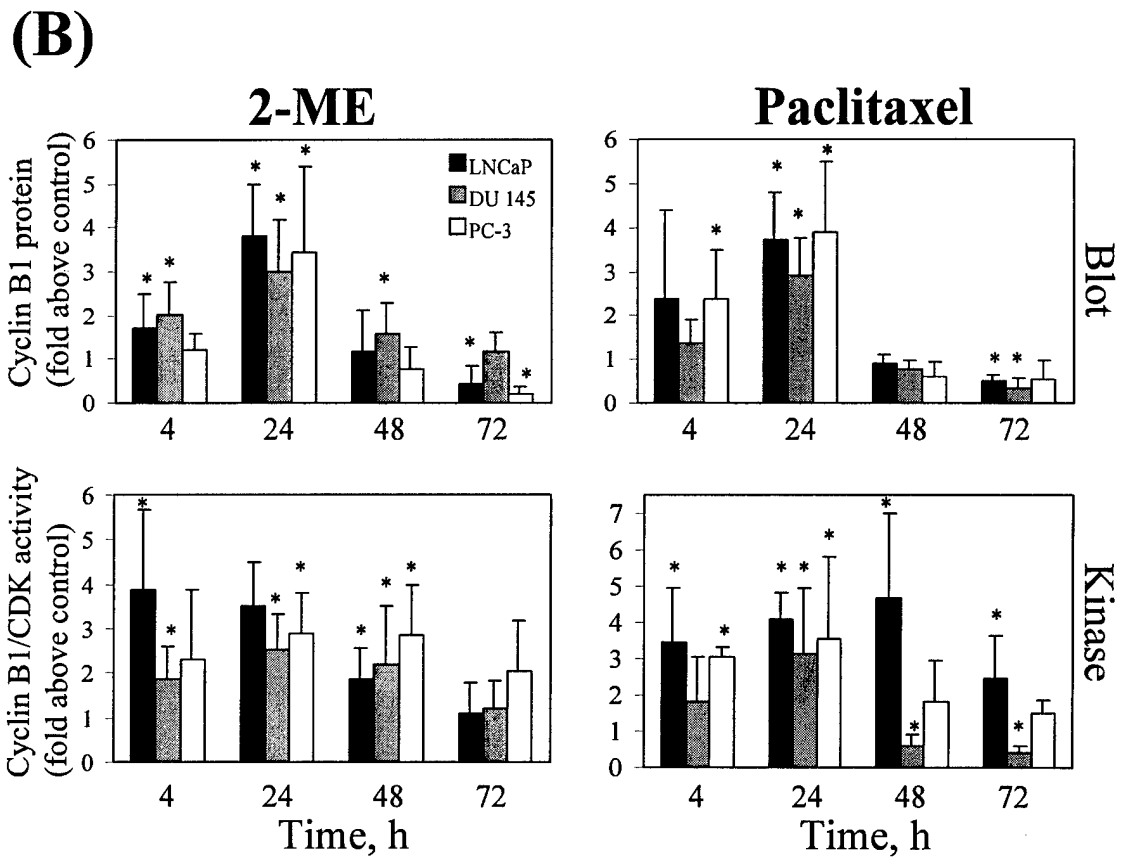
(B)
2-ME
Paclitaxel
Cyclin B1 protein (fold above control)
Cyclin B1/CDK activity (fold above control)
LNCaP
DU 145
PC-3
Blot
Kinase
Time, h
Time, h

Figure 5B

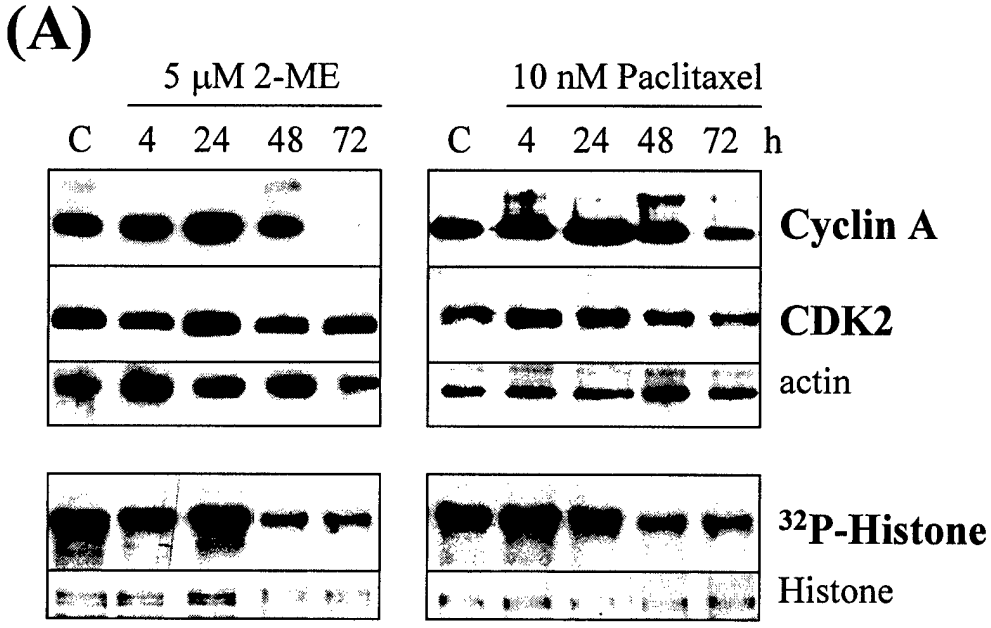
(A)
5 μM 2-ME
10 nM Paclitaxel
C 4 24 48 72
C 4 24 48 72 h
Cyclin A
CDK2
actin
32P-Histone
Histone

Figure 6A

(B)

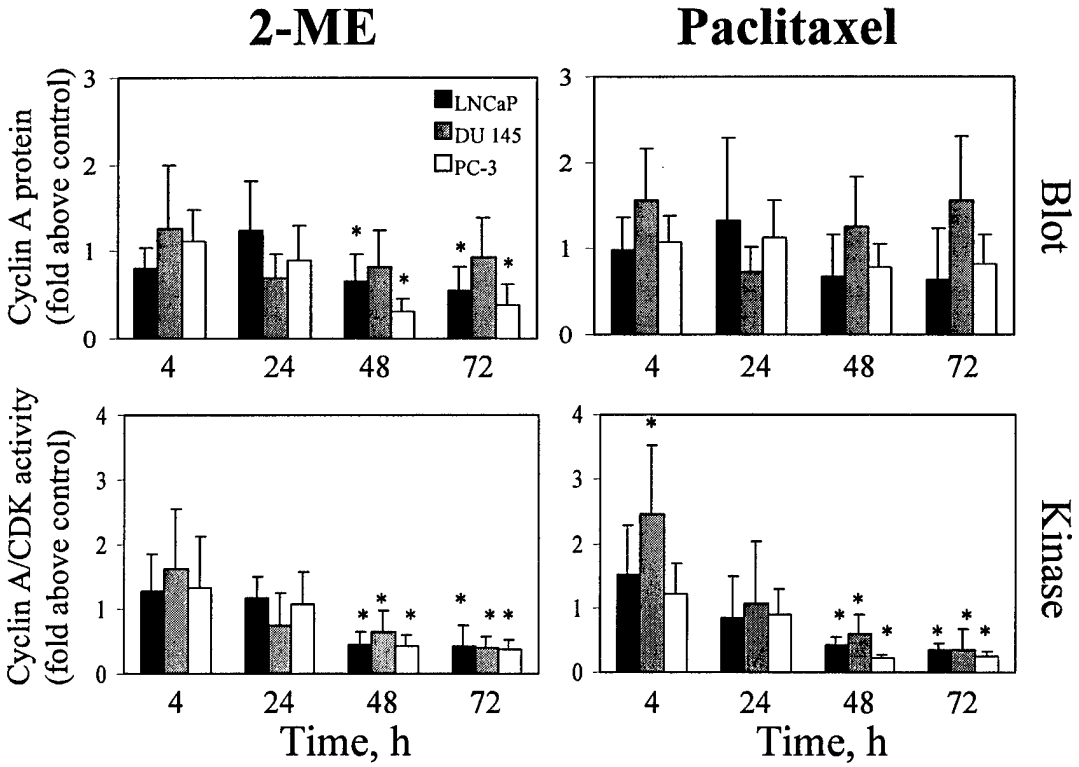
2-ME
Paclitaxel
Cyclin A protein (fold above control)
Cyclin A/CDK activity (fold above control)
LNCaP
DU 145
PC-3
Blot
Kinase
Time, h
Time, h

Figure 6B

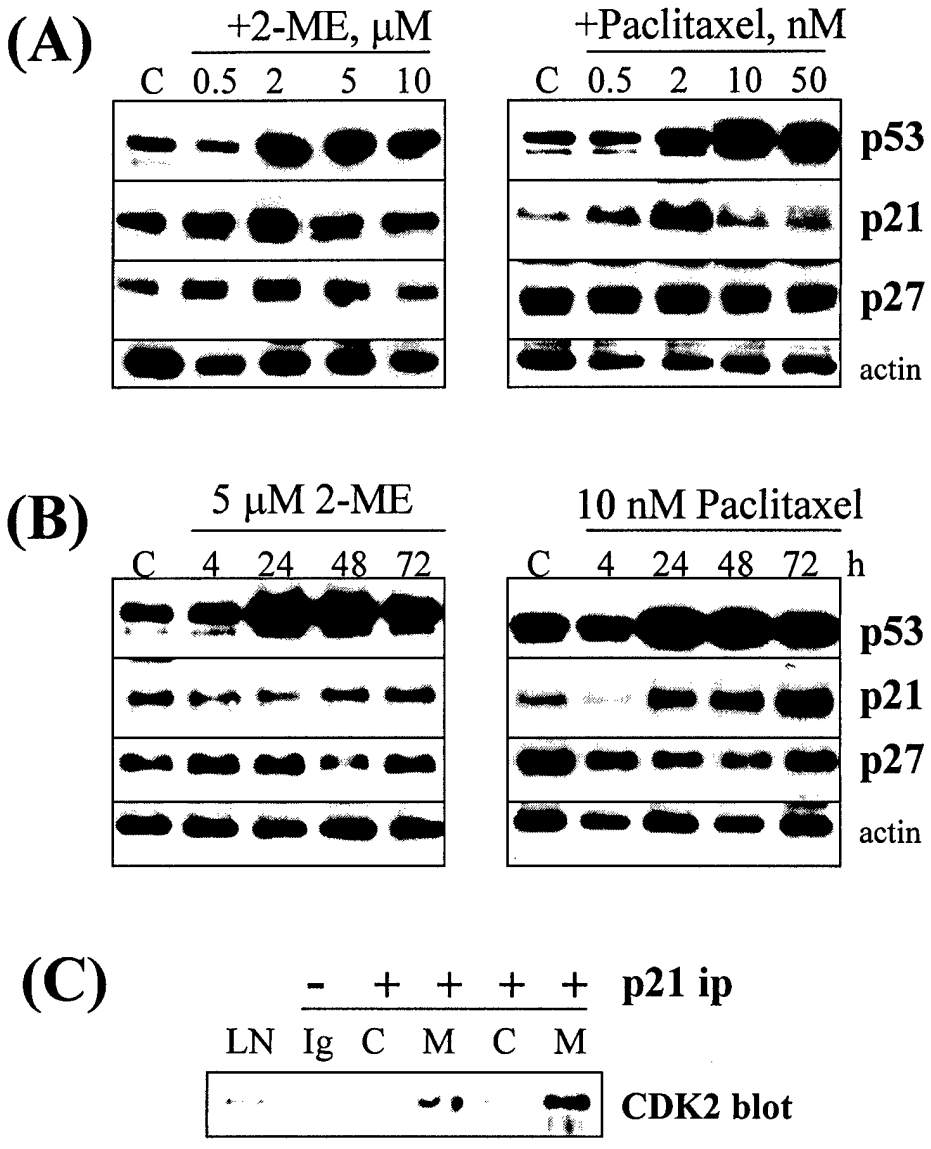
(A)
+2-ME, μM
C 0.5 2 5 10
+Paclitaxel, nM
C 0.5 2 10 50
p53
p21
p27
actin
(B)
5 μM 2-ME
C 4 24 48 72
10 nM Paclitaxel
C 4 24 48 72 h
p53
p21
p27
actin
(C)
- + + + + p21 ip
LN Ig C M C M
CDK2 blot

Figure 7

(A) LNCaP + 5 μM 2-ME

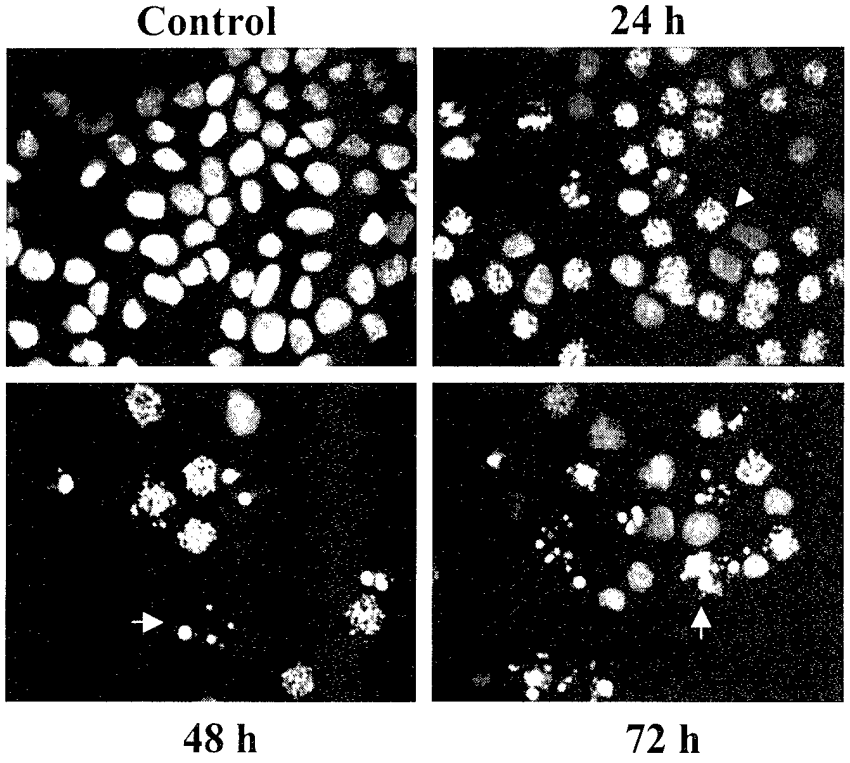

Figure 8A

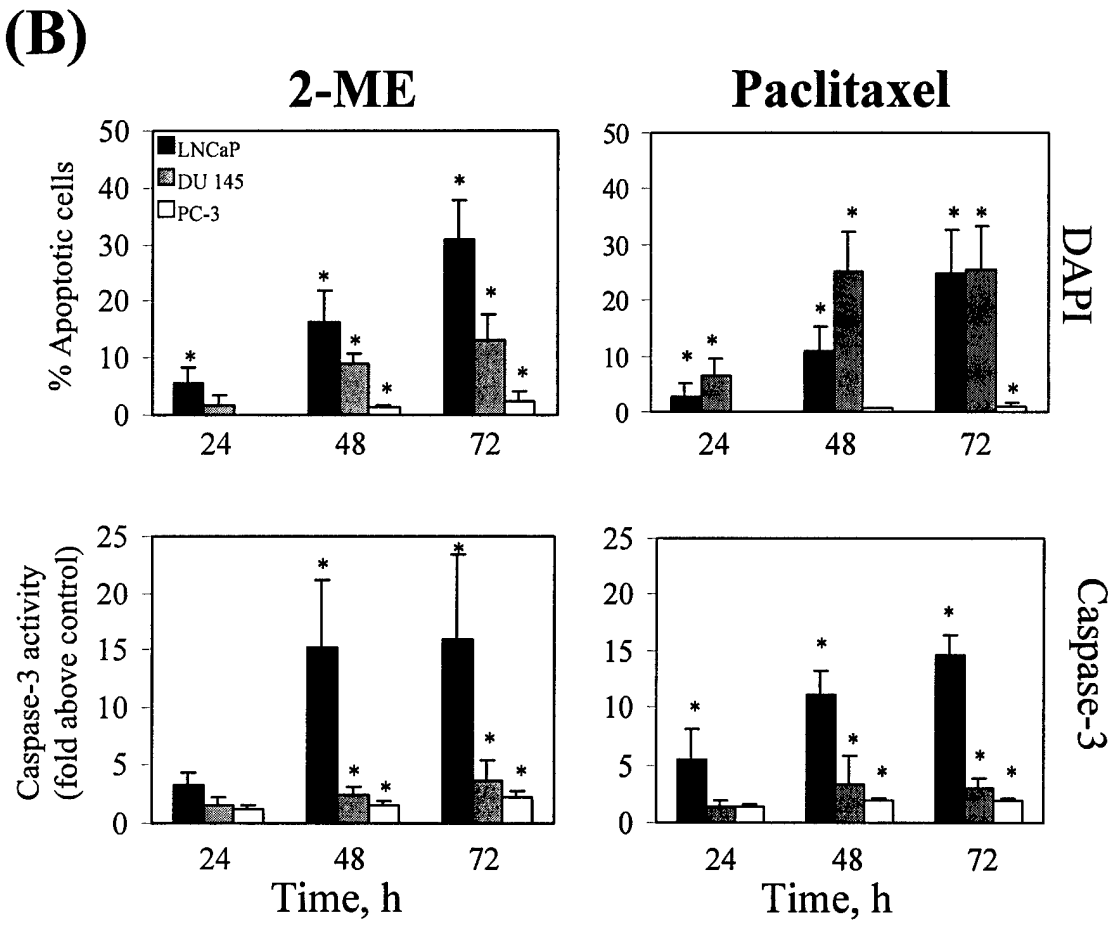
(B)
2-ME
Paclitaxel
LNCaP
DU 145
PC-3
% Apoptotic cells
DAPI
Caspase-3 activity
(fold above control)
Caspase-3
Time, h
Time, h

Figure 8B

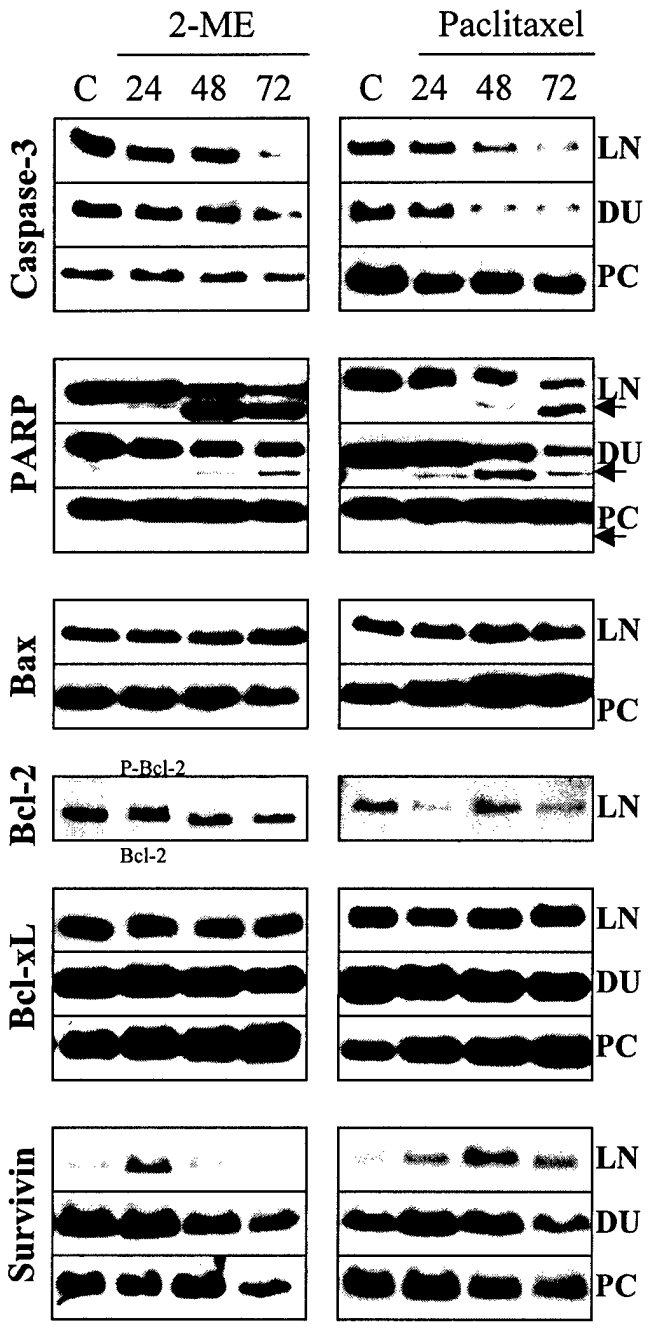
2-ME
Paclitaxel
C 24 48 72
C 24 48 72
Caspase-3
PARP
Bax
Bcl-2
P-Bcl-2
Bcl-2
Bcl-xL
Survivin
LN
DU
PC

Figure 9

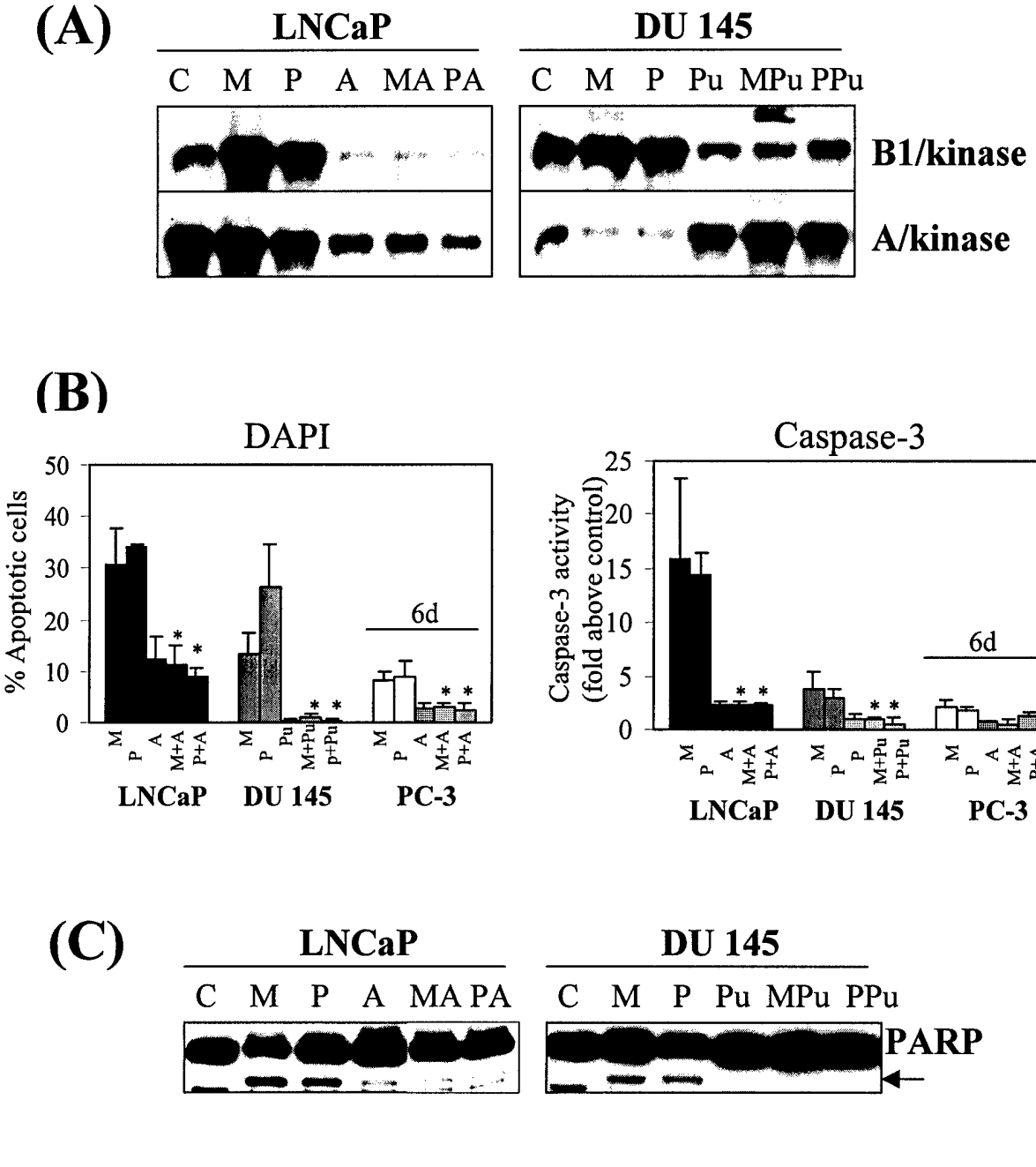

Figure 10A, B, C