Award Number:  DAMD17-03-1-0179

TITLE: 2-Methoxyestradiol as a Chemotherapeutic for Prostate Cancer

PRINCIPAL INVESTIGATOR: Carlos Perez-Stable, Ph.D.

CONTRACTING ORGANIZATION: University of Miami
Miami, FL 33101

REPORT DATE: April 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with promise for cancer chemotherapy, including advanced prostate cancer. Our hypothesis is one of the cancer-specific mechanisms whereby 2-ME exerts its anti-prostate cancer activity is the deregulated activation of cyclin B1/cdk1 kinase during the cell cycle, which results in the induction of apoptotic cell death. Several experimental results support this hypothesis: 1) there is a positive correlation between the levels of cyclin B1 protein and the ability of 2-ME to increase apoptosis in prostate cancer cells; 2) overexpression of cyclin B1 increases 2-ME-mediated apoptosis while inhibition of cdk1 activity lowers 2-ME-mediated apoptosis; 3) low doses of 2-ME and docetaxel can increase G2/M cell cycle arrest and apoptosis in prostate cancer cell lines and in the Gγ/T transgenic mouse model of prostate cancer greater than either drug alone. We conclude that 2-ME can increase apoptosis in prostate cancer cells because of the expression of cyclin B1 protein, which is minimally expressed in normal cells.
## Table of Contents

**Introduction** ........................................................................................................... 4

**Body** ....................................................................................................................... 4-10

**Key Research Accomplishments** ........................................................................ 9

**Reportable Outcomes** .......................................................................................... 9, 10

**Conclusions** ......................................................................................................... 10

**References** ............................................................................................................ 10

**Appendices** .......................................................................................................... 48 pages
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 third 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. A manuscript entitled “2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells” published in Cancer Letters summarizes some of our results and is included in the appendix [6]. Blocking the 2-ME and paclitaxel increase in cyclin B1/cdk activity with the potent cdk inhibitors purvalanol A and alsterpaullone resulted in decreased apoptosis. These results suggest that 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity is required for induction of apoptosis in prostate cancer cells.

In addition, a manuscript entitled “Increased Expression of Cyclin B1 Sensitizes Prostate Cancer Cells to Apoptosis Induced by Chemotherapy”, recently published in Molecular Cancer Therapeutics, summarizes our results included in the 2006 Annual Report [7]. Cyclin B1 is overexpressed in a variety of cancers compared to normal tissues and appears to be closely associated with early events in neoplastic transformation. In prostate cancer, it is not clear if the overexpression of cyclin B1 plays a role in disease progression and/or in resistance to chemotherapy. We found a positive correlation between cyclin B1 protein and apoptosis induced by chemotherapy in prostate cancer and non-transformed cells. Thus, despite its association with transformed cells, higher levels of cyclin B1 protein in prostate cancer may be a good prognostic marker for chemotherapy. We have submitted an application to the 2007 PCRP entitled “Role of Cyclin B1 in Sensitizing Prostate Cancer Cells to Apoptosis Induced By Chemotherapy” to continue our studies from our 2002 PCRP grant.

The following sections report our findings from 2006 to the present associated with each task in the approved statement of work.

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-161positions in the human prostate cancer cell lines LNCaP, DU 145, and PC-3 using quantitative Western blot (months 1-4).

   Completed and presented in the 2004 annual report.

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).
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 pursued this task because we have focused on other mechanisms.

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).

We presented data for this task in the 2005 annual report. We repeated the experiment using the NE-PER nuclear and cytoplasmic extraction reagents from Pierce Biotechnology (Rockford, IL). The results show that in control treated LNCaP cells, cyclin B1 is found predominantly in the nucleus; in PC-3 cells, however, there are similar levels of cyclin B1 in cytoplasm and nucleus. (Fig. 1). Treatment with 5 µM 2-ME showed an increase in cytoplasmic cyclin B1 in LNCaP but a decrease of cyclin B1 in PC-3 cells. In both LNCaP and PC-3 cells, there is an increase in nuclear cyclin B1 protein with 2-ME treatment. At this time, we do not know the significance of these results.

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).

We presented data for this task in the 2006 annual report, which is included in reference 7.


We presented data for this task in the 2006 annual report.

**Specific aim 2:** 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).

We presented data for this task in the 2006 annual report.

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 presented data for this task in the 2006 annual report, which is included in reference 7. To investigate molecular changes possibly involved in the ability of cyclin B1 kinase inhibitor purvalanol A to block 2-ME-mediated apoptosis in DU 145 AI-PC cells [6], we did a SuperArray analysis, as per the manufacture’s instructions (SuperArray Bioscience, Frederick, MD). DU 145 cells were treated with 5 µM 2-ME (induction of apoptosis) or 5 µM 2-ME plus 5 µM purvalanol A (antagonizes induction of apoptosis) for 72 h and biotinylated RNA probes hybridized to an apoptosis microarray specific for 112 genes involved in apoptosis (Fig. 2). Results show that AKT1 mRNA (spot 1), which is upregulated in most cancers including AI-PC and confers resistance to chemotherapeutic...
drugs [8], is decreased in 2-ME treated compared to 2-ME + purvalanol A treated DU 145 cells. Bak1 mRNA (spot 2), a pro-apoptotic member of Bcl-2 family [9], is increased in 2-ME compared to 2-ME + purvalanol A treated DU 145 cells. GADD45α mRNA (spot 4), a member of the growth arrest and DNA damage inducible family of proteins that inhibits CDK1 activity and is thought to play a role in G2/M checkpoint response to DNA damage [10], is increased in 2-ME compared to 2-ME + purvalanol A treated DU 145 cells. No differences in survivin and Mcl-1 mRNA were identified (spots 3 and 5).

Treatment of DU 145 cells with 2-ME (M) results in an increase in cleaved PARP, whereas addition of purvalanol A (P) antagonizes 2-ME increase in cleaved PARP (MP) (Fig. 3). To determine whether mRNA differences measured by SuperArray analysis correspond to differences in protein, we analyzed the expression of AKT1, Bak1, and GADD45α by Western blots. Results showed that 2-ME decreased AKT1 and increased Bak1 and GADD45α proteins compared to MP, P, and control treated DU 145 cells. Similar to the SuperArray mRNA results, there were no differences in the protein levels of survivin and Mcl-1 (not shown). We confirmed by Western blot that there is a consistent increase in Bak1 and decrease in AKT1 proteins in LNCaP and PC-3 cells treated with 2-ME (not shown). In Aim 2 of the 2007 PCRP application, we will further investigate whether Bak1 and AKT1 are important for cyclin B1 kinase increase in apoptosis mediated by 2-ME.

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 presented data for this task in the 2006 annual report, which is included in reference 7.

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).

We previously attempted to transfect the cyclin B1 expression plasmid pCMX/cyclin B1 (used in LNCaP/PC-3 stable clones; ref. 7) into NRP cells but failed to detect exogenous cyclin B1 by Western blot. In contrast, we were able to transfect other expression plasmids (Tag/pcDNA, pEGFP-F) into NRP cells and easily detect protein expression by Western blot and fluorescence microscopy. In specific aim 2 of our 2007 application (Determine the mechanisms of how cyclin B1 can enhance chemotherapy induced apoptosis in PC but not in non-transformed cells), we will determine if there is a difference in the stability of cyclin B1 protein between prostate cancer cells and non-transformed cells. NRP cells will be transfected with pCMX/cyclin B1 expression plasmid plus increasing doses of the proteasome inhibitor MG 132 and the levels of cyclin B1 measured by Western blot. We will also transfect mutant cyclin B1 expression plasmid (can not be ubiquinated and degraded by proteasome pathway into NRP cells and determine if cyclin B1 can be detected by Western blot. In addition, we will perform in vitro cyclin B1 degradation assays using lysates prepared from NRP and prostate cancer cells and recombinant cyclin B1 protein. These experiments will allow us to determine whether equal amounts of NRP/MSC lysates can degrade cyclin B1 more rapidly than LNCaP/PC-3 lysates.

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).

We presented data for this task in the 2005 annual report. LNCaP cell clones stably expressing siRNA specific for cyclin
B1 have lower levels of cyclin B1 and undergo less apoptosis compared to negative control clones when treated with 2-ME or docetaxel. This data is included in reference 7.

**Specific aim 3:** 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).

   We presented data for this task in the 2006 annual report.

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).

   We have not yet determined if 2-ME can also increase apoptosis in low-proliferating LNCaP cells.

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

   We presented data for docetaxel (Doc) inhibition of prostate cancer cell lines in the 2004 annual report.

4. Use isobolograph analysis of cell growth assays to determine if docetaxel, R-roscovitine, and etoposide can synergize with 2-ME/2-EE to inhibit prostate cancer cells in proliferating and non-proliferating in vitro conditions (months 14-18).

   We presented data for this task in the 2006 annual report. In addition to LN-AI and LN-AI/CSS cells, we now include the cell growth data for PC-3 and PC-3/AR (PC-3 cells stably expressing androgen receptor) (Fig. 4). The results show there is a greater than additive inhibition of cell proliferation in all prostate cancer cells using low doses of 2-ME (0.5-1 µM) and Doc (0.05-0.1 nM). We plan on using the CalcuSyn software from Biosoft (Cambridge, UK) to determine if the effect is synergistic. This will be Fig. 1 of our manuscript in preparation “Low Dose Combination of 2-Methoxyestradiol and Docetaxel Can Block Prostate Cancer Cells in Mitosis and Induce Apoptosis”.

5. Determine whether the drug that can act synergistically with 2-ME/2-EE to inhibit prostate cancer cell growth also has a synergistic effect on the cell cycle and apoptosis (months 16-20).

   We determined in LN-AI cells whether the combination of 2-ME and docetaxel has an effect on the cell cycle and apoptosis. LN-AI cells were treated with 0.5 µM 2-ME, 0.1 nM Doc, and their combinations and compared with control treated cells. After 24 h, the 2-ME/Doc combination results in G2/M block in the cell cycle whereas 2-ME and Doc (low dose) alone did not result in any difference in G2/M compared to control cells (Fig. 5A). After 72 h, the 2-ME/Doc combination results in a significant increase in apoptosis over that of single drug and control cells (Fig. 5B). Western blot analysis also shows greater levels of cleaved PARP in the 2-ME/Doc combination (Fig. 5C). These results suggest that lower doses of 2-ME and Doc can be used in combination to obtain the G2/M cell cycle block and increase in apoptosis. This will be figure 2 of the manuscript in preparation.
6. Using the most promising drug combination identified in vitro, test the in vivo anti-prostate cancer efficacy in the Gy/T-15 and TRAMP transgenic mice (10 mice per group x 6 groups = 60 Gy/T-15 and 60 TRAMP transgenic male mice) (months 20-36).

We presented data for this task in the 2006 annual report. In the Gy/T-15 mice, results showed that as single drugs, only the 150 mg/kg dose of 2-ME and the 5 mg/kg dose of Doc significantly inhibited primary tumors by 32% (P<0.05) and 39% (P<0.02), respectively compared to control treated mice. The low dose combination of 2-ME and Doc (75 + 2.5 mg/kg) significantly inhibited primary prostate tumors by 42% (P<0.02), despite each dose of the drug by itself having no significant effect. The single and combination doses that inhibited primary prostate tumors also increased mitotic cell cycle block as shown by flow cytometry and phospho-histone 3 immunohistochemistry (Fig. 6). All doses of 2-ME and Doc inhibit angiogenesis (CD31) whereas only the doses of 2-ME and Doc that result in mitotic block can increase apoptosis (cleaved caspase-3) (Figs. 7, 8). These results suggest that in order to achieve a chemotherapy effect in Gy/T-15 prostate tumors, it is more important to increase apoptosis than to decrease angiogenesis. We conclude that the combination of low doses of 2-ME and Doc can be utilized to inhibit prostate cancer by increasing mitotic block and inducing apoptosis. Figures 5-7 will be included in the manuscript in preparation.
Figure 8. Increase in apoptosis correlates with inhibition of primary prostate tumors. (A) Blood vessel density was determined by counting the number of CD-31 positive vessels per four random high powered fields (hpf, x400). The results demonstrate a significant inhibition of the number of blood vessels in all treated mice (n=4; *, P<2x10^-5) compared to controls (n=6). (B) Number of cells immunostaining for cleaved caspase-3 was determined per four random “hot spot” high powered fields (hpf, x400). The results demonstrate significant increases in M150, D5, and MD combinations compared to controls, M30, and M75 (n=3; *, P<0.05).

KEY RESEARCH ACCOMPLISHMENTS

- 2-ME increases cytoplasmic cyclin B1 protein in LNCaP cells (high apoptosis) but decreases cytoplasmic cyclin B1 protein in PC-3 cells (low apoptosis) (Fig. 1).
- SuperArray analysis of DU 145 cells treated with 2-ME compared to cells treated with 2-ME + purvalanol A (inhibitor of cyclin B1 kinase that blocks 2-ME apoptosis) identified differences in Bak1, AKT1, and GADD45α mRNA (Fig. 2).
- Western blot confirmed that 2-ME increases Bak1, GADD45α, and decreases AKT1 proteins, whereas 2-ME + purvalanol A blocks these changes (Fig. 3).
- Greater than additive inhibition of prostate cancer cell growth by the combination of 2-ME and Doc compared to the single doses (Fig. 4).
- Low dose combination of 2-ME + Doc increases G2/M cell cycle block and apoptosis in prostate cancer cells (Fig. 5).
- 2-ME and Doc doses that inhibit Gγ/T-15 primary prostate tumors are the same doses that block prostate cancer cells in mitosis (Fig. 6).
- Inhibition of angiogenesis (blood vessels) without increase in apoptosis does not inhibit primary prostate tumors. Only when there is an increase in apoptosis is there inhibition of primary prostate tumors (Figs 7, 8).

REPORTABLE OUTCOMES

Publications
1. Perez-Stable CM. 2006. 2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells. Cancer Letters, 231:49-64. Appended manuscript.
4. Gomez LA, A de las Pozas, T Reiner, K Burnstein, C Perez-Stable. 2007. Increased Expression of Cyclin B1...
CONCLUSIONS

Our data supports the hypothesis 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. First, there is a positive correlation between the levels of cyclin B1 protein and the ability of 2-ME to increase apoptosis in prostate cancer cells. Second, cyclin B1 protein levels is lowest in non-cancer MSC and NRP cells and this correlates with the smallest increase in apoptosis by 2-ME. Third, overexpression of cyclin B1 increases 2-ME-mediated apoptosis in LNCaP and PC-3 cells and dominant negative cdk1 lowered 2-ME-mediated apoptosis in LN-AI cells. Fourth, low doses of 2-ME and Doc can increase G2/M arrest and apoptosis in combination but not as single drugs in prostate cancer cells. This suggests that lower doses of 2-ME and Doc can be used in combination chemotherapy against prostate cancer. Fifth, the doses of 2-ME and Doc that result in mitotic cell cycle block are more effective inhibiting the growth of primary prostate tumors in Gy/T-15 mice. Sixth, only the doses of 2-ME, Doc, and their combinations that result in increase in apoptosis can inhibit the growth of primary prostate tumors in Gy/T-15 mice. Therefore, our data suggests the combination of 2-ME and Doc will be an effective chemotherapy regimen against prostate tumor.

REFERENCES

2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells

Carlos Perez-Stable\textsuperscript{a,b,*}

\textsuperscript{a}Geriatric Research, Education, and Clinical Center and Research Service, Veterans Affairs Medical Center, GRECC (11-GRC), 1201 NW 16 Street, Miami, FL 33125, USA
\textsuperscript{b}Department of Medicine and Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, Miami, FL 33101, USA

Received 29 July 2004; received in revised form 12 October 2004; accepted 14 January 2005

Abstract

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with promise for cancer chemotherapy, including advanced prostate cancer. We have focused on events related to cell cycle arrest (G1 and G2/M) and induction of apoptosis in human prostate cancer cells. Treatment with 2-ME increased cyclin B1 protein and its associated kinase activity followed by later inhibition of cyclin A-dependent kinase activity and induction of apoptosis. Similar results were obtained with paclitaxel (taxol), a clinically relevant agent used to treat advanced prostate cancer. Cyclin-dependent kinase inhibitors prevented 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity and blocked induction of apoptosis. Reduction of X-linked inhibitor of apoptosis (XIAP) protein by 2-ME and paclitaxel correlated with increased apoptosis. Lower doses of 2-ME and paclitaxel resulted in G1 (but not G2/M) cell cycle arrest in the p53 wild type LNCaP cell line, but with minimal induction of apoptosis. 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 subsequently leads to the induction of apoptotic cell death.

\textcopyright{} 2005 Elsevier Ireland Ltd. All rights reserved.

Keywords: 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 [1–4]. 2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer (AI-PC) utilizing a remarkable number of diverse mechanisms that include cell cycle arrest, induction of apoptosis, disruption of microtubules, inhibition of angiogenesis, and increasing oxidative damage [1–6]. 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 [2,7]. 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 [3]. 2-ME’s anti-prostate cancer activity, however, is not well 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 anticancer effect is the disruption of microtubule function and subsequent block in the G2/M phase of the cell cycle [1,2,4]. We have previously shown that 2-ME inhibits both androgen-dependent LNCaP and androgen-independent DU 145 and PC-3 human prostate cancer cells independent of the expression of androgen receptor and tumor suppressors p53 and Rb [5]. 2-ME blocks LNCaP, DU 145, and PC-3 prostate cancer cells in the G2/M phase of the cell cycle and induces apoptosis [5,6]. Specific mechanisms for 2-ME induced inhibition in prostate cancer cells are proposed to be mediated by activation of c-Jun N-terminal kinase (JNK) and inactivation of the anti-apoptosis proteins Bcl-2/Bcl-xl [8–10], up-regulation of the death receptor 5 and induction of the extrinsic pathway of apoptosis [11], and down-regulation of hypoxia-inducible factor-1 [12]. The effect of 2-ME on the components of the cell cycle, specifically the G2/M cyclins A and B1, and whether these effects are required for induction of apoptosis are not known.

Paclitaxel is a well studied chemotherapeutic agent that stabilizes microtubules and has clinical efficacy in a variety of cancers, including AI-PC [13]. Paclitaxel-mediated microtubule damage activates the mitotic checkpoint and blocks the degradation of cyclin B1, leading to a prolonged activation of cyclin B1-dependent kinase (cdk1) and mitotic arrest [14–16]. 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 [14]. 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 [17]. 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 [18]. The subsequent decrease in cyclin B1- cdk1 activity results in a decrease in the levels of survivin and increase in 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 function as anti-prostate cancer agents, we focused on the effect of these drugs on cyclin A and B1 proteins and their associated kinase activities. 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 a decrease in cyclin A-dependent kinase activity and induction of apoptosis in prostate cancer cells. Inhibition of cyclin B1-dependent kinase activity blocked subsequent induction of apoptosis by both agents. These results indicated that 2-ME inhibition of prostate cancer cells 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, and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Histone H1 protein was purchased from Roche Applied Sciences (Indianapolis, IN, USA). 4′,6-Diamidino-2-phenylindole (DAPI), purvalanol A, alsterpaullone, PD 98059, caspase-3 substrate (Ac-DEVD-pNA) and inhibitor (DEVD-CHO) were purchased from Calbiochem (San Diego, CA, USA). Annexin V-FITC was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

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

Human prostate carcinoma cell lines LNCaP-FGC [19], DU 145 [20], and PC-3 [21] 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). Prostate cancer cells were treated with different doses of 2-ME (0.5–10 µM), paclitaxel (0.5–50 nM), or DMSO (0.1%) control for varying times (4–72 h). In all the experiments, floating and trypsinized attached cells were pooled for further analysis.

2.3. Three-day cell growth assay for 2-ME and paclitaxel

LNCaP (10,000), DU 145 (2500), and PC-3 (3000) cells were seeded in 96-well plates. The next day, fresh media containing different doses of 2-ME (0.1–50 µM) and paclitaxel (0.1–50 nM) or control (0.1% DMSO) were added and cells incubated for 3 days. The CellTiter Aqueous cell proliferation colorimetric method (Promega, Madison, WI) was used to determine cell viability as per manufacturer’s instructions. Cell viability was normalized against the vehicle control and the data expressed as a percentage of control from three independent experiments done in triplicate.

2.4. Flow cytometric analysis

Propidium/hypotonic citrate method [22] 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 G1, S, and G2/M DNA content were determined by the ModFit program (Verity Software House, Topsham, ME, USA) from 6 to 8 samples analyzed from at least three independent experiments.

2.5. Western blot analysis

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 NaVO₄), 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% non-fat 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), survivin (FL-142), IAP-1 (H-83), IAP-2 (H-85), and actin (C-11) (Santa Cruz Biotechnology) were diluted 1/1000 in 5% non-fat dry milk, PBS, and 0.25% Tween-20 and incubated overnight at 4°C. Similarly, antibodies specific for poly ADP-ribose polymerase (PARP; C2-10), Bcl-xL (polyclonal) (BD Biosciences Pharmingen, San Diego, CA, USA), and XIAP (Cell Signaling Technology, Beverly, MA) were diluted 1/1500. Membranes were washed in PBS and 0.25% Tween-20 (3×10 min) 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 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 UNSCAN-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 (=1.0) in at least three different samples analyzed from 2 to 5 independent experiments. To determine the overall levels of 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 micrograms of total protein were incubated with 2 μg anti-cyclin A or B1 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. Immune-complexes were collected by centrifugation, washed 3× with NP40 cell lysis buffer, 3× with kinase buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM DTT), resuspended in kinase buffer containing 2 μg histone H1 substrate protein, 25 μM ATP, 5 μCi γ³²P-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 ³²P measured by scintillation counting. Changes in kinase activity of 2-ME and paclitaxel treated cells was determined by normalizing the ³²P-histone values to the scanned H1 protein value and comparing to values of control treated cells (= 1.0) in at least three different samples analyzed from 2 to 5 independent experiments.

2.7. p21 immunoprecipitation and cdk2 western blot

Four hundred micrograms of LNCaP total protein were incubated with 2 μg anti-p21 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. Immune-complexes were collected by centrifugation, washed 3× with NP40 cell lysis buffer, and analyzed by Western blot using cdk2 antibody.

2.8. Apoptosis assays

For the DAPI staining apoptosis assay, prostate cancer cells were resuspended in 0.6 ml 4% paraformaldehyde/PBS for 15 min, washed with PBS, and resuspended in 0.5 ml of DAPI (1 μg/ml)/PBS for 10 min. Cells were washed with PBS and 10 μl of concentrated cells 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 (<0.5%). For the annexin V apoptosis assay, prostate cancer cells were resuspended in 100 μl annexin binding buffer (10 mM Hepes, pH 7.9; 140 mM NaCl; 2.5 mM CaCl₂) followed by the addition of 2.5 μl of annexin V-FITC and 2 μl PI (50 μg/ml) and incubated for 20 min at room temperature. After the addition of 400 μl annexin binding buffer, the cells were read by flow cytometry and the percentage of early apoptotic cells determined by measuring the annexin-FITC positive/PI negative quadrant using WinMDI version 2.8.

2.9. Caspase-3 assay

Prostate cancer cells were 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 micrograms 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 (Ac-DEVD-pNA; 200 nM) and incubated at 37 °C. 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 (DEVD-CHO; 50 nM) was used to confirm specificity.

2.10. Purvalanol A and alsterpaullone cdk inhibitors

The potent cdk inhibitors purvalanol A and alsterpaullone [23,24] were used to investigate the effect on 2-ME and paclitaxel-mediated apoptosis in prostate cancer cells. 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. Differential growth inhibition of prostate cancer cells by 2-ME and paclitaxel

Using a 3-day cell growth assay, we showed that androgen-dependent LNCaP cells were more sensitive to inhibition by 2-ME compared to androgen-independent DU 145 and PC-3 prostate cancer cells (Fig. 1). The half-maximal inhibitory concentrations (IC50) with standard deviations in parenthesis were as follows: LNCaP, 1.35 μM (±0.25); DU 145, 2.0 μM (±0.41); and PC-3, 10.4 μM (±2.64). DU 145 cells were more sensitive to inhibition by paclitaxel compared to LNCaP and PC-3 cells. The IC50 for paclitaxel treated cells were as follows: DU 145, 1.53 nM (±0.3); LNCaP, 2.6 nM (±0.5); and PC-3, 7.0 nM (±1.8). Subsequent experiments sought to determine why LNCaP and DU 145 cells were more sensitive to inhibition by 2-ME and paclitaxel compared to PC-3 cells.

3.2. Low doses of 2-ME and paclitaxel increase LNCaP cells in the G1 phase of the cell cycle

To investigate the cell cycle effects of 2-ME compared to paclitaxel, we used flow cytometric analysis after treatment of prostate cancer cells with varying doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h (Fig. 2). Treatment of LNCaP cells with 2 μM 2-ME and 2 nM paclitaxel resulted in a significant (>13%) increase of cells in the G1 and decrease (>30%) in the S phase of the cell cycle. Similar doses of 2-ME and paclitaxel did not cause G1 accumulation in DU 145 (Fig. 2) and PC-3 (result not shown) cells, probably because their G1 cell cycle checkpoints are defective [25,26]. 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 suggested that lower doses of 2-ME and paclitaxel blocked LNCaP but not DU 145 or PC-3 cells in the G1 phase of the cell cycle.

3.3. Dose-specific changes in cyclins B1 and A in 2-ME and paclitaxel-treated prostate cancer cells

To investigate molecular changes involved in 2-ME and paclitaxel-mediated G1 and G2/M cell
cycle arrest in prostate cancer cells, we analyzed expression of cyclins B1 and A by Western blot and kinase assays (Fig. 3A). Cyclin A protein increases during the S and G2 phase of the cell cycle and is believed to be important for DNA replication [27]. 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 [28]. Treatment of LNCaP cells with 5 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 cells in G2/M with concomitant reduction in G1. Results are expressed as means ± standard deviation (error bars).

3.4. Time-dependent effects of the G2/M-promoting doses of 2-ME and paclitaxel on cell cycle distribution

To analyze the time-dependent effects 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) and paclitaxel (10 nM). 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 (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 with 2-ME but not 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 treated 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 cells.

3.5. Changes in cyclins B1 and A before and after 2-ME and paclitaxel-mediated block in G2/M

Since all prostate cancer cells were blocked at G2/M after 24 h treatment with ≥5 μM 2-ME and ≥10 nM paclitaxel, it was not surprising that there was a marked accumulation of cyclin B1 protein (Figs. 2 and 3). 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 (Figs. 4 and 5). This suggested that the increase of cyclin B1 protein and kinase activity 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 (Fig. 5A). Cyclin B1-dependent kinase activity also peaked at 24 h and decreased to control levels by 72 h 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. There was a 2–5-fold decrease in cyclin A protein and its associated kinase activity in all prostate cancer cell lines at 48 and 72 h, a time when apoptotic cells were increased (see Fig. 7A).

---

Fig. 4. Changes in cell cycle distribution with time after treatment of prostate cancer cells with 2-ME and paclitaxel. LNCaP and DU 145 cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the percentage of cells in the cell cycle phases (G1, S, and G2/M) analyzed by flow cytometry. In 2-ME treated DU 145 but not LNCaP 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 cells. Results are expressed as means ± standard deviation (error bars).
3.6. p53 and p21 proteins are increased in LNCaP cells treated with 2-ME and paclitaxel

To further investigate molecular changes involved in 2-ME and paclitaxel-mediated G1 and G2/M cell cycle arrest in LNCaP cells, we analyzed expression of p53 and p21 by Western blot (Fig. 6). The G1-promoting doses of 2-ME (2 μM) and paclitaxel (2 nM) that decreased cyclin A-dependent kinase activity (see Fig. 3) resulted in a 3–4-fold increase in
p53 protein (Fig. 6A); p53 is mutated in DU 145 cells and PC-3 cells and is non-functional [26]. Similarly, p53 protein levels were increased at 24 h (but not at 4 h) using the G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) and remained elevated at 48 and 72 h (Fig. 6B). Because p53 is known to increase transcription of the cdk inhibitor p21 gene [29], we also analyzed expression of p21 protein by Western blot. The G1-promoting dose of 2-ME (2 μM) and paclitaxel (2 nM) resulted in a small but significant two-fold increase in p21 protein, whereas the G2/M-promoting doses did not increase p21 (Fig. 6A). There was an increased association of p21 with cdk2 when the G1-promoting dose of 2-ME (2 μM) was utilized (Fig. 6C), suggesting a mechanism for inhibition of cyclin A-dependent kinase and blocking of LNCaP cells in G1. At 48 and 72 h, there was a 2–6-fold increase in p21 protein and this correlated with decreased cyclin A-dependent kinase activity (Figs. 5 and 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 (result not shown).

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

To 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 μM for LNCaP and DU 145; 10 μM for PC-3) and paclitaxel (10 nM) for 24, 48, and 72 h (Fig. 7A). In LNCaP, there was a significant increase of apoptotic cells from 6, 16, and > 25% after 24, 48, and 72 h treatment with 2-ME and paclitaxel, which also corresponded with a significant increase in caspase-3 activity (Fig. 7A). Less apoptotic 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 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, possibly due to caspase-independent events or other caspases were more active.

Similar results were obtained in DU145 and PC-3 using annexin V-FITC/PI flow cytometric detection of early apoptotic cells (Fig. 7B). The greater amount of apoptosis measured with annexin V compared to DAPI staining and caspase-3 assays on prostate cancer cells treated with 2-ME (5 μM for LNCaP and DU 145; 10 μM for PC-3) and paclitaxel (10 nM) for 24, 48, and 72 h (Fig. 7A). In LNCaP, there was a significant increase of apoptotic cells from 6, 16, and > 25% after 24, 48, and 72 h treatment with 2-ME and paclitaxel, which also corresponded with a significant increase in caspase-3 activity (Fig. 7A). Less apoptotic 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 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, possibly due to caspase-independent events or other caspases were more active.

Similar results were obtained in DU145 and PC-3 using annexin V-FITC/PI flow cytometric detection of early apoptotic cells (Fig. 7B). The greater amount of apoptosis measured with annexin V compared to DAPI staining and caspase-3 assays on prostate cancer cells treated with 2-ME (5 μM for LNCaP and DU 145; 10 μM for PC-3) and paclitaxel (10 nM) for 24, 48, and 72 h (Fig. 7A). In LNCaP, there was a significant increase of apoptotic cells from 6, 16, and > 25% after 24, 48, and 72 h treatment with 2-ME and paclitaxel, which also corresponded with a significant increase in caspase-3 activity (Fig. 7A). Less apoptotic 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 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, possibly due to caspase-independent events or other caspases were more active. 
the G2/M cell cycle block at 24 h. In contrast, treatment of LNCaP cells with G1-promoting doses (2 μM 2-ME and 2 nM paclitaxel) resulted in minimal (<1.5%) induction of apoptosis at all time points (not shown). In addition, blocking LNCaP and DU 145 cells in the S phase with 1 μM aphidicolin reduced 2-ME-mediated induction of apoptosis (result not shown), suggesting that G2/M block by 2-ME is important for induction of apoptosis.

3.8. 2-ME and paclitaxel decrease in XIAP correlates with increased apoptosis in prostate cancer cells

To investigate why 2-ME and paclitaxel-treated LNCaP cells undergo apoptosis greater than DU 145 and PC-3 cells, we sought to identify differences in the levels of proteins important in apoptosis by Western blot analysis. Cleavage of the PARP protein, indicative of apoptosis, occurred to a greater extent in 2-ME and
paclitaxel treated LNCaP and DU 145 compared to PC-3 cells (Fig. 8). There were no differences in the levels of the anti-apoptotic protein Bcl-xL. The levels of the anti-apoptotic protein survivin increased when the cyclin B1-cdk1 activity was elevated and subsequently decreased to a level similar to or below that of control cells (Fig. 8). 2-ME and paclitaxel increase in apoptosis in LNCaP (and in paclitaxel treated DU 145) correlated with a 2–3-fold decrease in the levels of XIAP, a member of the IAP family, at 48 and 72 h [30] (Fig. 8). There were no changes in the levels of other IAP family members IAP-1 and IAP-2 (result not shown). Another 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.

3.9. Cyclin-dependent kinase inhibitors block 2-ME and paclitaxel-mediated induction of apoptosis

To investigate whether the increase in cyclin B1-dependent kinase activity was required for 2-ME and paclitaxel-mediated induction of apoptosis, we utilized the potent cdk inhibitors purvalanol A and alsterpaullone [23,24]. Treatment of LNCaP cells with 5 μM alsterpaullone and DU 145 cells with 5 μM purvalanol A for 24 h resulted in an increase in G2/M (result not shown) and blocked the 2-ME and paclitaxel-mediated increase of cyclin B1-dependent kinase activity (Fig. 9 A). In addition to inhibiting cyclin B1-dependent kinase activity, alsterpaullone 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. 9A). At 72 h, alsterpaullone 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. 9B and C). Because treatment of PC-3 cells with 2-ME and paclitaxel induced minimal apoptosis at 72 h (1–2%; Fig. 7A), we chose treatment for 6 days when apoptosis increased to >8%. The results showed that 10 μM alsterpaullone similarly blocked the 2-ME and paclitaxel increase of apoptosis in PC-3 cells. Kinase inhibitors PD 98059 (20 μM; MAP kinase inhibitor) and lithium chloride (30 mM; glycogen synthase kinase 3β inhibitor) did not significantly block 2-ME-mediated induction of apoptosis in LNCaP and DU 145 cells (result not shown). These results suggested that 2-ME and paclitaxel-mediated increase in
in cyclin B1-dependent kinase activity was required for induction of apoptosis in prostate cancer cells.

4. Discussion

We analyzed in human prostate cancer cells the effects of the promising chemotherapeutic drugs 2-ME and paclitaxel on the cyclin proteins important in the G2/M phase of the cell cycle. Our results suggested a requirement for G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) to increase cyclin B1-dependent kinase activity in order to induce apoptosis. Furthermore, our results suggested that androgen-dependent LNCaP cells were sensitive to inhibition by 2-ME and paclitaxel because lower drug doses increased p53 and p21 proteins, inhibited cyclin A-dependent kinase activity, and resulted in a G1 block. In addition, G2/M-promoting doses of 2-ME and paclitaxel inhibited cyclin A-dependent kinase activity in all prostate cancer cells at the time apoptosis was increased. However, the differential induction of apoptosis by 2-ME and paclitaxel in prostate cancer cells is correlated with the ability to reduce the levels of XIAP, a member of the IAP family that inhibits caspase activity [30]. Overall, our results indicated that 2-ME has a similar mechanism as paclitaxel in the effects on the cell cycle 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 [14–16]. Our results showed that the increase in cyclin B1 protein and kinase activity in LNCaP cells occurred before significant changes in the cell cycle distribution (Figs. 4 and 5). This suggests that it is not just the mitotic spindle checkpoint that increases cyclin B1 protein and kinase activity. 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 [14,15]. 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 metaphase to anaphase transition during mitosis [31]. The end result of increasing cyclin B1 protein is the increase of its associated cdk1 activity, which has been shown to be important in the induction of mitotic catastrophe and many forms of apoptosis [32].

An issue is whether 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells requires the initial increase of cyclin B1-dependent kinase 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 and a substrate for cdk1 [17]. 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 cells compared to DU 145 and PC-3 cells (Figs. 5 and 7). 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 (Figs. 4, 7 and 8). These results indicated that decreased cyclin B1-dependent kinase activity and exit from mitotic block varied between 2-ME and paclitaxel treatment of prostate cancer cells. Therefore, we conclude that the initial 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity is more important than the subsequent decrease in activity for 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.

A 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 [33]. Our studies showed that a similar mechanism was also evident using lower doses of 2-ME and paclitaxel in LNCaP cells, which inhibited cyclin A-dependent kinase activity. Similar results were obtained in p53 wild type lung and breast cancer cell lines treated with 3−6 nM paclitaxel [34]. In order to maximize induction of apoptosis, however, G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) that increased cyclin B1-dependent kinase activity were required. In addition, the G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) inhibited cyclin A-dependent kinase activity at a time when apoptosis was maximized at 48 and 72 h. An important role for inhibition of cdk2 and induction of apoptosis in cancer but not normal cells was reported [35]. However, a recent report casts doubt on the importance of cdk2 inhibition in cancer therapy [36]. The cdk inhibitor purvalanol A as a single drug inhibited cyclin B1—but not cyclin A-dependent kinase activity in DU 145 cells, resulting in minimal apoptosis. Purvalanol A combined with 2-ME or paclitaxel blocked induction of apoptosis, indicating the importance of increased cyclin B1-dependent kinase activity (Fig. 9). In contrast, alsterpaullone as a single drug inhibited both cyclin B1- and cyclin A-dependent kinase activity and induced apoptosis in LNCaP cells. However, alsterpaullone blocked the 2-ME or paclitaxel blocked induction of apoptosis, indicating the importance of increased cyclin B1-dependent kinase activity (Fig. 9). We suggest that in addition to the early activation of cyclin B1-dependent kinase activity, later inhibition of cyclin A-dependent kinase activity plays an important role in 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). It is likely that the expression of wild type p53 in LNCaP but not in DU 145 and PC-3 cells plays an important role in the greater induction of apoptosis. An important role for p53 in 2-ME-mediated apoptosis has been demonstrated for LNCaP and lung cancer cells [10,37]. The ability of 2-ME...
and paclitaxel to decrease the levels of the IAP family member XIAP at 48 and 72 h correlated with increased apoptosis (Figs. 7 and 8). Inhibition of XIAP has been shown to increase apoptosis in cancer cells either directly or indirectly by sensitizing to other chemotherapeutic drugs [38,39]. A predominant role for members of the IAP family in the regulation of the induction of apoptosis in prostate cancer cells has been proposed [40]. Therefore, drugs that decrease the levels of anti-apoptotic proteins like XIAP should shift the overall balance towards apoptosis even in the most resistant AI-PC cells. In addition, higher levels of the anti-apoptotic proteins Bcl-xL and survivin in DU 145 and PC-3 compared to LNCaP may contribute to 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 anti-sense oligonucleotides can sensitize PC-3 cells to drug-mediated apoptosis [41,42].

In summary, our studies indicate that 2-ME is similar to paclitaxel in the early activation of cyclin B1- and later inhibition of cyclin A-dependent kinase activity and this may be an important mechanism for induction of apoptosis in prostate cancer cells. We are currently investigating in a transgenic mouse model of prostate cancer [43] whether these molecular changes are also occurring in vivo. 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 overexpression of cyclin B1 protein in cancer cells [44] and the differential sensitivity of cancer cells to the inhibition of cyclin A-dependent kinase [35]. We suggest that 2-ME and paclitaxel 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 [45], our results hold promise that 2-ME will have a similar efficacy in AI-PC.

Acknowledgements

We thank Alicia De Las Pozas and Adriana Gomez for excellent technical assistance, Ron Hamelik for help with flow cytometry, and Drs Bernard Roos and Awtar Krishan for review of this manuscript. This work was supported by V.A. Merit Review (026901) and Department of Defense (DAMD17-03-1-0179) to C. Perez-Stable.

References


Sequential combination of flavopiridol and docetaxel reduces the levels of X-linked inhibitor of apoptosis and AKT proteins and stimulates apoptosis in human LNCaP prostate cancer cells

Lourdes A. Gomez,1 Alicia de las Pozas,2 and Carlos Perez-Stable2,3
1South Florida Veterans Affairs Foundation; 2Geriatric Research, Education, and Clinical Center and Research Service, Veterans Affairs Medical Center; 3Department of Medicine and Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, Florida

Abstract
Clinical trials have shown that chemotherapy with docetaxel combined with prednisone can improve survival of patients with androgen-independent prostate cancer. It is likely that the combination of docetaxel with other novel chemotherapeutic agents would also improve the survival of androgen-independent prostate cancer patients. We investigated whether the combination of docetaxel and flavopiridol, a broad cyclin-dependent kinase inhibitor, can increase apoptotic cell death in prostate cancer cells. Treatment of DU 145 prostate cancer cells with 500 nmol/L flavopiridol and 10 nmol/L docetaxel inhibited apoptosis probably because of their opposing effects on cyclin B1–dependent kinase activity. In contrast, when LNCaP prostate cancer cells were treated with flavopiridol for 24 hours followed by docetaxel for another 24 hours (FD), there was a maximal induction of apoptosis. However, there was greater induction of apoptosis in DU 145 cells when docetaxel was followed by flavopiridol or docetaxel. These findings indicate a heterogeneous response depending on the type of prostate cancer cell. Substantial decreases in X-linked inhibitor of apoptosis (XIAP) protein but not survivin, both being members of the IAP family, were required for FD enhanced apoptosis in LNCaP cells. Androgen ablation in androgen-independent LNCaP cells increased activated AKT and chemo resistance to apoptosis after treatment with FD. The proteasome inhibitor MG-132 blocked FD-mediated reduction of XIAP and AKT and antagonized apoptosis, suggesting that the activation of the proteasome pathway is one of the mechanisms involved. Overall, our data suggest that the docetaxel and flavopiridol combination requires a maximal effect on cyclin B1–dependent kinase activity and a reduction of XIAP and AKT prosurvival proteins for augmentation of apoptosis in LNCaP cells. [Mol Cancer Ther 2006;5(5):1216–26]

Introduction
Prostate cancer is the most frequently diagnosed noncutaneous malignancy and the second leading cause of cancer-related deaths among men in the United States (1). The principal therapy for men with advanced disease is androgen ablation, but most of these patients eventually progress to an androgen-independent disease (2). Docetaxel (Taxotere), a semisynthetic derivative of paclitaxel (Taxol) originally derived from the yew tree (3), is a promising anticancer drug shown to inhibit a wide variety of tumor cells, including prostate cancer cells by diverse mechanisms that include cell cycle arrest, induction of apoptosis, stabilization of microtubules, and inhibition of angiogenesis (4, 5). Studies showing that treatment with docetaxel combined with prednisone can improve survival of patients with androgen-independent prostate cancer have been recently reported (6). It is likely that docetaxel combined with other novel chemotherapeutic drugs would also result in improved patient survival. The ability of chemotherapeutic drugs, such as docetaxel, to induce apoptotic cell death in prostate cancer cells is probably one of the chief mechanisms involved in improved survival. However, the precise mechanisms of how docetaxel in combination with other drugs induces apoptosis in prostate cancer cells are not known.

Flavopiridol, a semisynthetic flavonoid derived from an indigenous plant from India, is a broad inhibitor of cyclin-dependent kinases (cdk) and is being tested in clinical trials (7, 8). Treatment of cancer cells with flavopiridol results in a decrease in cyclins D1 and B1 leading to a cell cycle arrest in G1 and G2-M phases (9, 10). Flavopiridol also reduces the levels of the antiapoptotic proteins Bcl-2, Bcl-xl, Mcl-1, and X-linked inhibitor of apoptosis (XIAP) and sensitizes cancer cells to apoptosis after subsequent treatment with other chemotherapeutic agents (10–13). However, results from phase II clinical trials of flavopiridol as a single agent have been reported to be unsatisfactory,

Received 11/10/05; revised 2/3/06; accepted 3/2/06.
Grant support: Aventis Pharmaceuticals grant GIA 60025, Veterans Affairs Merit Review grant 026901, and Department of Defense grant DAMD17-03-1-0179 (C. Perez-Stable).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Carlos Perez-Stable, Geriatric Research, Education, and Clinical Center and Research Service, Veterans Affairs Medical Center, 11-GRC, 1201 Northwest 16 Street, Miami, FL 33125, Phone: 305-324-4455, ext. 4391; Fax: 305-575-3365. E-mail: cperez@med.miami.edu
Copyright © 2006 American Association for Cancer Research. doi:10.1158/1535-7163.MCT-05-0467
indicating that flavopiridol may work best as an anticancer agent when combined with other agents (14, 15). Whether flavopiridol and docetaxel can enhance apoptotic cell death in prostate cancer cells has not been investigated previously.

One of the proposed mechanisms for the anticancer effect of docetaxel is the stabilization of microtubules, activation of the mitotic checkpoint, and blockade of the degradation of cyclin B1, which leads to a prolonged activation of its associated kinase cdk1, mitotic arrest, and induction of apoptosis (4, 5, 16, 17). An increase in cdk1 activity results in phosphorylation and stabilization of survivin, a member of the IAP family and a substrate for cdk1 (18). Therefore, it has been proposed that the subsequent decrease in cyclin B1-cdk1 activity results in a decrease in the levels of survivin and an increase in sensitivity to induction of apoptosis (19). Preclinical studies in human gastric and breast cancer cell lines have shown that the greatest increase in apoptosis occurs when docetaxel is followed by flavopiridol (16). The hypothesis of this regimen is that flavopiridol treatment after the docetaxel-mediated mitotic block results in the inhibition of cyclin B1-cdk activity, a decrease in phosphorylated survivin, a more rapid exit from mitosis, and an increase in apoptosis. Whether this mechanism is generally applicable to androgen-dependent and androgen-independent prostate cancer cells is not known.

Treatment with single chemotherapeutic agents, such as docetaxel and flavopiridol, will not cure most cancers, including androgen-independent prostate cancer. Therefore, the purpose of the present study was to determine whether the combination of docetaxel and flavopiridol could increase apoptotic cell death in prostate cancer cells. Our results show that in androgen-dependent and androgen-independent prostate cancer cells, including androgen-independent prostate cancer (22 –26), and confers resistance to chemotherapy in most types of cancer, including androgen-independent prostate cancer (22–26), and confers resistance to chemotherapy in most types of cancer, including androgen-independent prostate cancer and androgen-independent prostate cancer cells is not known.

Materials and Methods

Reagents

Flavopiridol and docetaxel were obtained from Aventis Pharmaceuticals (Bridgewater, NJ). Propidium iodide (PI) and DMSO were purchased from Sigma (St. Louis, MO). Histone H1 protein was purchased from Roche Applied Sciences (Indianapolis, IN). 4',6-Diamidino-2-phenylindole (DAPI), MG-132, LY 294002, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole, alsterpaullone, epoxomicin, and parthenolide were purchased from Calbiochem (San Diego, CA). Annexin V-FITC was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Human prostate carcinoma cell lines LNCaP (29) and DU 145 (30) were obtained from the American Type Culture Collection (Rockville, MD). LN-AI is an androgen-independent derivative of the human prostate cancer cell line LNCaP, which was spontaneously derived in our laboratory (31). These cells express androgen receptor and prostate-specific antigen similar to LNCaP. LNCaP, LN-AI, and DU 145 were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (Hyclone, Logan, UT), 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin (Invitrogen). Unlike androgen-dependent LNCaP, the LN-AI cells are able to grow for long-term in RPMI 1640 with 5% charcoal-stripped fetal bovine serum and are called LN-AI/CSS. The normal rat prostate basal epithelial cell line NRP-152 (provided by Dr. David Danielpour, Case Western Reserve University, Cleveland, OH) was maintained in HEPES-free DMEM/F12 (1:1, v/v) with 5% fetal bovine serum, antibiotic/antimycotic, 20 ng/mL epidermal growth factor, 10 ng/mL cholera toxin, 5 µg/mL insulin, and 0.1 µmol/L dexamethasone (32). Human mesenchymal stromal cells derived from bone marrow were obtained from Gianluca D'Ippolito (University of Miami, Miami, FL) and cultured in DMEM (low glucose) with 5% fetal bovine serum and antibiotic/antimycotic (33).

Treatment with Flavopiridol and Docetaxel

For treatment with flavopiridol or docetaxel, 7 × 10⁵ LNCaP cells, 5 × 10⁵ LN-AI, 10 × 10⁵ LN-AI/CSS, 3 × 10⁶ DU 145 cells, 1 × 10⁶ NRP-152 cells, and 2 × 10⁵ mesenchymal stromal cells were seeded per 6-cm dish and allowed to attach overnight. The next day, fresh medium containing different doses of flavopiridol (10–500 nmol/L), docetaxel (2–50 nmol/L), or DMSO (0.1%) control was added and the cells were cultured for varying times (24–72 hours). For the sequential combinations of flavopiridol and docetaxel, after 24 hours treatment with 500 nmol/L flavopiridol or 10 nmol/L docetaxel, floating cells were removed, centrifuged, resuspended in the appropriate medium containing flavopiridol, docetaxel, or DMSO, added back to the attached cells, and incubated for an additional 24 hours. In all the experiments, floating and trypsinized attached cells were pooled for further analysis. Similar experiments were conducted using alsterpaullone (5 µmol/L), LY 294002 (20 µmol/L), MG-132 (5 µmol/L), and epoxomicin (1 µmol/L).

Flow Cytometric Analysis

Propidium/hypotonic citrate method (34) was used to study cell cycle distribution of flavopiridol- and docetaxel-treated prostate cancer cells. After harvesting and washing cells with PBS, the cell pellets were resuspended in 0.5 mL PI staining solution (0.1% sodium citrate, 0.03% NP40, 50 μg/mL PI) and vortexed to release nuclei, and DNA distribution histograms were generated by analysis of 10,000 nuclei in a Coulter (Miami, FL) XL flow cytometer.
percentage of cells in the G1, S, and G2–M DNA content was determined by the ModFit program (Verity Software House, Topsham, ME) from six to eight samples analyzed from at least three independent experiments.

**Western Blot Analysis**

Cell pellets were resuspended in NP40 cell lysis buffer [1% NP40, 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L EDTA, protease inhibitor tablet, 50 mmol/L NaF, 0.1 mmol/L NaVO₄] lysed by vortex, left on ice for 30 minutes, and centrifuged, and the protein concentrations of the supernatant were determined with the Bio-Rad (Hercules, CA) protein assay. After separation of 25 to 50 μg protein by SDS-PAGE, proteins were transferred by electrophoresis to Immobilon-P membrane and incubated in 5% nonfat dry milk, PBS, and 0.25% Tween 20 for 1 hour. Antibodies specific for cyclin B1 (GNS1), cyclin A (H-432), survivin (FL-142), Mcl-1 (S-19; Santa Cruz Biotechnology), poly(ADP-ribose polymerase) (PARP; C2-10), Bcl-xL (polyclonal; BD Biosciences PharMingen, San Diego, CA), XIAP, cleaved caspase-3, phosphorylated AKT (Ser473; 587F11), and AKT (9272; Cell Signaling Technology, Beverly, MA) were diluted 1:1,000 to 1:3,000 in 5% nonfat dry milk, PBS, and 0.25% Tween 20 and incubated overnight at 4°C. Membranes were washed in PBS and 0.25% Tween 20 and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (1:1,000 dilution; Santa Cruz Biotechnology) for 1 hour, washed in PBS and 0.25% Tween 20, and analyzed by exposure to X-ray film using Enhanced Chemiluminescence Plus (Amersham Pharmacia Biotech, Piscataway, NJ). Phosphorylated AKT blots were stripped and reprobed with AKT antibody.

Cyclins B1– and A–Dependent Kinase Assay

Total protein (400 μg) was incubated with 2 μg anti–cyclin B1 (H-433; Santa Cruz Biotechnology) or cyclin A antibody for 3 hours on ice followed by the addition of 20 μL protein A/G-agarose (Santa Cruz Biotechnology) and incubation overnight at 4°C. with agitation. Immune complexes were collected by centrifugation, washed thrice with NP40 cell lysis buffer, thrice with kinase buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L MgCl₂, 0.5 mmol/L DTT], resuspended in kinase buffer containing 2 μg histone H1 substrate protein, 25 μmol/L ATP, 5 μCi [γ-³²P]ATP, and incubated for 30 minutes at 30°C. Reactions were stopped with SDS gel loading buffer, samples were electrophoresed on SDS-PAGE, electrophobed to Immobilon P membranes, and analyzed by autoradiography. Coomassie blue staining of membranes revealed similar loading of histone proteins. Changes in kinase activity were determined as described previously (35) from at least four different samples analyzed from two to three independent experiments.

**RNase Protection Assay**

RNA from prostate cancer cells sequentially treated with flavopiridol and docetaxel was isolated using QiAshredder and RNeasy miniprep kit (Qiagen, Valencia, CA). The hAPO-5c human apoptosis multiprobe template set (BD Biosciences Pharmingen) was used for T7 RNA polymerase (Ambion, Austin, TX) synthesis of ³²P-labeled antisense RNA probes specific for XIAP, survivin, and glyceraldehyde-3-phosphate dehydrogenase. Total RNA (10 μg) was hybridized to antisense RNA probes at 56°C overnight followed by digestion with RNase mixture (Ambion) at 30°C for 45 minutes. RNase digestion products were analyzed by electrophoresis on 5% polyacrylamide-urea gels followed by autoradiography.

**Transfection of XIAP Small Interfering RNA**

LN-AI/CSS cells (2 × 10⁵) were seeded in 12-well plates and transfected the next day with 200 nmol/L small interfering RNA (siRNA) SMART pool specific for XIAP and siCONTROL nontargeting pool (Dharmacon, Lafayette, CO) using Oligofectamine (Invitrogen) following the manufacturer’s instructions. After 72 hours, cells were harvested and analyzed for expression of XIAP by Western blot as described above. Subsequently, LN-AI/CSS cells were transfected with XIAP and control siRNA for 24 hours followed by treatment with FD with or without 20 μmol/L LY 294002 for an additional 48 hours. The numbers of apoptotic cells were determined by DAPI staining as described above. Changes in apoptosis were determined in at least eight different samples analyzed from three independent experiments.

**Apoptosis Assays**

For the DAPI staining apoptosis assay, cells were resuspended in 0.6 mL of 4% paraformaldehyde/PBS for 15 minutes, washed with PBS, and resuspended in 0.5 mL DAPI (1 μg/mL)/PBS for 10 minutes. Cells were washed with PBS and 10 μL 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 (Melville, NY) fluorescence microscope with a DAPI filter. The number of apoptotic cells in at least 200 total cells was determined from at least four random microscope fields. Changes in apoptosis from flavopiridol- and docetaxel-treated cells were determined as percentage of apoptotic cells in at least five different samples from three independent experiments. Only minimal apoptosis was detected in control-treated cells (<0.5%). For the Annexin V apoptosis assay, LN-AI and DU 145 prostate cancer cells were resuspended in 100 μL Annexin V binding buffer [10 mmol/L HEPES (pH 7.9), 140 mmol/L NaCl, 2.5 mmol/L CaCl₂] followed by the addition of 2.5 μL Annexin V-FITC and 2 μL PI (50 μg/mL) and incubated for 20 minutes at room temperature. After the addition of 400 μL Annexin V binding buffer, the cells were read by flow cytometry and the percentage of early apoptotic cells was determined by measuring the Annexin V-FITC–positive/PI-negative quadrant using WinMDI version 2.8.

**Combination Chemotherapy in Prostate Cancer**

Mol Cancer Ther 2006;5(5). May 2006
Statistical Analysis

Statistical differences between drug-treated and control cells were determined by two-tailed Student’s *t* test with *P* < 0.05 considered significant.

Results

To evaluate the effect of flavopiridol and docetaxel as single drugs and in combination, we used various human prostate cancer cell lines (LNCaP, LN-AI, LN-AI/CSS, and DU 145), a nontransformed rat prostate cell line (NRP-152), and a primary human mesenchymal stromal cell line. These cells provide useful *in vitro* models of the different stages of progression of human prostate cancer, from normal nontransformed to androgen-independent prostate cancer compared with a primary nontransformed nonprostate cell.

**G1 and G2-M Cell Cycle Effects of Flavopiridol- and Docetaxel-Treated Prostate Cancer Cells**

To evaluate the cell cycle effects of flavopiridol and docetaxel on LNCaP and DU 145 prostate cancer cells, we did flow cytometric analysis after treatment with varying doses of flavopiridol (10–500 nmol/L) and docetaxel (0.5–50 nmol/L) for 24 hours (Fig. 1). The treatment of LNCaP and DU 145 cells with increasing doses of flavopiridol (≥50–100 nmol/L) results in an increase in cells in G1 and G2-M with a decrease in S phase (Fig. 1). These results reflect the ability of flavopiridol to inhibit multiple cdks important in the G1 and G2-M phases of the cell cycle. Flow cytometric analysis of LNCaP and DU 145 cells treated with varying doses of docetaxel (2–50 nmol/L) shows that ≥10 nmol/L docetaxel results in an increase in G2-M and a decrease in G1. As expected, these results are similar to those after treatment with 10 nmol/L paclitaxel (35) and indicate that docetaxel blocks prostate cancer cells in the G2-M phase of the cell cycle.

**Opposite Effects of Flavopiridol and Docetaxel on Cyclin B1–Dependent Kinase Activity**

To evaluate molecular changes involved in the mediated effects on the cell cycle in LNCaP and DU 145 cells by flavopiridol and docetaxel, we analyzed the expression of cyclins B1 and A proteins by Western blot and kinase analysis (Fig. 2). Cyclin A protein increases during the S and G2 phase of the cell cycle and is believed to be important for DNA replication (36). The transition from the G2 to the M phase of the cell cycle requires an accumulation of cyclin B1 and activation of its associated kinase, cdk1. The end of the G2-M transition and exit from mitosis requires the proteolysis of cyclin B1 and a reduction of cdk1 activity (37). Increased doses of flavopiridol (≥250 nmol/L) decrease both cyclins B1 and A proteins and their associated kinase activities in DU 145 cells (Fig. 2). In contrast, the treatment of DU 145 cells with the G2-M-promoting doses of docetaxel (≥10 nmol/L) for 24 hours results in an increase in cyclin B1 protein and kinase activity but not cyclin A protein and kinase activity (Fig. 2). Similar results were obtained in flavopiridol- and docetaxel-treated LNCaP cells (data not shown). These results indicate that (a) flavopiridol inhibits both cyclins B1 and A-dependent cdk activity probably explaining the G1 and G2-M effects on the cell cycle and (b) docetaxel increases cyclin B1–dependent kinase activity, which correlates with increased G2-M.

**Differential Induction of Apoptosis by Flavopiridol and Docetaxel in Prostate Cancer Cells**

The induction of apoptosis is a requirement for the effect of chemotherapeutic drugs on prostate cancer cells (38). To measure the induction of apoptosis by flavopiridol and docetaxel, we did a DAPI staining assay in LNCaP, LN-AI,
LN-AI/CSS, DU 145, NRP-152, and mesenchymal stromal cells treated with 500 nmol/L flavopiridol and 10 nmol/L docetaxel for 72 hours (Fig. 3A and B). We selected these doses because of the increased effect on cyclin B1–dependent kinase (Fig. 2), which was shown previously to be important in the induction of apoptosis (35). The results indicate that both flavopiridol- and docetaxel-mediated apoptosis was greatest in LN-AI (38–49%) followed by LNCaP (14–25%) cells. A removal of androgens results in a decrease in flavopiridol- and docetaxel-mediated apoptosis in LN-AI/CSS (8–10%) compared with LN-AI cells. The levels of apoptosis in DU 145 (5–12%) are similar to those in LN-AI/CSS cells. Flavopiridol induces a similar degree of apoptosis in nontumorigenic NRP-152 and mesenchymal stromal cells (9%) compared with LN-AI/CSS and DU 145 cells, whereas docetaxel induces less apoptosis in NRP-152 and mesenchymal stromal cells (2–5%) compared with prostate cancer cells. These results indicate that flavopiridol and docetaxel are most effective in producing apoptosis in LN-AI cells and that the removal of androgens reduces the ability of flavopiridol and docetaxel to produce apoptosis in LN-AI/CSS cells.

**Treatment with Flavopiridol and Docetaxel Antagonizes Induction of Apoptosis**

If the effect of flavopiridol and docetaxel on cyclin B1–dependent kinase activity is important for induction of apoptosis, then it can be predicted from the previous results (Fig. 2) that treatment with flavopiridol (inhibits cyclin B1–dependent kinase) and docetaxel (increases cyclin B1–dependent kinase) should antagonize each other and result in less than additive induction of apoptosis. The treatment of DU 145 cells with 500 nmol/L flavopiridol and 10 nmol/L docetaxel for 72 hours results in only 2% apoptosis, which is a decrease from flavopiridol (5%) and docetaxel (12%) alone (Fig. 3C). This result was confirmed by Western blot showing less cleaved PARP (signifying apoptosis) in the flavopiridol and docetaxel combination compared with docetaxel alone (data not shown). In LNCaP cells, treatment with flavopiridol and docetaxel results in no additional apoptosis above each drug alone (data not shown). These results indicate that simultaneous treatment of prostate cancer cells with flavopiridol and docetaxel results in antagonism with respect to induction of apoptosis probably due to opposing effects on cyclin B1–dependent kinase activity.

**Flavopiridol followed by Docetaxel Produces the Greatest Induction of Apoptosis in LNCaP cells**

We used the DAPI apoptosis assay in LNCaP cells to identify the sequential combination of flavopiridol and docetaxel that can induce apoptosis to a greater extent than each drug alone (Fig. 4). Previous studies have shown that docetaxel followed by flavopiridol (DF) was more effective in inducing apoptosis in gastric cancer cells (16). Our results indicate that the best sequence for induction of apoptosis is 500 nmol/L flavopiridol for 24 hours followed by 10 nmol/L docetaxel for 24 hours. Similar results with the FD sequence were obtained in LN-AI and LN-AI/CSS cells, although there was less apoptosis in LN-AI/CSS (10%) compared with LN-AI (46%) cells (data not shown). In DU 145 cells, the DF sequence (9%) induces greater apoptosis compared with the FD sequence (2%). However, there was no additional apoptosis in DF-treated DU 145 cells compared with docetaxel followed by docetaxel (DD)–treated cells (Fig. 4). In LN-AI cells, a greater apoptosis in the FD sequence compared with the DF sequence was confirmed by flow cytometry using the Annexin V-FITC/PI assay, whereas in DU 145 cells there was no difference in any sequence of flavopiridol and docetaxel (Fig. 4B).

Interestingly, when flavopiridol was followed by vehicle control in LNCaP, LN-AI, and LN-AI/CSS cells, there was

---

**Figure 3.** Induction of apoptosis by flavopiridol and docetaxel as single drugs and in combination. **A**, percentage of apoptotic cells were determined by DAPI in LNCaP, LN-AI, LN-AI/CSS, and DU 145 prostate cancer cells treated with 500 nmol/L flavopiridol for 72 h and compared with treatment of nontransformed NRP-152 and mesenchymal stromal cells. **B**, percentage of apoptotic cells after treatment for 72 h with 10 nmol/L docetaxel. Flavopiridol and docetaxel as single drugs were most effective in inducing apoptosis in LN-AI cells and removal of androgens reduced their ability to induce apoptosis in LN-AI/CSS cells. There was minimal apoptosis (<0.5%) detected by DAPI in the control-treated cells (data not shown). **C**, treatment of DU 145 cells with the combination of 500 nmol/L flavopiridol and 10 nmol/L docetaxel added simultaneously for 72 h (FP + Doc) results in less apoptosis (DAPI) compared with each drug alone (FP and Doc). Columns, mean of three independent experiments (n = 6); bars, SD. *, P < 0.001.
A greater induction of apoptosis compared with sequential use of flavopiridol (FF; Fig. 4C). Similar results were obtained when LNCaP cells were treated with the cdk inhibitor alsterpaullone (5 μmol/L; ref. 39) for 24 hours followed by vehicle control for 24 hours (data not shown), suggesting that increased apoptosis was due to inhibition of cdk activity by flavopiridol. In contrast, the treatment of LNCaP cells with the cdk9 inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (75 μmol/L; required for transcription elongation; ref. 40) for 24 hours followed by vehicle control or 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole resulted in low apoptosis (data not shown).

These results indicate that the optimal sequence combination in LNCaP cells is flavopiridol followed by docetaxel and that a continuous treatment of LNCaP cells with flavopiridol for 48 hours is less effective in inducing apoptosis compared with a 24-hour treatment followed by no drug.

Increased Apoptosis Is Correlated with Decreased XIAP but not Survivin Protein

To investigate in LNCaP cells why the FD sequence induced greater apoptosis than the DF, FF, and DD sequences, we sought to identify the differences in the levels of proteins important for apoptosis by Western blot analysis. The results in LNCaP and LN-AI cells show that FF treatment contained more cleaved (activated) caspase-3 and PARP (Fig. 5) compared with the DF, FF, and DD sequences. There was much less cleaved caspase-3 in LN-AI/CSS compared with LN-AI cells using the FD sequence. In DU 145 cells, there was a greater cleaved capase-3 in the DD and DF sequence compared with the FF and FD sequence. These results correlate with those obtained with the apoptosis assays (Fig. 4). There were no significant differences in the levels of the antiapoptotic protein Bcl-xL in any drug sequence.

In contrast, all of the FF- and DF-treated cells show a substantial reduction in the antiapoptotic protein Mcl-1 compared with FD and DD sequence (Fig. 6); however, these results did not correlate with greater apoptosis in the FD sequence. The FD sequence reduces the levels of XIAP and survivin, members of the IAP family (20, 21), in LNCaP, LN-AI, and LN-AI/CSS compared with DU 145 cells (Fig. 6). However, the FD sequence decreases XIAP to a greater extent in LNCaP (7-fold) and LN-AI (10-fold) compared with LN-AI/CSS (3-fold) cells and this correlates with increased apoptosis. In DU 145, there was a 2-fold decrease in XIAP protein in DD- and DF-treated cells. These results suggest that the greatest levels of apoptosis induced by the FD sequence may require a substantial decrease in XIAP protein.

Reduction of XIAP Protein by siRNA Increases Apoptosis in FD-Treated LN-AI/CSS Cells

To determine if a reduction of XIAP protein can increase FD-mediated apoptosis in LN-AI/CSS cells, we used siRNA specific for XIAP. The results show that XIAP siRNA caused a 7-fold decrease in XIAP protein in LN-AI/CSS cells compared with cells transfected with control siRNA (Fig. 7A). Subsequently, LN-AI/CSS cells were transfected with XIAP and control siRNA for 24 hours followed by treatment with FD for an additional 48 hours and the effect on apoptosis was determined by DAPI staining. The results indicate that in the presence of XIAP siRNA there was a small but significant increase in apoptotic LN-AI/CSS cells compared with control siRNA transfected cells (Fig. 7B). This suggests that lowering XIAP protein levels sensitizes prostate cancer cells to FD treatment.

Decrease in XIAP Protein Is Not Due to Decreased mRNA

One of the mechanisms proposed for flavopiridol inhibition of cancer cells is its ability to inhibit cdk7 and
cdk9, which are required for phosphorylation of RNA polymerase II and transcription elongation (40). We therefore did RNase protection assay using the hAPO-5c human apoptosis multiprobe template set to determine whether the FD-mediated decrease in XIAP protein was due to a decrease in mRNA. The results show that this treatment of LNCaP, LN-AI, LN-AI/CSS, and DU 145 cells with FD did not reduce the levels of XIAP mRNA compared with control-treated cells (Fig. 8). In contrast, FD treatment of LNCaP, LN-AI, and LN-AI/CSS but not DU 145 cells results in a decrease in survivin mRNA, which may explain the decrease in survivin protein. In addition, there was a decrease in mRNA for XIAP in FF- and DF-treated cells, which possibly corresponds to a decrease in XIAP protein. These results indicate that decreased XIAP protein in FD-treated LNCaP and LN-AI cells was due to post-transcriptional mechanisms and not to decreased XIAP mRNA.

FD Treatment Decreases Total AKT Protein in LN-AI Cells

To investigate molecular changes possibly involved in the reduction of XIAP protein in FD-treated LNCaP cells, we analyzed the expression of activated and total AKT by Western blot. In addition to phosphorylation and inactivation of the proapoptotic protein Bad (22), AKT has recently been shown to phosphorylate and stabilize XIAP (41).

Results indicate that LN-AI/CSS cells contain a higher level of activated (phosphorylated at Ser473) AKT compared with LN-AI cells (Fig. 9A). Because activated AKT has been shown to protect cells from apoptosis (22), this may explain why LN-AI/CSS cells are more resistant to apoptosis induced by FD treatment compared with LN-AI cells. We then analyzed the levels of activated and total AKT in FD-treated LN-AI compared with LN-AI/CSS cells (Fig. 9B). The results show that FD treatment greatly reduces the levels of total AKT protein (and therefore activated AKT) in LN-AI cells with few changes in LN-AI/CSS cells. Interestingly, the treatment of LN-AI but not LN-AI/CSS cells with FF results in a 5-fold increase in activated AKT compared with control-treated cells. Inhibition of AKT activity with the phosphatidylinositol 3-kinase inhibitor LY 294002 (20 μmol/L inhibits Ser 473 phosphorylated AKT) increases apoptosis (Fig. 9C) and decreases XIAP protein levels (data not shown) in FF- and FD-treated LN-AI/CSS cells. Finally, the combination of a reduction in XIAP protein with siRNA and an inhibition of AKT activity with LY 294002 cause a greater increase in apoptosis than a reduction of XIAP or a decrease in AKT activity alone (Fig. 9D). Overall, these results suggest that (a) an increase in activated AKT in LN-AI/CSS cells may explain its greater resistance to apoptosis; (b) FD treatment reduces...
total AKT protein levels in LN-AI but not LN-AI/CSS cells, possibly explaining differentially decreased XIAP and increased apoptosis; and (c) a reduction of XIAP protein and an inhibition of AKT activity greatly increases apoptosis in FD-treated LN-AI/CSS cells.

Inhibition of the Proteasome Pathway Antagonizes FD-Induced Apoptosis

A possible mechanism explaining FD decrease in XIAP protein without a reduction in XIAP mRNA may be the increased degradation by the ubiquitin-proteasome pathway (42). To address this hypothesis, we treated LNCaP cells with flavopiridol (500 nmol/L), proteasome inhibitor MG-132 (5 μmol/L), and combination of flavopiridol and MG-132 for 24 hours. The results show that MG-132 blocks the ability of flavopiridol to reduce the protein levels of cyclin B1 and Mcl-1 (Fig. 10A). This suggests that flavopiridol activates the proteasome pathway to decrease these proteins. The addition of MG-132 (5 μmol/L) in the FD sequence combination results in lesser cleavage of PARP in LNCaP cells, which correlates with a block in FD-mediated decrease in XIAP and AKT (Fig. 10B). Conversely, FD lowers cleavage of PARP mediated by MG-132, suggesting antagonism relative to proteasome degradation activity. MG-132 also blocks FD-mediated apoptosis as determined by DAPI staining (data not shown). Similar results were obtained using the proteasome inhibitor epoxomicin (1 μmol/L; data not shown). In addition, treatment of LNCaP cells with FD and the nuclear factor-κB inhibitor parthenolide (20 μmol/L) had no effect on apoptosis, suggesting that an inhibition of nuclear factor-κB activity was not important for the ability of MG-132 to block FD-mediated apoptosis (data not shown). These results suggest that FD increases the degradation of cyclin B1, XIAP, and AKT proteins by stimulating the proteasome pathway and this augments apoptosis in LNCaP cells.

Discussion

We analyzed in human prostate cancer cells whether the combination of flavopiridol and docetaxel can enhance apoptosis more than either drug alone. Our results indicate that the sequential addition of flavopiridol followed by docetaxel was required for maximal induction of apoptosis in LNCaP cells. The opposing effects on cyclin B1–dependent kinase activity by flavopiridol (a decrease) and docetaxel (an increase) likely plays an important role in the requirement for a sequential combination regimen and an induction of apoptosis. Our data also suggest that the FD-mediated decrease in XIAP and AKT proteins, both being inhibitors of apoptosis, is an important factor for increased apoptosis in LNCaP and LN-AI compared with LN-AI/CSS and DU 145 prostate cancer cells. A possible mechanism for flavopiridol-mediated decrease in XIAP, AKT, cyclin B1, and Mcl-1 proteins may be the activation of the proteasome pathway of protein degradation. Overall, our data suggest that the sequential regimen of flavopiridol and docetaxel that leads to the greatest decrease in XIAP and AKT protein also results in the greatest increase in apoptosis.

The deregulated increase of cyclin B1–dependent kinase activity by docetaxel is an important mechanism for the induction of apoptosis in prostate cancer cells (35, 43). However, it is not clear if an inhibition of cyclin B1–dependent kinase activity by flavopiridol is also important for induction of apoptosis. Our data show that when flavopiridol and docetaxel were added simultaneously
there was an antagonism of apoptosis compared with either drug alone (Fig. 3C). This suggests that inhibition by flavopiridol of cyclin B1–dependent kinase activity is important for the induction of apoptosis in prostate cancer cells. Therefore, to maximize apoptosis, flavopiridol and docetaxel should not be added simultaneously because of their opposing effects on cyclin B1–dependent kinase activity.

In contrast to results in gastric cancer cell lines (16), our results show that in LNCaP cells treatment with FD is more effective than that with DF in inducing apoptosis (Fig. 4). Flavopiridol can decrease proteins that inhibit apoptosis, such as Bcl-2, Bcl-xL, Mcl-1, and XIAP (10–13). Therefore, it is possible that in LNCaP an initial treatment with flavopiridol decreases inhibitors of apoptosis and sensitizes cells for subsequent treatment with docetaxel. In contrast to LNCaP cells, DF is more effective for induction of apoptosis than FD in DU 145 cells as determined by DAPI and greater cleavage of caspase-3 and PARP (Figs. 4 and 5). However, overall apoptosis in DF-treated DU 145 cells were not different from that in DD-treated cells (Fig. 4). Therefore, the FD or DF sequence that optimally induces apoptosis may depend on the type of prostate cancer cells (i.e., androgen-dependent or androgen-independent) as well as on the expression of androgen receptor or p53 proteins. Because prostate cancers growing in vivo consist of a heterogeneous mixture of different cell types (44, 45), our results suggest that either the FD or the DF sequence regimen may work in inhibiting tumor growth and inducing apoptosis. In fact, we have shown in the Cg7/T-15 transgenic mouse model of androgen-independent prostate cancer (31) that DF or FD treatment can inhibit the growth of primary and metastatic prostate tumors more effectively than either drug alone by increasing apoptotic cell death (46).

In addition to inhibition of cyclin B1–dependent kinase activity and a decrease in inhibitors of apoptosis, another potential mechanism for flavopiridol is its ability to inhibit transcription elongation (40). It is thought that blocking transcription results in a loss of mRNAs with short half-lives (e.g., Mcl-1 and cycin D1) and therefore a loss of protein (9, 47). Our results show that FD treatment of LNCaP cells did not decrease XIAP mRNA (Fig. 8) and therefore cannot be an explanation for decreased XIAP protein (Fig. 6). FD treatment results in decreased mRNA for survivin and therefore is a likely explanation for decreased survivin protein in LNCaP cells (Figs. 6 and 8). However, decreased survivin protein does not seem to play a major role in FD-mediated induction of apoptosis because there is less apoptosis in LN-AI/CSS compared with LN-AI cells despite that both have decreased survivin protein and mRNA (Figs. 6 and 8).
XIAP protein was higher in FD-treated LN-AI/CSS compared with LN-AI and LNCaP cells and a reduction of XIAP protein with siRNA increased apoptosis (Fig. 7), suggesting a more important role for XIAP. A recent report shows that lowering XIAP protein can sensitize prostate cancer cells to a variety of chemotherapeutic agents (27). In addition, treatment of LN-AI cells with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (75 μmol/L) did not result in increased apoptosis (data not shown), indicating that the ability of flavopiridol to inhibit transcription is not required for induction of apoptosis in FD-treated LN-AI cells.

Another potential mechanism for FD-mediated apoptosis is the differential effect on the prosurvival AKT protein in LN-AI compared with LN-AI/CSS cells (Fig. 9). Our results show that the greater resistance to apoptosis in LN-AI/CSS compared with LN-AI cells was possibly due to higher levels of activated AKT (Fig. 9A). It has been shown previously that androgen ablation can increase AKT activation in LNCaP cells and support survival and proliferation in conditions of androgen deprivation (48). Treatment with FD reduces total AKT protein levels in LN-AI but not LN-AI/CSS cells possibly by increased proteasome degradation of AKT. This may be an explanation why FD treatment induced greater apoptosis in LN-AI compared with LN-AI/CSS cells. Because activated AKT has recently been shown to phosphorylate and stabilize XIAP (41), lower AKT protein (and therefore reduced activated AKT) in FD-treated LN-AI and LNCaP cells may be another explanation for lower XIAP protein. In addition, our results suggest that a 24-hour treatment with flavopiridol followed by no drug is more effective in inducing apoptosis than a continuous 48-hour treatment (Fig. 4C) possibly because of the effect on total AKT protein, which is higher in continuous compared with noncontinuous treatment (Fig. 9B; data not shown). The addition of the phosphatidylinositol 3-kinase inhibitor LY 294002 enhanced apoptosis in FD-treated LN-AI/CSS cells, especially when XIAP protein was reduced with siRNA (Fig. 9C and D), further suggesting the importance of decreasing AKT activity to augment apoptotic cell death. Drug combinations that reduce XIAP protein and activated AKT are more likely to increase apoptosis (22), especially in prostate cancer cells, such as LNCaP, which has a mutated PTEN and a constitutively active AKT (49). However, this strategy may not be effective in prostate cancer cells, such as DU 145, in which PTEN is not mutated and AKT is not constitutively active (50).

A potential explanation for decreased XIAP protein without decreased mRNA in FD-treated LNCaP and LN-AI cells is the activation of the proteasome degradation pathway by flavopiridol. Our results show that the proteasome inhibitor MG-132 can block the ability of flavopiridol to decrease cyclin B1 and McI-1 proteins in LNCaP cells (Fig. 10A). This suggests that flavopiridol can decrease these proteins using the proteasome pathway. In FD-treated LNCaP cells containing MG-132, there was less PARP cleavage and higher levels of XIAP and AKT proteins, implying an antagonism between proteasome inhibition and FD treatment (Fig. 10B). These results are in contrast to synergistic induction of apoptosis in leukemic cells treated with flavopiridol and MG-132, which is associated with disruption of the nuclear factor-κB pathway (51). Thus, treatment strategies that are synergistic in one type of tumor may be antagonistic in another type of tumor.

In summary, our studies suggest that the FD sequence combination in LNCaP and LN-AI prostate cancer cells results in the activation of the proteasome pathway, degradation of prosurvival proteins XIAP and AKT, and induction of apoptosis. The FD sequence induces less apoptosis in the LN-AI/CSS cells possibly because of failure to substantially decrease XIAP and AKT proteins. However, in the DU 145 cells, the DF and DD sequence combinations are more effective than the FD and FF regimens, suggesting a heterogeneous response depending on the type of prostate cancer cell. Phase I clinical trials on the DF sequence in patients with lung cancer have shown few responses, although there was some disease stabilization (8). Because of the heterogeneous nature of prostate cancers, we suggest that the efficacy of the DF or FD sequence may depend on the relative levels of the different types of prostate cancer cells, such as LNCaP (sensitive to FD) and DU 145 (sensitive to DF), present in tumors.

Acknowledgments
We thank Dr. Andrew Schally (Veterans Affairs Medical Center, Miami, FL) and Dr. Diane Brassard (Aventis Pharmaceuticals) for review of this article, Dr. Gianluca D’Ippolito for mesenchymal stromal cells, and Dr. Daniel Danielpour for NRP-152 cells.

References


Perez-Stable C, 2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells. Cancer Lett 2006;231:49 –64.


Yang T, Buchan HL, Townsend K, Craig RW. MCL-1, a member of the BLC-2 family, is induced rapidly in response to signals for cell differentiation or death, but not to signals for cell proliferation. J Cell Physiol 1996;166:523 –36.


Sequential Combinations of Flavopiridol and Docetaxel Inhibit Prostate Tumors, Induce Apoptosis, and Decrease Angiogenesis in the Gγ/T-15 Transgenic Mouse Model of Prostate Cancer

Teresita Reiner, Alicia de las Pozas, and Carlos Perez-Stable

1 Geriatric Research, Education, and Clinical Center and Research Service, VA Medical Center, Miami, Florida
2 Department of Medicine and Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, Florida

BACKGROUND. We investigated whether sequential combinations of flavopiridol and docetaxel can increase apoptotic cell death and inhibit the growth of primary and metastatic prostate tumors in the Gγ/T-15 transgenic mouse model of prostate cancer.

METHODS. Transgenic males were treated and the weights of primary and metastatic prostate tumors determined. Immunohistochemistry and Western blot was performed to evaluate the differences in apoptosis, proliferation, and angiogenesis.

RESULTS. Docetaxel was slightly more effective than flavopiridol in inhibiting primary prostate tumors, but neither drug alone inhibited metastases. Single drug treatments decreased angiogenesis but did not increase apoptosis. Both sequential combinations resulted in greater inhibition of primary and metastatic prostate tumors, increased apoptosis, and decreased angiogenesis compared to control mice.

CONCLUSIONS. Flavopiridol and docetaxel sequence combinations were effective in inhibiting prostate tumors in the Gγ/T-15 transgenic mice. An increase in apoptosis and a decrease in angiogenesis resulted in the greatest inhibition of prostate cancers.

KEY WORDS: prostate cancer; transgenic mice; metastases; apoptosis; angiogenesis

INTRODUCTION

Prostate cancer is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer-related deaths among men in the United States [1]. The principal therapy for men with advanced disease is androgen ablation, but most of these patients eventually progress to an androgen-independent disease [2]. Studies showing that treatment with docetaxel combined with prednisone can improve survival of patients with androgen-independent prostate cancer (AI-PC) have been recently reported [3]. It is likely that docetaxel combined with other novel chemotherapeutic drugs might also result in improved patient survival. Determining the efficacy of docetaxel combinations in pre-clinical animal models of prostate cancer is necessary before doing clinical trials. However, although traditional animal models based on prostate cancer cell lines xenografted subcutaneously into immunocompromised mice frequently respond to anti-cancer drugs, these drugs often do not show activity in clinical trials [4].
Targeting of oncogenes like SV40 T antigen (Tag) to the prostate has resulted in the development of animal models of prostate cancer that represent a more natural history of tumor development, that is, the cancer originates from normal cells in their natural microenvironment and progresses through multiple stages, as in the case of human prostate cancer [5,6]. The TRAMP and LADY models (probasin/Tag) have been used by a variety of investigators to study the molecular events in the carcinogenesis of the prostate and in the pre-clinical testing of new therapies [7–16]. We have developed a unique transgenic mouse model of AI-PC (Gγ/T-15) that targets a subset of basal epithelial cells by using the human fetal Gγ-globin promoter linked to Tag [17–19]. The progression of prostate cancer in the Gγ/T-15 transgenic mice is similar to progression in humans, that is, it originates from high-grade prostate intraepithelial neoplasia (PIN) and progresses to advanced metastatic carcinomas. We have utilized the Gγ/T-15 transgenic mice to test the efficacy against prostate cancer using EB 1089 (a less calcemic and more advanced metastatic carcinomas. We have utilized the Gγ/T-15 transgenic mice to test the efficacy against prostate cancer using EB 1089 (a less calcemic and more potent analog of 1,25-dihydroxyvitamin D3) [19] and prostate cancer using EB 1089 (a less calcemic and more potent analog of 1,25-dihydroxyvitamin D3) [19] and 2-methoxyestradiol, an estrogen metabolite [20]. It remains to be determined whether the outcome of drug efficacy in transgenic mouse models will be more predictive of clinical outcome and therefore reduce the clinical failure rates of novel chemotherapeutic drugs and their combinations.

Docetaxel (taxotere), a semi-synthetic derivative of paclitaxel (taxol) that is derived from the yew tree [21], is a promising anti-cancer drug shown to inhibit a wide variety of tumor cells including prostate cancer cells by diverse mechanisms that include cell cycle arrest, induction of apoptosis, stabilization of microtubules, and inhibition of angiogenesis [22,23]. Flavopiridol, a semi-synthetic flavonoid derived from an indigenous plant from India, is a broad inhibitor of cyclin-dependent kinases (cdk) and it is being tested in clinical trials [24,25]. However, results of Phase II clinical trials of flavopiridol as a single agent have been reported to be unsatisfactory, indicating that flavopiridol may work best as an anti-cancer agent when combined with other agents [26,27]. Studies in a pre-clinical model of xenografted human gastric cancer cells have shown that the greatest increase in apoptosis occurs when docetaxel is followed by flavopiridol [28]. Whether flavopiridol and docetaxel can enhance apoptotic cell death in a transgenic mouse model of prostate cancer has not been previously investigated.

Treatment with single chemotherapeutic agents like flavopiridol and docetaxel will not cure most cancers, including AI-PC. We have recently reported that the human prostate cancer cell lines LNCaP and DU 145 show a heterogeneous response to the sequential combination of flavopiridol and docetaxel [29]. The purpose of the present study was to determine whether the combination of flavopiridol and docetaxel could inhibit the growth of primary and metastatic prostate tumors in the Gγ/T-15 transgenic mice better than either drug alone. Our results show that the sequences of flavopiridol followed by docetaxel (FD) or docetaxel followed by flavopiridol (DF) produce greater apoptosis and inhibit angiogenesis better than either drug alone, leading to a significant inhibition of primary and metastatic prostate tumors. Our findings suggests that both flavopiridol and docetaxel sequence combinations should provide greater efficacy against prostate cancer than either drug alone.

MATERIALS AND METHODS

Reagents

Flavopiridol and docetaxel were obtained from Sanofi-Aventis Pharmaceuticals (Bridgewater, NJ). Flavopiridol was resuspended in PBS at 3 mg/ml, dissolved by adjusting the pH to 3.25, aliquoted, and stored at −20°C. Docetaxel was dissolved in ethanol, Tween-80 (Sigma, St. Louis, MO), PBS (1:1:18, v/v/v) (final concentration of docetaxel was 1.5 mg/ml), and it was stored at −20°C. Vehicle controls (PBS for flavopiridol and ethanol/Tween-80/PBS for docetaxel) were stored at +4°C.

Treatment of Gγ/T-15 Transgenic Males With Flavopiridol and Docetaxel

We utilized the Gγ/T-15 transgenic mouse model of AI-PC [17–19] to evaluate the anti-tumor effects of flavopiridol and docetaxel in vivo as single drugs and in sequence-specific combinations (flavopiridol followed by docetaxel (FD) and docetaxel followed by flavopiridol (DF)). Transgenic mice (CBA × C57) were identified by DNA slot blot analysis as previously described [17,18]. These mice, bred to homozygosity, begin to develop prostate tumors at 13 weeks of age, an earlier time than in hemizygous mice (16 weeks). Homozygous male transgenic mice were palpated three times per week in the urogenital region starting at 13 weeks to detect prostate tumor mass. Mice with palpable prostate tumors were randomly divided into experimental and control groups and injected i.p. every 3 days with 0.1 ml of flavopiridol (F, 10 mg/kg; n = 7), docetaxel (D, 5 mg/kg; n = 7), or the appropriate vehicle controls (PBS, n = 7; ethanol/Tween-80/PBS, n = 7) for 2 weeks (five injections). Treatment with higher doses of docetaxel (15 and 30 mg/kg) resulted in deaths before the 2-week period. For sequence-specific combinations, mice with palpable prostate tumors were injected i.p. with flavopiridol followed by docetaxel 24 hr later (FD; n = 9), docetaxel followed
by flavopiridol 24 hr later (DF; n = 10), or the appropriate vehicle controls (PBS followed by ethanol/Tween-80/PBS; n = 6; ethanol/Tween-80/PBS followed by PBS, n = 6). This regimen was repeated a total of five times over 2 weeks. On day 15, mice were anesthetized, blood collected by cardiac puncture, and serum stored at −80°C. Primary prostate tumors and visible metastases to the pelvic lymph nodes were removed and weighed determined. A portion of primary prostate tumor was stored at −80°C for Western blot analysis and another portion was fixed overnight in formalin for histology (H&E) and immunohistochemistry. Statistical differences between drug and control (combined controls, n = 26) primary and metastatic prostate tumor weights were determined by two-tailed student t-test, with P < 0.05 considered significant. All animal studies were carried out with the approval of the Institutional Animal Care and Use Committee of the Miami VA Medical Center (AAALAC accredited) and conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

**Change in Body Weight and Serum Chemistries**

The body weights for the drug-treated and control mice were determined at the end of the study. Serum from transgenic mice with prostate tumors treated with drugs and controls were analyzed for albumin, alanine aminotransferase (ALT) (markers of liver function), aspartate aminotransferase (AST, marker of soft tissue damage), lactate dehydrogenase (LDH), creatine phosphokinase (CPK) (markers of cardiac and skeletal muscle damage), and alkaline phosphatase (ALKP, marker of kidney, intestine, liver, and bone damage) using a Vitros 250 chemistry analyzer (Ortho Clinical Diagnostics, Rochester, NY). Bilirubin (liver function) were measured by radioimmunoassay (MP Biomedicals, Orangeburg, NY).

**Histology and Immunohistochemistry**

After fixation of primary prostate tumor overnight in 10% buffered formalin, samples were dehydrated, embedded in paraffin, sectioned at 5 μm, and baked at 55°C overnight. Control and drug-treated prostate tumor sections were stained with H&E. For immunostaining, endogenous peroxidases were blocked using 3% H2O2 in methanol for 5 min. Antigen retrieval was done by incubating sections in hot 10 mM citrate buffer (pH 6.0) for 20 min. Immunostaining for PCNA was performed using a 1/100 dilution of mouse monoclonal antibody to PCNA (PC10; Santa Cruz Biotechnology, Santa Cruz, CA) and the Vector Mouse on Mouse (M.O.M.) Peroxidase Kit (Vector Laboratories, Burlingame, CA) following the manufacturer instructions. Immunostaining for cleaved caspase-3 (Cell Signaling Technology, Beverly, MA) and Ki67 (NCL-Ki67p; Vision BioSystems, Inc., Norwell, MA) was performed using a 1/100 dilution of rabbit polyclonal antibody and a 1/100 dilution of biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories). Specific color was developed with the Vector ABC kit and 3,3’-diaminobenzidine (DAB) substrate kit (Vector Laboratories) and the sections were counterstained with hematoxylin, dehydrated, and glass cover slipped.

For the negative controls, we used the same concentration of mouse and rabbit IgG (Santa Cruz Biotechnology) instead of specific primary antibodies, which resulted in no immunostaining. For the TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridinetriphosphate (dUTP) nick end-labeling) technique, we used hot citrate antigen retrieval and the in situ Cell Death Detection Kit, POD (Roche Applied Sciences, Indianapolis, IN) following the manufacturer instructions.

**Western Blot Analysis**

Total protein lysates from prostate tumors was prepared by resuspending frozen tissues in NP40 cell lysis buffer (1% NP40, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, protease inhibitor tablet, 50 mM NaF, and 0.1 mM NaVO4), lysed in a glass homogenizer, left on ice for 30 min, and centrifuged. The protein concentrations of the supernatant were determined with the Bio-Rad protein assay. After separation of 50–100 μg protein by SDS–PAGE, proteins were transferred by electrophoresis to Immobilon-P membrane and incubated in 5% non-fat dry milk, PBS, and 0.25% Tween-20 for 1 hr. Antibodies specific for cleaved caspase-3, XIAP (Cell Signaling Technology), Bcl-xL (polyclonal; BD Biosciences Pharmingen, San Diego, CA), survivin (FL-142), and Bcl-2 (N-19) (Santa Cruz Biotechnology) were diluted 1/3,000 in 5% non-fat dry milk, PBS, and 0.25% Tween-20 and incubated overnight at 4°C. Membranes were washed in PBS and 0.25% Tween-20 and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1/3,000 dilution; Santa Cruz Biotechnology) for 1 hr, washed in PBS and 0.25% Tween-20, and analyzed by exposure to X-ray film using enhanced chemiluminescence plus (ECL plus, Amersham Pharmacia Biotech, Arlington Heights, IL). Antibodies specific for β-tubulin (TU-02; 1:3,000 dilution; Santa Cruz Biotechnology) were used as protein loading controls. 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). The changes in cleaved caspase-3, XIAP, survivin, Bcl-xL, and Bcl-2 protein levels in drug-treated and
control prostate tumors were determined by normalizing the values to tubulin from the same Western blot.

**Blood Vessel Density**

Sections of prostate tumor were processed as described above and immunostained for CD31 (PECAM) using a 1/50 dilution of goat polyclonal antibody (M20; Santa Cruz Biotechnology) and a 1/100 dilution of biotinylated rabbit anti-goat IgG secondary antibody (Vector Laboratories). Specific color was developed as described above. Blood vessel density was determined by counting the number of CD31-positive vessels from four random high-powered fields (400x) of each section. Four to five tumors were analyzed for each group.

**Statistical Analysis**

Statistical differences between drug-treated and control prostate tumors were determined by two-tailed student t-test with \( P < 0.05 \) considered significant.

**RESULTS**

**Primary and Metastatic Prostate Tumors in \( G_G/T-15 \) Transgenic Mice**

The \( G_G/T-15 \) transgenic mice contain the human fetal \( G_G \)-globin promoter linked to SV40 T antigen, which targets a subset of basal epithelial cells present in the normal prostate. These mice develop AI-PC and metastasis to the pelvic lymph nodes [17–19]. At between 13 and 26 weeks of age, >75% of the transgenic males develop palpable primary prostate tumors. The peak of tumor incidence occurs at 19 weeks and the weights of the palpable prostate tumors range from 0.1 to 0.2 g. After a period of 2–3 weeks, the primary prostate tumors grow rapidly and metastasize to the pelvic lymph nodes to form visible lesions (Fig. 1). Using the \( G_G/T-15 \) transgenic mouse system, we previously determined the anti-tumor efficacy of vitamin D analog EB 1089 [19] and 2-methoxyestradiol [20].

**Flavopiridol and Docetaxel as Single Chemotherapeutic Drugs in \( G_G/T-15 \) Mice**

We used the \( G_G/T-15 \) mice to test the efficacy of flavopiridol and docetaxel as single chemotherapeutic drugs and in combination. \( G_G/T-15 \) males with palpable prostate tumors were treated every 3 days with flavopiridol (F, 10 mg/kg), docetaxel (D, 5 mg/kg), or the appropriate controls for a period of 2 weeks. The primary and metastatic prostate tumors were weighed and the results are shown in Figure 2. Treatment of mice with flavopiridol caused a 16% decrease in the weights of primary prostate tumors compared to control mice (\( n = 7; \ P = 0.33 \)) and treatment with docetaxel resulted in a 32% decrease in the weights of primary prostate tumors (\( n = 7; \ P = 0.09 \)). Neither drug decreased the weights of metastatic prostate tumors compared to control mice. Although inhibition of primary tumors by single drugs was not statistically significant, there was a trend for docetaxel to be more effective than flavopiridol, similar to what is observed in the treatment of human AI-PC [30].

**Flavopiridol and Docetaxel Sequence Combinations Inhibit Primary and Metastatic Prostate Tumors in \( G_G/T-15 \) Mice**

We have shown that the simultaneous addition of flavopiridol and docetaxel to human DU 145 prostate cancer cells results in antagonism with respect to induction of apoptosis, compared to each drug alone [29]. Therefore, a sequential addition of flavopiridol and docetaxel is required for maximal induction of apoptosis. Our in vitro data indicate that treatment of human LNCaP prostate cancer cells with flavopiridol for 24 hr followed by docetaxel for another 24 hr (FD) induces apoptosis more effectively than docetaxel followed by flavopiridol (DF). In contrast, the DF

---

**Fig. 1.** Primary and metastatic prostate tumors in the \( G_G/T-15 \) transgenic mice. Two weeks after first detecting palpable prostate tumors in the urogenital region, primary and lymph node metastatic lesions were removed and weighed in order to measure effect of chemotherapeutics.
sequence is more effective to induce apoptosis compared to the FD sequence in the DU 145 prostate cancer cells [29]. The results show that the DF sequence decreases the weights of primary prostate tumors in G\textsubscript{G}/T-15 mice by 61% compared to control mice (n = 10; P < 0.0003) and the FD sequence reduces primary prostate tumors by 43% (n = 9; P < 0.02). Although the average weights of the primary prostate tumors were smaller in the DF sequence compared to the FD sequence, the differences were not significant (P = 0.08). There was a significant difference between DF (but not FD) treatment and the single drug treatments with flavopiridol and docetaxel (P < 0.04).

Only one of nine FD-treated mice (11%) developed metastatic prostate tumors, resulting in a 96% decrease in tumor weights compared with control mice (P < 0.05). In DF-treated mice, five of ten (50%) developed metastatic prostate tumors, but the overall average tumor weights were not significantly different from control mice (P = 0.20). Metastatic prostate tumors developed in 93% of control mice (24 of 26) and 86% of F- and D-treated mice (12 of 14). Overall, the results in the G\textsubscript{G}/T-15 mice indicate that: (1) the DF sequence was more effective in inhibiting primary prostate tumors; (2) the FD sequence more effectively suppressed metastatic prostate tumors; and (3) both the DF and FD sequences had greater efficacy against prostate cancer compared with single drugs alone.

**Treatment With Flavopiridol and Docetaxel Is not Toxic in G\textsubscript{G}/T-15 Mice**

To determine in the G\textsubscript{G}/T-15 mice if treatment with flavopiridol, docetaxel, and their sequence combinations is toxic, we measured the body weights and serum chemistry, and compared these values to those in control mice. There were no significant differences in final body weights in drug-treated compared to control mice (not shown). In addition, serum chemistry measuring soft tissue damage (liver, muscle, and kidney) shows no significant differences between drug-treated and control mice (Table I). One significant difference was lower serum ALKP in mice treated with flavopiridol, possibly due to a specific effect on the ALKP gene or the tissues synthesizing ALKP (liver, bone, etc.). Overall, these results indicate that treatment of the G\textsubscript{G}/T-15 mice with flavopiridol, docetaxel, and their sequence combinations does not result in acute toxicity. However, differences in pharmacokinetics between mice and humans make it difficult to extrapolate these toxicity results clinically.

**Increase in Apoptosis Without a Decrease in Cell Proliferation in G\textsubscript{G}/T-15 Prostate Tumors Treated With FD and DF**

We investigated whether the decrease in the weights of the primary prostate tumors in the FD and DF treatment groups were due to increases in apoptosis and/or decreases in cell proliferation. Immunostaining for the DNA proliferation marker PCNA revealed no obvious differences between drug-treated and control prostate tumors (Fig. 3). A prostate duct embedded within the control tumor showed no immunostaining for PCNA. Similar results were also obtained by immunostaining for the proliferation marker Ki67 (not shown). Results with the in situ TUNEL peroxidase method showed a greater number of apoptotic cells in the DF compared to the control tumors (Fig. 3). Finally, a greater number of cells in FD-treated G\textsubscript{G}/T-15
TABLE I. Serum Chemistry From Gγ/T-15 Transgenic Males Treated With Flavopiridol (F), Docetaxel (D), Flavopiridol Followed by Docetaxel (FD), Docetaxel Followed by Flavopiridol (DF), and Vehicle Controls

<table>
<thead>
<tr>
<th>Mice + drug</th>
<th>N(^a)</th>
<th>Albumin g/dl</th>
<th>AST U/L</th>
<th>LDH U/L</th>
<th>CPK U/L</th>
<th>ALKP U/L</th>
<th>ALT U/L</th>
<th>Bile acids μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>19</td>
<td>2.23 (0.49)</td>
<td>377 (213)</td>
<td>8,335 (5,563)</td>
<td>3,911 (3,961)</td>
<td>58.8 (16.7)</td>
<td>63.9 (31.9)</td>
<td>7.5 (8.65)</td>
</tr>
<tr>
<td>F, 10 mg/kg</td>
<td>7</td>
<td>1.86 (0.45)</td>
<td>273 (167)</td>
<td>6,425 (2,597)</td>
<td>2,224 (2,563)</td>
<td>*42.3 (12.4)</td>
<td>78.9 (80.7)</td>
<td>5.89 (4.18)</td>
</tr>
<tr>
<td>D, 5 mg/kg</td>
<td>7</td>
<td>1.9 (0.47)</td>
<td>372 (149)</td>
<td>5,868 (3,084)</td>
<td>3,354 (2,582)</td>
<td>63.7 (25.6)</td>
<td>70.4 (29.6)</td>
<td>4.93 (3.26)</td>
</tr>
<tr>
<td>FD</td>
<td>8</td>
<td>2.45 (0.64)</td>
<td>337 (188)</td>
<td>6,251 (1,764)</td>
<td>4,034 (4,890)</td>
<td>47.1 (34.3)</td>
<td>65.8 (34.5)</td>
<td>4.13 (1.64)</td>
</tr>
<tr>
<td>DF</td>
<td>8</td>
<td>2.05 (0.59)</td>
<td>294 (131)</td>
<td>7,700 (6,608)</td>
<td>4,110 (4,309)</td>
<td>46.8 (18)</td>
<td>61.5 (29)</td>
<td>17.9 (25.3)</td>
</tr>
</tbody>
</table>

\(^a\)Numbers of mice.
\(^b\)Numbers in parenthesis refers to standard deviations.
*P < 0.03.

Fig. 3. An increase in apoptosis without a significant decrease in cell proliferation in FD- and DF-treated Gγ/T-15 prostate tumors. Immunostaining of primary prostate tumors with PCNA shows no obvious differences between drug-treated (DF) and control (Co) (hematoxylin, 200×). TUNEL staining shows a greater number of apoptotic cells in the DF compared to the control tumors (hematoxylin, 200×). A greater number of cells in FD-treated prostate tumors were positive for cleaved (activated) caspase-3 compared to control prostate tumors (hematoxylin, 400×).
prostate tumors were immunostained by an antibody specific for the cleaved and activated form of caspase-3 (Fig. 3). Overall, these results suggest that the FD- and DF-treated primary prostate tumors were smaller because there was greater apoptosis without significant changes in cell proliferation.

**Decreases in XIAP and Bcl-xL Proteins in DF-Treated Gγ/T-15 Prostate Tumors**

To further investigate why the FD- and DF-treated Gγ/T-15 primary prostate tumors had decreased weights compared to controls, we sought to identify by Western blot analysis the differences in the levels of proteins important in apoptosis (Figs. 4 and 5). The results showed a 6.5- and 2.1-fold greater level of cleaved caspase-3 in FD- and DF-treated prostate tumors, respectively, compared to control prostate tumors. In F- and D-treated prostate tumors, there was a 1.7- and 1.4-fold increase in cleaved caspase-3 compared to control prostate tumors. The levels of cleaved caspase-3 detected by Western blot correlated with the levels identified by immunohistochemistry.
XIAP is a member of the inhibitor of apoptosis (IAP) family that binds caspase-3, inhibits its activity, and blocks apoptosis [31,32]. Prostate tumors treated with D and DF showed a small but consistent decrease (27 and 29%; $P < 0.05$) in total XIAP protein, whereas there was a 58% increase in F- ($P < 0.01$) and no change in FD-treated prostate tumors (Figs. 4 and 5). There were no significant changes in the levels of survivin, which is also a member of the IAP family [31,32], in any treatment groups (not shown). We also measured the levels of Bcl-xL and Bcl-2, anti-apoptotic members of the Bcl-2 family that increase with progression of prostate cancer [33]. F- and DF-treated prostate tumors show a small but consistent decrease (18 and 22%; $P < 0.02$) in total Bcl-xL protein, whereas there were no significant changes in prostate tumors treated with D and FD. There were no significant modifications in Bcl-2 protein in any treatment group (not shown). Overall, these results suggest that the inhibition of the growth of primary prostate tumors induced by the DF regimen correlates with lower levels of XIAP and Bcl-xL proteins and the inhibition of the growth of metastases in the FD combination can be correlated with higher levels of activated (cleaved) caspase-3 in primary prostate tumors.

### Inhibition of Angiogenesis in DF- and FD-Treated G\textsubscript{T}/T-15 Prostate Tumors

We investigated whether treatment of G\textsubscript{T}/T-15 mice with F, D, FD, and DF results in the inhibition of angiogenesis by immunostaining for CD31 (PECAM) (Fig. 6). Results show that treatment with F and D as single drugs significantly inhibited the number of blood vessels compared to control prostate tumors (F, $6.0 \pm 1.9$ and D, $7.1 \pm 2.3$ vs. control, $14 \pm 4.8$ CD31-positive blood vessels per high-powered field; $P < 6 \times 10^{-5}$). Treatment with FD and DF also inhibited the number of blood vessels compared to controls (FD, $4.0 \pm 1.3$; DF, $4.7 \pm 1.6$; $P < 5 \times 10^{-7}$). There were significantly fewer blood vessels in FD-treated compared to F- and D-treated prostate tumors ($P < 0.002$). In DF-treated prostate tumors, there were significantly less blood vessels compared to D-treated ($P < 0.006$) but not compared to F-treated ($P = 0.06$) prostate tumors. These results indicate that the combination of
FD and DF inhibits angiogenesis more than each drug alone.

**DISCUSSION**

We evaluated in the G\(\gamma\)/T-15 transgenic mouse model of AI-PC whether the combination of flavopiridol and docetaxel can inhibit the growth of primary and metastatic prostate tumors better than either drug alone. Our results indicate that the regimen docetaxel followed by flavopiridol (DF) is more effective in inhibiting primary prostate tumors and that the flavopiridol followed by docetaxel (FD) sequence is better at decreasing metastases (Fig. 2). An increase in apoptosis is a likely reason for smaller prostate tumors in the FD and DF regimens (Fig. 3). The highest levels of cleaved (activated) caspase-3 in the FD group could be correlated with a reduction in metastases and a decrease in the anti-apoptosis proteins XIAP and the Bcl-xL in the DF group correlated with smaller primary prostate tumors (Figs. 4 and 5). There was a better inhibition of angiogenesis in the FD- and DF-treated groups compared to single drugs (Fig. 6). Overall, our results indicate that in the G\(\gamma\)/T-15 transgenic mice both sequence combinations of flavopiridol and docetaxel are more effective in reducing primary and metastatic prostate tumors than either drug alone.

There is a strong probability that docetaxel combined with other chemotherapeutic drugs would result in an improved inhibition of prostate cancer cell growth. A previous study using the subcutaneous xenografts of MKN-74 human gastric cancer cells in nude mice showed that the DF regimen is more effective than FD in decreasing tumor growth [28]. These results are similar to the flavopiridol and paclitaxel or epothilone B regimens in gastric and breast cancer cell lines in vitro, that is, paclitaxel or epothilone B followed by flavopiridol can increase apoptosis more than the inverse sequence combination [34,35]. In contrast, our in vitro studies show that the LNCaP prostate cancer cells are more sensitive to the DF regimen, whereas the DU 145 prostate cancer cells are more sensitive to the DF sequence combination [29]. These results suggest a heterogeneous response to the DF or FD combination sequence depending on the type of prostate cancer cell. In the G\(\gamma\)/T-15 transgenic mouse prostate tumors, the expression of Tag results in the inactivation of p53 and Rb, a cellular phenotype similar to that in DU 145 cells [36]. Although G\(\gamma\)/T-15 prostate tumors are poorly differentiated, these tumors express androgen receptors (AR), similar to LNCaP cells [19]. It is likely that in G\(\gamma\)/T-15 prostate tumors, a heterogeneous mixture of prostate cancer cells like DU 145 (sensitive to DF) and LNCaP (sensitive to FD) is present. This offers an explanation why both sequence combinations have efficacy against prostate cancer. This heterogeneous mixture of cancer cells is most likely absent in xenograft models of cancer cell lines grown in vitro.

The induction of apoptosis is a requirement for the effect of chemotherapeutic drugs on prostate cancer cells [37]. Our results in the G\(\gamma\)/T-15 mice demonstrate that the greatest efficacy against prostate cancer occurs when apoptosis increases in the FD and DF regimens (Figs. 4 and 5). Although the FD and DF sequence combinations inhibit proliferation of prostate cancer cells and have an effect on the cell cycle in vitro [29], neither sequence combination had a significant effect on cell proliferation in G\(\gamma\)/T-15 prostate tumors (Fig. 3). Most anti-cancer drugs appear to exert their therapeutic effect by decreasing proliferation and increasing apoptosis [38]. Anti-cancer regimens like FD and DF that are not dependent on a significant decrease in cell proliferation to increase apoptosis in vivo may have a therapeutic advantage in slow growing tumors like prostate cancer [39]. In addition, there is a correlation with an increase in activated caspase-3 and a decrease in metastases in the FD treatment group (Figs. 2–4). This suggests that chemotherapy that increases apoptosis will result in a decrease in metastases [40]. Transgenic models of prostate cancer like G\(\gamma\)/T-15 are more useful in testing the effect of combination chemotherapy on metastases, compared to subcutaneous xenograft models, which rarely metastasize.

We have recently shown that a substantial decrease in XIAP, but not in survivin protein, both being members of the IAP family, correlates with enhancement of apoptosis by FD in LNCaP prostate cancer cells [29]. In the G\(\gamma\)/T-15 mice, the D and DF regimens show a small, but significant decrease in total XIAP protein in primary prostate tumors (Figs. 4 and 5), whereas there were no significant changes in survivin protein in any treatment group (not shown). XIAP is upregulated in most types of cancer, including AI-PC, and confers resistance to chemotherapeutic drugs [41,42]. Targeted downregulation of XIAP increases the sensitivity of cancer cells, including that of AI-PC cells, to a variety of chemotherapeutic drugs [41–44]. The combination of chemotherapeutic drugs that results in the greatest decrease in XIAP protein may lead to the highest efficacy against prostate cancer. However, the greatest decrease of XIAP protein in single DF-treated mice did not always correlate with an increase in activated caspase-3 or with the smallest prostate tumors, suggesting that other factors are also important in mediating the effect against prostate cancer.

Flavopiridol reduces the levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL and sensitizes cancer cells to apoptosis after subsequent treatment with other
chemotherapeutic agents [35]. Our results show that treatment of G7/T-15 mice with F and DF results in a small, but significant decrease in Bcl-xL (but not Bcl-2) proteins in primary prostate tumors (Figs. 4 and 5). However, similarly to results with XIAP, a decrease in Bcl-xL protein in single DF-treated mice did not always correlate with higher cleaved caspase-3 and smaller prostate tumors, suggesting that other factors may be important. Our results do not provide a specific mechanism to distinguish the differential effects of the DF and FD regimens on primary and metastatic prostate tumors. The molecular identification of the factors that mediate the inhibition of primary and metastatic prostate tumors in FD- and DF-treated G7/T-15 mice could be better addressed by gene expression microarray studies.

The inhibition of angiogenesis is an important mechanism in increasing the anti-cancer efficacy of chemotherapeutic drugs [45]. In G7/T-15 mice, both flavopiridol and docetaxel as single drugs significantly inhibited angiogenesis compared to controls (Fig. 6). These results support previous results showing that flavopiridol and docetaxel can inhibit angiogenesis and suggest that this may be an important mechanism for inhibiting tumor growth [46,47]. However, the inhibition of angiogenesis without an increase in apoptosis did not result in a significant reduction in the weights of primary and metastatic prostate tumors in flavopiridol- and docetaxel-treated G7/T-15 mice. The FD and DF regimens increase apoptosis and further inhibit angiogenesis, resulting in a significant reduction in the weights of primary and metastatic prostate tumors. These results suggest that drug combinations that increase apoptosis and decrease angiogenesis to the greatest extent will provide the greatest anti-tumor effect.

Two of the features of cancer that are essential for the progressive growth and expansion of solid tumors are the evasion of apoptosis and the formation of new blood vessels by angiogenesis [48]. Our results in the G7/T-15 transgenic mouse model of AI-PC indicate that either the FD or DF regimen can simultaneously increase apoptosis and inhibit angiogenesis, providing the greatest efficacy against prostate cancer.

ACKNOWLEDGMENTS

We thank Dr. Andrew Schally and Dr. Diana Brassard for the review of this manuscript and helpful suggestions. We also thank Carolyn Cray for assistance and advice on the chemistry of mouse serum. This research was supported by grants from Sanofi-Aventis Pharmaceuticals, VA Merit Review, and the Department of Defense, all to C. Perez-Stable.

REFERENCES


Increased expression of cyclin B1 sensitizes prostate cancer cells to apoptosis induced by chemotherapy

Lourdes A. Gomez, Alicia de las Pozas, Teresita Reiner, Kerry Burnstein, and Carlos Perez-Stable

1Geriatric Research, Education, and Clinical Center and Research Service, VA Medical Center; 2Department of Medicine, 3Department of Molecular and Cellular Pharmacology, and 4Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine; and 5South Florida VA Foundation, Miami, Florida

Abstract
Chemotherapeutic drugs ideally should take advantage of the differences between transformed and normal cells and induce apoptosis only in cancer cells. One such difference may be the overexpression of cyclin B1 protein in cancer cells, which is required for the proper progression through mitosis. Previously, we showed that treatment of human prostate cancer cells with 2-methoxyestradiol (2-ME) or docetaxel results in an accumulation of cyclin B1 protein and an increase in cyclin B1 kinase activity, followed by induction of apoptotic cell death. Inhibition of cyclin B1 kinase lowers apoptosis induced by 2-ME and docetaxel. In this study, we established a positive correlation between cyclin B1 protein and apoptosis induced by chemotherapy in prostate cancer cells. There is minimal cyclin B1 and induction of apoptosis by chemotherapy in nontransformed cells. LNCaP and PC-3 prostate cancer cells stably overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy. LNCaP cells expressing cyclin B1 small interfering RNA to lower cyclin B1 protein or dominant negative cyclin-dependent kinase 1 to inhibit cyclin B1 kinase show a decrease in apoptosis. Increased sensitivity to apoptosis by overexpression of cyclin B1 may be due to lower Bcl-2, higher p53, and decreased neuroendocrine differentiation. We suggest that a cancer-specific mechanism whereby 2-ME and docetaxel may exert anti–prostate cancer activity is the deregulated activation of cyclin B1 kinase, leading to the induction of apoptotic cell death. Our results also suggest that higher levels of cyclin B1 in prostate cancer cells may be a good prognostic marker for chemotherapy. [Mol Cancer Ther 2007;6(5):1534–43]

Introduction
One of the features that distinguish cancer cells from normal cells is uncontrolled cell division, likely resulting from the overexpression of cyclins and the abnormal control of cyclin-dependent kinases (CDK; ref. 1). Cyclins are a family of proteins whose levels vary during the cell cycle to activate specific CDKs required for the proper progression through the cell cycle. Cyclin B1, which is essential for cell cycle progression through mitosis, is overexpressed in a variety of cancers compared with normal cells and tissues (2–4). The deregulated expression of cyclin B1 seems to be closely associated with early events in neoplastic transformation (5).

Antibodies to cyclin B1 have been detected in patients with a variety of cancers including prostate cancer and, therefore, cyclin B1 is considered to be a tumor-specific antigen (6, 7). In addition, the overexpression of cyclin B1 has been identified as a prognostic marker for poor patient outcome in some cancers (8, 9). However, in a recent study, patients with follicular lymphoma expressing higher levels of cyclin B1 showed a better outcome after chemotherapy compared with those with lower cyclin B1 (10). In patients with prostate cancer, high cyclin B1 expression correlated with tumor grade and DNA ploidy, but not with disease recurrence (11). However, in another study, the most powerful predictor of time to relapse of prostate cancer was a high ratio of cyclins A and B to the proliferation marker Ki67 (i.e., the higher the ratio, the longer the time to relapse; ref. 3). Gene microarray studies indicate that the overexpression of cyclin B1 mRNA correlates with undifferentiated metastatic prostate cancer with poor prognosis (12–14). Overall, it is not clear in prostate cancer if the overexpression of cyclin B1 plays a role in disease progression and/or resistance to chemotherapy.

Because the overexpression of cyclins and their associated kinases correlates with increased proliferation of cancer cells, small-molecule inhibitors of CDK activity have been identified and are being investigated in multiple clinical trials as potential chemotherapeutic agents (15). However, some reports cast doubts on the importance of CDK2 inhibition in cancer therapy (16). In addition, knockout mice without cyclin D, cyclin E, CDK2, CDK4, or CDK6 have normal fetal development, suggesting that these genes are not strictly required for cell proliferation (17). Therefore, inhibitors of CDK2, CDK4, and CDK6, which primarily function in the G1-to-S transition of the cell cycle,
may not be appropriate for cancer therapy. In contrast, deletion of the S and G2-M cyclin A2 or cyclin B1, which are the partners for CDK1, in knockout mice is associated with an embryonic lethal phenotype, suggesting they are required for cell proliferation (18, 19). Therefore, more recent studies have focused on inhibition of cyclin B1 and CDK1 activity in cancer therapy.

Reduction of cyclin B1 protein in the HeLa cervical carcinoma cell line using small interfering RNA (siRNA) results in decreased proliferation and increased sensitivity to apoptosis induced by paclitaxel (20–22). Other studies have shown that the levels of cyclin B1 can mediate γ-radiation–induced apoptosis and increase sensitivity to paclitaxel and that the specific inhibition of cyclin B1-CDK1 is required for induction of apoptosis by chemotherapy (23–26). Overall, these studies suggest that chemotherapeutic drugs that target cyclin B1-CDK1 may be more appropriate. Whether the levels of cyclin B1 have an effect on apoptosis in prostate cancer and increase sensitivity to chemotherapeutic drugs is not clear.

We have recently shown that treatment of human prostate cancer cell lines with the promising chemotherapeutic drugs 2-methoxyestradiol (2-ME) and docetaxel increases cyclin B1 protein and its associated kinase activity following induction of apoptosis (27, 28). Small-molecule inhibitors of CDK1 prevent 2-ME– and docetaxel-mediated increase in cyclin B1–dependent kinase activity and block induction of apoptosis. We hypothesize that 2-ME– and docetaxel-mediated activation of cyclin B1–dependent kinase and G2-M cell cycle arrest is required for induction of apoptosis in prostate cancer cells. The purpose of the present study was to determine whether altering the levels of cyclin B1 in prostate cancer cells has an effect on apoptosis induced by 2-ME and docetaxel. Our results show that cyclin B1 protein is highest in androgen-dependent and androgen-independent LNCaP prostate cancer cells, which are more sensitive to apoptosis induced by 2-ME and docetaxel, compared with androgen-independent DU-145 and PC-3 prostate cancer cells. An elevation of cyclin B1 protein in LNCaP and PC-3 cells increases apoptosis induced by 2-ME and docetaxel, whereas reduction of cyclin B1 or inhibition of CDK1 activity decreases apoptosis. These results suggest that prostate cancer cells that express higher cyclin B1 protein should be more responsive to apoptosis induced by chemotherapy compared with prostate cancer cells expressing lower levels of cyclin B1.

Materials and Methods
Reagents
2-ME was obtained from EntreMed, Inc. and docetaxel was obtained from Aventis Pharmaceuticals. 2-ME and docetaxel were resuspended in DMSO and aliquots stored at −20°C. 4′-6-Diamidino-2-phenylindole (DAPI) was purchased from Calbiochem. Protease inhibitor cocktail tablets were purchased from Roche Applied Sciences. Coomassie blue was purchased from EMD Chemicals, Inc.

Cell Culture
Human prostate carcinoma cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (29). LN-AI is an androgen-independent subline of LNCaP, which was spontaneously derived in our laboratory (28). These cells express androgen receptor (AR) and prostate-specific antigen, similar to LNCaP. PC-3/AR cells stably expressing AR and PC-3/AI are the negative control cells (30). LNCaP, LN-AI, DU-145, PC-3, PC-3/AI, and PC-3/Neo cells were maintained in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (Hyclone), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin (Invitrogen). Unlike androgen-dependent LNCaP, the LN-AI cells are able to grow for long term in RPMI 1640 with 5% charcoal-stripped fetal bovine serum (Hyclone) and are referred to as LN-AI/CSS. Nontransformed human mesenchymal stromal cells (MSC) derived from bone marrow were obtained from G. D’Ippolito (University of Miami, Miami, FL) and cultured in DMEM (low glucose) with 5% fetal bovine serum and antibiotic/antimycotic (31). The normal rat prostate basal epithelial cell line NRP-152, provided by D. Danielpour, was maintained in HEPES-free DMEM/F12 (1:1, v/v) with 5% fetal bovine serum, antibiotic/antimycotic, 20 ng/mL epidermal growth factor, 10 ng/mL cholera toxin, 5 μg/mL insulin, and 0.1 μmol/L dexamethasone (32).

Treatment with 2-ME and Docetaxel
LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 cells were seeded in 6-cm dishes and allowed to attach overnight. The next day, fresh medium containing 2-ME (1, 2, or 5 μmol/L), docetaxel (0.5, 1, or 10 nmol/L), or DMSO (0.1%) control was added and the cells were cultured for varying times (24–72 h). In all the experiments, floating and trypsinized attached cells were pooled for further analysis.

Western Blot Analysis
Preparation of protein lysates and Western blot analysis was done as previously described (28). We used antibodies specific for cyclin B1 (GNS1), CDK1 (17), Bax (N-20), AR (N-20), Mcl-1 (S-19), survivin (FL-142), p53 (DO-1; Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP; C2-10), E-cadherin (clone 36), Bcl-xL (polyclonal; BD Biosciences PharMingen), cleaved PARP (Asp214), cleaved caspase-3 (9661), X-linked inhibitor of apoptosis (XIAP; Cell Signaling Technology), neuron-specific enolase (clone 5E2; Upstate), and synaptophysin (Zymed Laboratories). Antibodies specific for α-tubulin (TU-02, Santa Cruz Biotechnology) or Coomassie blue staining of total protein on membranes were used for protein loading controls. 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.). The scanned bands from the same blot were normalized to scanned total protein.

DAPI Apoptosis Assay
For the DAPI staining apoptosis assay, cells were resuspended in 0.6-mL 4% paraformaldehyde/PBS for 15 min, washed with PBS, and resuspended in 0.5 mL of DAPI (1 μg/mL)/PBS for 10 min. Cells were washed with
PBS and 10 µL of concentrated cells added on a microscope slide followed by placement of a coverslip. Cells containing densely stained and fragmented chromatin were identified as end-stage apoptotic using a Nikon fluorescence microscope with a DAPI filter. The number of apoptotic cells in at least 200 total cells was determined from at least four random microscope fields. Changes in apoptosis from 2-ME– and docetaxel-treated cells were determined as percentage of apoptotic cells in at least five different samples from three independent experiments. Minimal apoptosis was detected in control treated cells (<0.5%).

**Stable Transfection of Cyclin B1, siRNA Cyclin B1, and Dominant Negative CDK1**

To overexpress cyclin B1 in prostate cancer cells, we obtained the pCMX/cyclin B1 expression plasmid from Jonathan Pines (Wellcome Trust/Cancer Research UK; ref. 33). LNCaP cells (90% confluent) were cotransfected with pCMX/cyclin B1 and pCMVneo (for drug selection) using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The negative control was transfection with pCMVneo alone. Cells were grown in media with 400 µg/mL G418, colonies selected, and clones that overexpressed cyclin B1 compared with pCMVneo negative control clones were identified by Western blot. In PC-3, we used FuGene 6 (Roche Applied Sciences) for stable transfection. To lower the cyclin B1 levels, LNCaP cells were cotransfected with pKD-cyclin B1-v2 (siRNA expression plasmid specific for cyclin B1; Upstate) and pCMVneo, and clones containing lower levels of cyclin B1 protein compared with negative control transfected cells (pKD-NegCon-v1; Upstate) were identified. Finally, we isolated LN-AI clones overexpressing dominant negative CDK1, obtained from Sander van den Heuvel (Harvard Medical School, Boston, MA; ref. 34). An LN-AI clone containing the pCMVneo plasmid was used as the negative control.

**Transient Transfection of Cyclin B1 siRNA**

LN-AI cells were seeded in 12-well plates and transfected the next day with 100 nmol/L siRNA SMARTpool specific for cyclin B1 and siCONTROL nontargeting pool (Dharmacon) using Oligofectamine (Invitrogen), following the manufacturer’s instructions. After 48 h, cells were harvested and analyzed for expression of cyclin B1 by Western blot as described above. Subsequently, LN-AI cells were transfected with cyclin B1 for 48 h to reduce cyclin B1 protein, reseeded, retransfected in the presence or absence of 5 µmol/L 2-ME for an additional 48 h, and proteins analyzed by Western blot and compared with siCONTROL siRNA–transfected cells.

**Statistical Analysis**

Statistical differences between drug-treated and control cells were determined by two-tailed Student’s t test, with P < 0.05 considered significant.

**Results**

2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer cells, using a number of diverse mechanisms (35–37). The induction of apoptosis is a requirement for chemotherapeutic drugs to be effective against prostate cancer cells (38). To evaluate the effect of cyclin B1 on apoptosis induced by 2-ME, we used various human prostate cancer cell lines (LNCaP, LN-AI, LN-AI/CSS, DU-145, and PC-3). LNCaP are androgen-dependent and LN-AI, LN-AI/CSS, DU-145, and PC-3 are androgen-independent prostate cancer cells. The results were compared with a primary nontransformed human MSC and rat prostate epithelial NRP-152 cell lines.

**Cyclin B1 Protein Level Correlates with Induction of Apoptosis by Chemotherapy**

To measure the induction of apoptosis by 2-ME, we did a DAPI staining assay in LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 cells treated with 5 µmol/L 2-ME for 72 h (Fig. 1). We selected this dose because of the increased effect on cyclin B1–dependent kinase, which is important in the induction of apoptosis (27). The results indicated that apoptosis induced by 2-ME was greatest (39%) in LN-AI followed by LNCaP cells (26%). Removal of androgens resulted in decreased apoptosis in LN-AI/CSS (6%) compared with LN-AI cells. The levels of apoptosis in DU-145 (10%) and PC-3 (5%) were similar to those in LN-AI/CSS cells. 2-ME induced minimal apoptosis in non-tumorigenic MSC (1%) and NRP-152 (3.5%) compared with tumorigenic cells. Similar results were obtained with docetaxel and flavopiridol (a pan-CDK inhibitor; ref. 28). These results indicate that 2-ME, docetaxel, and flavopiridol are more effective in inducing apoptosis in LNCaP and

![Figure 1.](image)

Figure 1. Cyclin B1 protein levels correlate with induction of apoptosis by 2-ME in prostate cancer and nontransformed cells. Top, percentage of apoptotic cells determined by DAPI in LNCaP, LN-AI, LN-AI/CSS, DU-145, and PC-3 prostate cancer cells treated with 5 µmol/L 2-ME for 72 h and compared with treatment of nontransformed MSC and NRP-152 cells. There was minimal apoptosis (<0.5%) detected by DAPI in the 0.1% DMSO control treated cells (not shown). Columns, mean; bars, SD. Bottom, Western blot analysis showing the levels of cyclin B1 and Cdk1 in LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 cells (no treatment). Coomassie blue stain of total protein is loading control. Similar results were obtained from three independent experiments.
LN-AI cells as compared with androgen-independent prostate cancer and nontumorigenic cells. Cyclin B1 protein is overexpressed in cancer cells compared with normal nontumorigenic cells (3, 4, 6). To determine if the levels of cyclin B1 vary between androgen-dependent and androgen-independent prostate cancer cells, we did Western blot analysis of LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 proteins when cells were 50% to 60% confluent (Fig. 1). The results indicate that cyclin B1 protein levels are greatest in LN-AI followed by LNCaP cells. Removal of androgens results in decreased cyclin B1 protein in LN-AI/CSS cells compared with LN-AI cells and is similar to the levels in DU-145 and PC-3 cells. The levels of cyclin B1 in MSC and NRP-152 cells are very low. In contrast, the levels of CDK1 are similar between the different prostate cancer and NRP-152 cell lines and very low in MSC cells. These results indicate that the levels of cyclin B1 protein correlate with the ability of 2-ME, docetaxel, and flavopiridol to induce apoptosis in prostate cancer cells and in nontransformed cells.

**Higher Expression of Cyclin B1 in LNCaP Cells Increases Apoptosis Induced by Chemotherapy**

To determine if increased cyclin B1 protein has an effect on apoptosis induced by chemotherapy, we isolated three stably transfected LNCaP clones (LNCaP/B1-15, 23, and 4-1) with increased cyclin B1 protein compared with negative control LNCaP cells (Fig. 2A). To determine if the LNCaP/B1 clones are more sensitive to apoptosis compared with LNCaP/Neo cells, we treated cells with low doses of 2-ME (1 and 2 μM). Western blot analysis indicated a significantly greater cleavage of intact PARP to an 85-kDa fragment and an increase in cleaved caspase-3 (measure of apoptosis) in the LN/B1-23 cells compared with the LNCaP/Neo cells (Fig. 2B). This result was also confirmed by DAPI analysis (Fig. 2C). Similar results were obtained when LNCaP/B1 clones 15, 23, and 4-1 were treated with low doses of docetaxel (0.5 and 1 nmol/L), flavopiridol (250 nmol/L), doxorubicin (0.25 μmol/L), and camptothecin (10 μmol/L; result not shown). Therefore, these results suggest that higher cyclin B1 protein levels can sensitize LNCaP prostate cancer cells to apoptosis induced by a variety of chemotherapeutic drugs.

**Higher Expression of Cyclin B1 in PC-3 Androgen-Independent Prostate Cancer Cells Increases Apoptosis Induced by 2-ME**

We investigated in PC-3 cells whether higher expression of cyclin B1 can also sensitize androgen-independent prostate cancer cells to increased apoptosis induced by chemotherapy. PC-3 cells are aggressive undifferentiated androgen-independent prostate cancer cells that are resistant to apoptosis induced by chemotherapy (29). Because most androgen-independent prostate cancer cells in humans express AR, we used PC-3/AR cells that stably express AR (30, 39). Interestingly, PC-3/AR cells express 4- to 5-fold greater cyclin B1 protein compared with PC-3/Neo and parental cells (Fig. 3A). PC-3/AR cells undergo greater apoptosis when treated with 5 μmol/L 2-ME, as determined by Western blot (cleaved PARP and caspase-3; Fig. 3B) and DAPI analysis (not shown). To determine if PC-3/AR cells are more sensitive to apoptosis induced by 2-ME because of greater expression of cyclin B1 protein, we isolated stably transfected PC-3 clones expressing higher cyclin B1 protein. The results show that the PC-3/B1-8 clone expresses 2- to 3-fold greater cyclin B1 compared with PC-3/Neo cells and undergoes greater apoptosis when treated with 5 μmol/L 2-ME (Fig. 3B). These results suggest that in androgen-independent LNCaP cells, higher cyclin B1 protein levels can sensitize androgen-independent PC-3 cells to apoptosis induced by 2-ME.

**Reduction of Cyclin B1 Protein with siRNA Decreases Apoptosis Induced by 2-ME**

To determine if reduction of cyclin B1 protein has an effect on apoptosis induced by 2-ME, we transiently
transfected siRNA specific for cyclin B1 into LN-AI cells. Cyclin B1 protein was reduced 7-fold compared with LN-AI cells transfected with control siRNA (Fig. 4A). In the presence of cyclin B1 siRNA, there was greater intact PARP in 2-ME–treated LN-AI cells compared with control siRNA (Fig. 4B). There was no effect on PARP cleavage in control treated cells in the presence of cyclin B1 or control siRNA. These results suggest that lowering cyclin B1 protein levels decreases apoptosis induced by 2-ME in LN-AI prostate cancer cells.

To further support a role for cyclin B1 in apoptosis induced by 2-ME and docetaxel, we isolated three stably transfected cyclin B1 siRNA LNCaP clones (LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29) that express 3- to 50-fold less cyclin B1 protein compared with negative control LNCaP cells (Fig. 5A). The results indicated that there was slightly less cleaved PARP and caspase-3 in LNCaP/B1siRNA clones 3, 27, and 29 treated with 5 μmol/L 2-ME compared with control and 10 nmol/L docetaxel compared with negative control LNCaP cells (Fig. 5C). These results provide further evidence that lowering cyclin B1 protein levels in LNCaP cells reduces apoptosis induced by 2-ME and docetaxel.

**Dominant Negative CDK1 Reduces Apoptosis Induced by 2-ME**

Our previous results suggest that the increase in cyclin B1–dependent kinase activity mediated by 2-ME and docetaxel is required for induction of apoptosis in prostate cancer cells (27, 28). Cyclin B1 associates with CDK1 to form the active kinase complex that is the key initiator of mitosis (2). To further investigate the role of deregulated CDK1 in apoptosis induced by 2-ME, we isolated two stably transfected LN-AI clones overexpressing dominant negative CDK1 (LN-AI/dnCDK-8 and LN-AI/dnCDK-13; Fig. 6A). The results indicated that there was less cleaved PARP and apoptosis in LN-AI/dnCDK1 clones 8 and 13 treated with 2-ME compared with a LN-AI negative control (Fig. 6B and C). These results suggest that inhibition of CDK1 activity lowers apoptosis induced by 2-ME and supports the hypothesis that deregulated cyclin B1-CDK1 activity is important for apoptosis induced by chemother-apy in prostate cancer cells.

**Overexpression of Cyclin B1 Decreases Bcl-2 and Increases p53 in LNCaP Cells**

To investigate why LNCaP cells overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy, we sought to identify differences in the levels of proteins important for apoptosis. Cancer cells resistant to chemotherapeutic drugs often overexpress Bcl-2 and Bcl-xL, which act on the mitochondrial membrane to prevent caspase activation by interfering with cytochrome c release (40). In addition, overexpression of inhibitors of apoptosis (IAP) family members like XIAP and survivin blocks apoptosis and increases drug resistance (41). p53, the most commonly mutated gene in human cancers, can also mediate the apoptosis response to chemotherapy (42). The results showed that LNCaP/B1 clones expressed 7- to 15-fold less Bcl-2 protein and 4- to 6-fold more p53 protein compared with LNCaP/Neo negative control cells (Fig. 7). There were no changes in the levels of Bcl-xL, Bax, or XIAP.
These results suggest that LNCaP cells overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy because they express less Bcl-2 and more p53 proteins.

Overexpression of Cyclin B1 Decreases Neuroendocrine and Epithelial Differentiation in LNCaP Cells

To investigate the effect of overexpressing cyclin B1 on differentiation of prostate cancer cells, we did Western blot analysis of epithelial and neuroendocrine proteins. The normal prostate epithelium consists of secretory luminal, basal, and rare neuroendocrine cells. Intermediate cells coexpressing luminal, basal, and/or neuroendocrine cell proteins have been identified in normal adult prostate and in prostate cancer (43, 44). Studies have shown that hormonal therapy induces neuroendocrine differentiation of prostate cancer, which may contribute to progression into chemotherapy-resistant androgen-independent prostate cancer (45). LNCaP cells are an example of a luminal/neuroendocrine intermediate prostate cancer cell (46). Our results showed that, compared with LNCaP/Neo cells, LNCaP/B1 clones 23, 15, and 4-1 expressed lower levels of neuron-specific enolase and synaptophysin, both being markers of neuroendocrine cells (Fig. 8A). Similar to DU-145 and PC-3 androgen-independent prostate cancer cells, which are more undifferentiated compared with LNCaP cells, there was a decrease in intact and an increase in fragments of the epithelial marker E-cadherin (47) in the LNCaP/B1-23 and LNCaP/B1-15 clones but no changes in the levels of cytokeratins and AR (Fig. 8A and B). E-cadherin fragments have previously been reported in prostate cancer and may be a mechanism to reduce protein levels (48). These results suggest that an overexpression of cyclin B1 decreases neuroendocrine and epithelial differentiation in LNCaP cells.

Figure 5. Stable LNCaP cyclin B1 siRNA clones expressing lower cyclin B1 protein are less sensitive to apoptosis induced by 2-ME and docetaxel. A, three stably transfected cyclin B1 siRNA LNCaP clones (3, 27, and 29) expressing lower cyclin B1 protein compared with a LNCaP clone expressing negative control (NC) siRNA, as determined by Western blot. B, slightly less cleavage of PARP and caspase-3 in LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29 compared with negative control LNCaP cells when treated with 5 μmol/L 2-ME for 48 h, as determined by Western blot. Coomassie blue stains of total proteins are loading controls. C, percentage of apoptotic cells determined by DAPI in LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29 compared with negative control LNCaP when treated with 5 μmol/L 2-ME for 48 h, as determined by DAPI. Coomassie blue stains of total proteins are loading controls. Columns, mean; bars, SD. These results suggest that LNCaP cells overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy because they express less Bcl-2 and more p53 proteins.

Figure 6. Increased expression of dominant negative CDK1 reduces apoptosis induced by 2-ME in LN-AI cells. A, two stably transfected dominant negative CDK1 LN-AI clones (8 and 13) overexpress CDK1 compared with LN-AI clone transfected with the negative control pCMV/Neo plasmid (C), as determined by Western blot. B, less cleaved PARP in LN-AI/dnCDK1-8 and LN-AI/dnCDK1-13 compared with LN-AI/cmv negative control cells when treated with 2 μmol/L 2-ME for 72 h. Coomassie blue stains of total proteins are loading controls. C, percentage of apoptotic cells determined by DAPI in LN-AI/dnCDK1-8 and LN-AI/dnCDK1-13 compared with LN-AI/cmv negative control cells treated with 2 μmol/L 2-ME for 72 h. Expression of dominant negative CDK1 in LN-AI/dnCDK1-8 cells lowers apoptosis induced by 2-ME compared with LN-AI/cmv cells (n = 6, three independent experiments; *, P < 0.001, Student’s t test). Columns, mean; bars, SD.
Discussion

Overexpression of cyclin B1 is associated with transformed cells and is a marker of poor prognosis for a variety of cancers (8, 9). Our results in prostate cancer cells indicate a positive correlation between the levels of cyclin B1 protein and apoptosis induced by chemotherapeutic drugs. The higher the expression of cyclin B1 protein, like in LNCaP and LN-AI prostate cancer cells, the greater the induction of apoptosis by 2-ME, docetaxel, or flavopiridol. In nontransformed human MSC and rat NRP-152 cells, there is minimal expression of cyclin B1 and essentially no induction of apoptosis by 2-ME and docetaxel. In nontransformed cells, the overexpression of cyclin B1 protein is less sensitive to induction of apoptosis by chemotherapy, whereas clones that decrease cyclin B1 protein or overexpress dominant negative CDK1 are less sensitive to apoptosis. Among possible mechanisms for increased sensitivity to apoptosis in LNCaP clones overexpressing cyclin B1 may be lower levels of the apoptosis inhibitor Bcl-2 and higher levels of the apoptosis promoter p53. In addition, an overexpression of cyclin B1 in LNCaP cells decreases neuroendocrine differentiation, which is associated with resistance to chemotherapy and poor patient outcome (45). Overall, our results suggest that despite its association with transformed cells, higher levels of cyclin B1 protein in prostate cancer may be a good prognostic marker for chemotherapy.

Immunohistochemical analysis has shown that overexpression of cyclin B1 is a marker for poor prognosis in non–small-cell lung and head and neck carcinomas (8, 9). However, cyclin B1 is not a useful prognostic marker for gastric and colorectal carcinomas (49, 50). In prostate cancer, the results are less clear. In one study, the ratio of cyclins B1 and A to Ki67, considered to be associated with proliferating cells, was correlated with better patient outcome by delaying the recurrence, whereas another study showed that cyclin B1 did not correlate with the outcome (3, 11). Several gene microarray studies, however, suggest that high cyclin B1 mRNA correlates with poor patient outcome (12–14). A recent study in follicular lymphoma shows that high cyclin B1 protein correlates with better response to chemotherapy compared with low cyclin B1 (10). The standard chemotherapy combination for follicular lymphoma includes vincristine, which is a microtubule inhibitor and causes G2-M cell cycle arrest, similar to 2-ME and docetaxel. Therefore, an increase in expression of cyclin B1 protein may promote greater induction of apoptosis by microtubule inhibitors and result in a better patient outcome. It has also been suggested that rapidly proliferating cancer cells respond better to

![Figure 7](image_url)

**Figure 7.** Overexpression of cyclin B1 in LNCaP cells decreases Bcl-2 and increases p53 proteins. The levels of Bcl-2, Bcl-xL, Bax, XIAP, and p53 (proteins important in apoptosis) in LNCaP/B1-23, 15, and 4-1 cyclin B1–overexpressing clones without drug treatment were determined by Western blot, normalized to Coomassie blue–stained protein (not shown), and compared with LNCaP/Neo negative control cells (C). There is a decrease in Bcl-2 and an increase in p53 protein in cyclin B1–overexpressing clones compared with negative control cells. No significant differences were notable in Bcl-xL, Bax, and XIAP proteins.

![Figure 8](image_url)

**Figure 8.** Overexpression of cyclin B1 in LNCaP cells decreases neuroendocrine and epithelial differentiation. A, the levels of differentiation markers neuron-specific enolase (NSE), synaptophysin (Syp), E-cadherin (E-cad), cytokeratins (CK), and AR in LNCaP/B1-23, 15, and 4-1 cyclin B1–overexpressing clones without drug treatment were determined by Western blot, normalized to Coomassie blue–stained protein (not shown), and compared with LNCaP/Neo negative control cells (C). There is a decrease in neuron-specific enolase and synaptophysin in cyclin B1–overexpressing clones compared with negative control cells. In LNCaP/B1 clones 23 and 15, there is a decrease in 120-kDa E-cadherin (arrow) but an increase in E-cadherin fragments (~80, 70, and 65 kDa). No significant differences were notable in cytokeratin and AR proteins when normalized to total proteins. B, Western blot showing lower 120-kDa E-cadherin in DU-145 and PC-3 androgen-independent prostate cancer cells compared with LNCaP, LN-Al, and LN-Al/CSS cells. Similar to LNCaP/B1-23 and 15 cells, there is an increase in E-cadherin fragments in DU-145 and PC-3 cells.
apoptosis induced by chemotherapy. However, our results suggest this is not the case in prostate cancer because 2-ME, docetaxel, and flavopiridol induced less apoptosis in the more rapidly proliferating DU-145 and PC-3 cells. In addition, MSC and NRP-152 cells are also rapidly proliferating cells, but 2-ME and docetaxel induce very little apoptosis (Fig. 1). We suggest that 2-ME and docetaxel induce apoptosis in prostate cancer cells and not in normal cells because of increased expression of cyclin B1 protein. Additional trials will be required to determine if a higher expression of cyclin B1 protein correlates with a better response to chemotherapy in prostate cancer.

In the HeLa cervical carcinoma cell line, the lowering of cyclin B1 protein with siRNA results in an increase in apoptosis, even without any drug treatment, suggesting a potential gene therapy strategy (20–22). In contrast, our results showed that lowering cyclin B1 protein in LNCaP or LN-AI prostate cancer cells did not increase apoptosis without drug treatment (Fig. 4). There may be distinct differences between cancer cell types because lowering cyclin B1 protein will signal pathways leading to apoptosis in cervical carcinoma cells, but not in prostate cancer cells. In addition, it is often the case that treatment strategies that are effective in one type of cancer may be ineffective in another type. Therefore, gene therapy strategies that reduce cyclin B1 protein are not likely to be effective for all types of cancer.

The molecular mechanisms of cyclin B1 overexpression in cancer and restricted to minimal levels in proliferating normal cells are not clear. It is likely that one of the mechanisms in cancer cells is by deregulation of the cyclin B1 promoter so that there is a greater initiation of transcription (51). Another possible mechanism in cancer cells is the inhibition of cyclin B1 protein degradation, which is critical for progression through mitosis (2). Our results in prostate cancer showed that when androgens were removed and LN-AI cells converted into LN-AI/CSS cells, cyclin B1 protein levels decreased (Fig. 1). In addition, cyclin B1 protein levels are lower in DU-145 and PC-3 cells, which do not express AR and are not responsive to androgens. Interestingly, in PC-3/AR cells, which stably express AR, cyclin B1 protein levels are 4- to 5-fold higher than in PC-3/Neo or parental cells (Fig. 3A). These results suggest that in prostate cancer cells, AR and androgen signaling may increase cyclin B1 protein levels. Whether this is the result of increasing cyclin B1 transcription and/or decreasing protein degradation is currently being investigated in our laboratory. Positive androgen regulation of cyclin B1 mRNA and protein has previously been shown in the CWR22 xenograft model of prostate cancer (52). Other reports have shown that p53 can negatively regulate cyclin B1 promoter and is important in G2 checkpoint control (53, 54). Our results, however, indicate that overexpression of cyclin B1 in LNCaP cells (wild-type p53) increases p53 protein levels (Fig. 7). In addition, cyclin B1 protein is lower in DU-145 and PC-3 cells, which contain mutations in p53 that render it nonfunctional (29). Therefore, wild-type p53 in prostate cancer cells may increase expression of cyclin B1, although the pathways of how this occurs are not known.

The mechanisms how chemotherapeutic drugs that deregulate (2-ME and docetaxel) or inhibit (flavopiridol) cyclin B1-CDK1 activity can induce apoptosis in cancer cells are poorly understood. Changes in the phosphorylation status of key CDK1 substrates mediated by chemotherapy may be important in the induction of apoptosis. For example, CDK1 phosphorylates the proapoptotic protein BAD to mediate apoptosis in primary neurons (55). However, mutation of the CDK1 phosphorylation site of BAD suggests that it has no role in paclitaxel-mediated apoptosis in nonneuronal cells, including breast cancer (56). CDK1 can also phosphorylate and stabilize survivin, a member of the IAP family (57). The subsequent decrease in cyclin B1-CDK1 activity results in a decrease in the levels of survivin and an increase in sensitivity to induction of apoptosis. However, our previous results suggest that the initial 2-ME– and paclitaxel-mediated increase in cyclin B1–dependent kinase activity is more important than the subsequent decrease in activity for the induction of apoptosis (27). We have preliminary data suggesting that the 2-ME stimulation of cyclin B1 kinase can increase proapoptotic Bak1 and decrease prosurvival AKT in prostate cancer cells (not shown). Further work is required to determine if Bak1 and AKT are important for 2-ME–mediated apoptosis.

What is clear from our results is that the levels of cyclin B1 protein in prostate cancer cells are important for increasing apoptosis by chemotherapy, although the exact downstream mechanisms are not known. One of the proposed mechanisms in LNCaP cells is a decrease in Bcl-2 protein in cyclin B1–overexpressing clones (Fig. 7). This may facilitate the release of mitochondrial proteins to initiate the process of apoptosis through activation of caspase-3 and PARP cleavage (40). Another potential mechanism in LNCaP cells is the increase in wild-type p53 protein in cyclin B1–overexpressing clones (Fig. 7), which can mediate apoptosis induced by 2-ME, docetaxel, and flavopiridol. It is not known whether the overexpression of cyclin B1 has a direct effect on the Bcl-2 and p53 gene promoters and/or protein stability or if it is a consequence of an effect on cell differentiation. Interestingly, an overexpression of cyclin B1 decreases neuroendocrine differentiation in LNCaP cells as shown by lower levels of neuron-specific enolase and synaptophysin (Fig. 8A). Neuroendocrine cells in prostate cancer have been shown to express higher levels of Bcl-2 protein and are known to be more resistant to chemotherapy (58, 59). In addition, a higher neuroendocrine differentiation in patients correlates with poor outcome (45). Therefore, higher levels of cyclin B1 in LNCaP cells promote a cell phenotype that has less neuroendocrine differentiation and is more responsive to chemotherapy. However, a higher level of cyclin B1 also seems to have an effect on lowering epithelial differentiation by decreasing the levels of E-cadherin. This may reflect the association of higher cyclin B1 with a more undifferentiated prostate cancer cell phenotype.
Anticancer chemotherapeutic agents ideally should take advantage of the molecular differences between transformed and normal cells and induce apoptosis only in cancer cells. Our studies suggest that an overexpression of cyclin B1 protein in cancer cells is a reason for the greater induction of apoptosis by 2-ME and docetaxel in malignant but not in normal cells. Our studies also indicate that increased expression of cyclin B1 in prostate cancer cells can mediate a greater induction of apoptosis by chemotherapy. Therefore, it is possible that evaluating the cyclin B1 protein levels in patients with prostate cancer will be an important prognostic marker for use of chemotherapy. However, larger clinical trials will be required to determine if chemotherapy can increase survival in patients with greater cyclin B1 protein.

Acknowledgments

We thank Dr. Andrew Schally for the review of this manuscript and helpful suggestions; Dr. Gianluca D’Ippolito for MSC cells; Dr. Jonathan Pines for cyclin B1 expression plasmid; Dr. Sander van den Heuvel for dnCDK1 suggestions; Dr. Gianluca D’Ippolito for MSC cells; Dr. Jonathan Pines for cyclin B1 expression plasmid; and Ricardo Parrondo for technical assistance.

References


