Cancer Therapy: Preclinical

Natura-Alpha Targets Forkhead Box M1 and Inhibits Androgen-Dependent and -Independent Prostate Cancer Growth and Invasion

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Abstract

Purpose: The development of new effective therapeutic agents with minimal side effects for prostate cancer (PC) treatment is much needed. Indirubin, an active molecule identified in the traditional Chinese herbal medicine—Qing Dai (Indigo naturalis), has been used to treat leukemia for decades. However, the anticancer properties of Natura-alpha, an indirubin derivative, are not well studied in solid tumors, particularly in PC.

Experimental Design: The growth kinetics and invasion ability of on human PC cell lines with or without Natura-alpha treatment were measured by cell proliferation and invasion assays. The antitumor effects of Natura-alpha were examined in nude mice tumor xenograft models, and in a patient with advanced hormone-refractory metastatic PC. Signal network proteins targeted by Natura-alpha were analyzed by using proteomic pathway array analysis (PPAA) on xenografts.

Results: Natura-alpha inhibited the growth of both androgen-dependent (LNCaP) and androgen-independent (LNCaP-AI, PC-3, and DU145) PC cells with IC50 between 4 to 10 mmol/L, and also inhibited invasion of androgen-independent PC cells. Its antitumor effects were further evident in in vivo tumor reduction in androgen-dependent and androgen-independent nude mice tumor xenograft models and reduced tumor volume in the patient with hormone refractory metastatic PC. PPAA revealed that antiproliferative and antiinvasive activities of Natura-alpha on PC might primarily be through its down-regulation of Forkhead box M1 (FOXM1) protein. Forced overexpression of FOXM1 largely reversed the inhibition of growth and invasion by Natura-alpha.

Conclusion: Natura-alpha could serve as a novel and effective therapeutic agent for treatment of both hormone-sensitive and hormone-refractory PC with minimal side effects. Clin Cancer Res; 17(13); 4414–24. ©2011 AACR.

Introduction

Prostate cancer (PC) is the most common cancer in men in the United States and was expected to cause 217,730 new cases and 32,050 deaths in 2010 (1). Androgen ablation is the most common therapy for advanced PC. The treatment failure of PC lies in the fact that, after androgen ablation therapy, the disease inevitably progresses from androgen-dependence to androgen-independence. For patients who are not cured by local treatment with ensuing metastases, neither androgen ablation nor chemotherapy can extend their survival time. Thus, the development of new effective therapeutic agents with minimal side effects is highly warranted.

Cancer is increasingly being viewed as a cell-cycle disease because deregulation in the cell-cycle machinery can be found in most cancers (2–4). Major components in the cell-cycle machinery are cyclin-dependent kinases (cdk) and their interacting partners, the cyclins and the endogenous inhibitors (e.g., cdki). Defects have been described in the components of the cell-cycle machinery itself, or the checkpoint components that ensure orderly advancement through the cell-cycle phases, or in upstream signaling that triggers cell-cycle events (5–6). Strategies have been developed and intensified in the last few years by directly or indirectly targeting cdks and these have been reviewed extensively (3, 7–9). The first two cdk
Proliferation and invasion by Natura-alpha. Further study indicated that the suppression of Forkhead Evaluation Criteria in Solid Tumors (RECIST; ref. 17). Approximately 26% by using guidelines of Response cancer achieved stable disease in response to Natura-alpha. The patient with hormone-refractory metastatic prostate advanced hormone refractory metastatic PC. Our findings revealed that antiproliferation and antiinvasion activities of Natura-alpha on PC might primarily be through its downregulation of Forkhead box M1 protein. This study provides comprehensive evidence to support that Natura-alpha could serve as a novel and effective therapeutic agent for treatment of both hormone-sensitive and hormone-refractory PC in near future with minimal side effects.

Translational Relevance
Prostate cancer (PC) is the most common cancer in men in the United States. The development of new effective therapeutic agents with minimal side effects for PC treatment is much needed. In this study, we show anti-PC properties of Natura-alpha in vitro, in vivo by using nude mice xenograft models, and in a patient with advanced hormone refractory metastatic PC. Our findings revealed that antiproliferation and antiinvasion activities of Natura-alpha on PC might primarily be through its downregulation of Forkhead box M1 protein. This study provides comprehensive evidence to support that Natura-alpha could serve as a novel and effective therapeutic agent for treatment of both hormone-sensitive and hormone-refractory PC in near future with minimal side effects.

Inhibitors. Flavopiridol and UCN-01, have been in clinical trials either alone, or in combination with other chemotherapeutic agents and have shown promising results with evidence of antitumor activity (10–12).

Indirubin, an active molecule identified in the traditional Chinese herbal medicine—Qing Dai (Indigo naturalis), has been used to treat leukemia for decades (13–14). In recent years, there has been a dramatic renewal of the interest in indirubin due to the discovery of its great pharmacologic potential (15). Increasing evidences show that indirubin, and its derivatives and analogs, target various important signal pathways involved in cancer, including inhibition of cdks (16). Natura-alpha (NTI-Onco2008-1), an indirubin derivative, shows an ability to arrest leukemia cells at G1 phase, inhibit expression of the oncogene c-Myb, and induce cell differentiation and maturation at low concentration, in which cell growth is completely inhibited without decrease in cell viability. At higher concentration, this agent blocks tumor cells at M–G2 phases.

To further evaluate its potential clinical application and to explore mechanisms of its anticancer action, in this study, we examined therapeutic activities of Natura-alpha on androgen-dependent and androgen-independent prostate cancer in vitro and in vivo, and in a patient with advanced hormone-refractory metastatic PC. Natura-alpha caused strong inhibition of cell proliferation and invasion in various human PC cell lines and tumor growth in nude mouse xenografts. Most importantly, the patient with hormone-refractory metastatic prostate cancer achieved stable disease in response to Natura-alpha with his liver metastatic tumors reduced by approximately 26% by using guidelines of Response Evaluation Criteria in Solid Tumors (RECIST; ref. 17). Further study indicated that the suppression of Forkhead box M1 (FOXM1) was the primary target of inhibition of proliferation and invasion by Natura-alpha.

Materials and Methods

Reagents
The chemical name of Natura-alpha is N-methyl-Δ3,3’-dihydroindole-2,2’-diketone. Natura-alpha was provided by Natrogen Therapeutics International, Inc. It was synthesized under cGMP conditions, and structure confirmed by IR, NMR, and mass spectrometry with a purity of 98.00% or more.

Cell culture and cell proliferation assays
LNCaP and DU145 cells were maintained in RPMI 1640 (Gibco and PC3 cells were cultured in 50% RPMI 1640 and 50% F2 Gibco) with 10% heat-inactivated FBS. The androgen-independent LNCaP-AI cells, a derivative of LNCaP (18–20), were maintained in RPMI 1640 medium containing 10% charcoal-stripped, heat-inactivated FBS (CSFBS; Hyclone Laboratories, Inc.) and 5 μg/mL of insulin, as described previously (18). Cell proliferation was determined by MTT as described previously (21–22). Anchorage-independent cell growth in soft agar was done in triplicate with cells (1 × 10⁴) suspended in 2 mL of medium containing 0.35% agar (Becton Dickinson) spread on top of 5 mL of 0.7% solidified agar (23). Colony volume was calculated from the average radius of representative colonies.

Matrigel invasion assays
Effect of Natura-alpha on invasive activity of LNCaP and LNCaP-AI cells was determined via BD Matrigel invasion assay (Growth Factor Reduced Matrigel Invasion Chamber) as described (24). After rehydration of the insert with medium for 2 hours, LNCaP and LNCaP-AI cells at their exponential growth phases were added to the upper chamber at the density of 1 × 10⁶ cells in 500 μL medium in the presence or absence of indicated concentration of Natura-alpha and incubated at 37°C for 48 hours. Data were adjusted by growth condition and expressed as mean of migrating cells in 3 fields ± SD.

Western blot analysis
Whole cell or cell fraction extracts were subjected to SDS-PAGE and transferred to a nitrocellulose membrane for Western blot analysis. Blots were incubated with primary antibodies including FOXM1, cyclin D1, cyclin B, cyclin E, and β-actin (Cell Signaling Technology, Inc.) for 2 hours at room temperature, washed with TBS-T, and incubated for 1.5 hours with horseradish peroxidase-conjugated secondary antibody (1:5,000; Amersham Biosciences). The protein bands were detected by an enhanced chemiluminescence kit (Amersham Biosciences).

Nude mice xenografts
Androgen-dependent LNCaP and androgen-independent LNCaP-AI PC cells, mixed with Matrigel (Becton Dickinson) at a ratio of 1:1 were inoculated into the bilateral flanks of 4- to 5-week-old male Nu/Nu BALB/c athymic nude mice by s.c. injection. The tumor growth and
volume were monitored every 3 days. When the tumor grew to a diameter of 4 to 8 mm (4–5 weeks), animals were randomly divided into 2 groups, 10 mice each, according to tumor size. One group of animals was treated with drug vehicle only as control, and another group was treated with Natura-alpha at dose of 100 mg/kg by gavage, once a day, 5 days a week until the diameter of tumors in control group reached approximately 15 mm. The tumor growth was monitored daily and tumor size recorded every 3 days. The tumor volume was calculated as $l \times d \times h \times 0.52$ (25).

Proteomic pathway array analysis

Total cellular proteins were extracted from xenograft tumors (3 mm$^3$) by using a lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 20 mmol/L sodium pyrophosphate, 40 mmol/L B-glycerophosphate, 30 mmol/L sodium fluoride, 2 mmol/L EGTA, 10 mmol/L NaCl, and 0.5% NP-40. The lysate was sonicated 3 times for 15 seconds each time, and then centrifuged (14,000 rpm, 30 minutes, and 4°C). The tubes were kept on ice throughout the process. The protein concentration was determined with the BCA Protein Assay Kit (PIERCE). Isolated proteins were separated by SDS-PAGE (10% acrylamide). Three hundred micrograms of protein extracts were loaded in a well across the entire width of gel for SDS-PAGE, followed by electrotransferring to a nitrocellulose membrane. The membrane was then blocked for 1 hour with 5% milk or 3% bovine serum albumin and clamped on to a Mini-PROTEAN II Multiscreen apparatus that isolates 20 channels across the membrane (Bio-Rad). Two or 3 antibodies were added to each channel and incubated overnight at 4°C. Different sets of antibodies were used for each membrane after stripping the previous set of antibodies. Antibodies were purchased either from Cell Signaling Technology, Inc., or from Santa Cruz Biotechnology, Inc. (Supplementary Table S1). The pathway array analysis was run in duplicate for each sample in each set of antibodies and protein levels were normalized by using β-actin and glyceraldehyde-3-phosphate dehydrogenase as standards. Chemiluminescence signals were captured by using the ChemiDoc XRS System. Differences in protein levels were determined by densitometric scanning and normalized to internal standards.

FDA- and IRB-approved single patient clinical trial

An 86-year-old patient with advanced androgen-independent metastatic PC was consented for the Natura-alpha trial therapy for his disease with approval from the Food and Drug Administration (FDA; IND# 104191) and Institutional Review Board (IRB; IntegReview NTI-2007-1-PC). Natura-alpha was administered orally with increasing dose (Dm), and normalized to internal standards.

Statistical analysis

The growth inhibition (1 – T/C, Effect), median effect dose (Dm), and combination indexes (CI) were calculated and analyzed by using the computer program, CalcuSyn, of Biosoft edited by T.C. Chou, Memorial Sloan-Kettering Cancer Center, New York, and M.P. Hayball (Biosoft; refs. (21, 26). The CI was used to evaluate the results of the combinations. A CI greater than 1 indicates the combination is antagonistic, CI equal to 1 indicates the combination is additive, and CI smaller than 1 indicates that the combination is synergistic (26).

Results

Effects of Natura-alpha on PC growth and invasion in vitro

A response of different human PC cells to the treatment of Natura-alpha was obtained by using colorimetric MTT and/or SRB methods after 3 days of exposure. Table 1 shows the IC$_{50}$ of Natura-alpha on hormone-dependent and hormone-independent human PC cell lines ranged between 3.96 to 9.39 μmol/L. IC$_{50}$ data pertain to the concentration of the tested drug that inhibits 50% cell growth in in vitro growth kinetic studies. The data of Table 1 are mean ± SD from 3 independent experiments.

To explore if Natura-alpha is able to enhance activity of clinically available chemotherapeutic drugs used for PC, the commonly used antimicrotubule agent, paclitaxel (Taxol), was combined with Natura-alpha in 3 different sequential exposures. In the first combination, LNCaP-AI cells were treated with Natura-alpha and Taxol simultaneously for 6 days. In the second combination, the cells were treated with Natura-alpha for the first 3 days followed by treatment with Taxol for additional 3 days (NTI → Taxol). In the third regimen, the cells were treated with Taxol for the first 3 days followed by treatment with Natura-alpha for additional 3 days (Taxol → NTI). Exposure of the cells to either Natura-alpha or Taxol alone served as controls. After treatment, cell growth was determined by MIT assays with the growth inhibition (1 – T/C, Effect), Dm, and CI calculated and analyzed as described in the Materials and Methods.

The concurrent combination of Natura-alpha with the antimicrotubule agent, Taxol, achieved a strong synergistic effect on LNCaP-AI PC growth. For example, the calculated Dm of Natura-alpha and Taxol alone against LNCaP-AI cells was found to be approximately 7.54 μmol/L and 41.57 nmol/L, respectively. However, when the 2 drugs were applied to the cancer cells simultaneously at the ratio of

| Table 1. IC$_{50}$ of Natura in hormone-dependent and hormone-independent human cancer cell lines |
|-----------------|---------------------|
| **Cell lines**  | **IC$_{50}$**       |
| LNCaP           | 4.54 ± 0.73         |
| LNCaP-AI        | 6.89 ± 0.57         |
| DU145           | 9.39 ± 0.23         |
| PC-3            | 3.96 ± 0.58         |


1,000:1 (Natura-alpha:Taxol), Dm was significantly reduced to 0.78 μmol/L and 0.78 nmol/L, respectively (Supplementary Table S2) and CI was well below 1 (Fig. 1A and B). One-way ANOVA test showed \( P < 0.001 \) indicating that there is statistically significant difference between the combination and the 2 individual agents.

Interestingly, the effects of the combination are highly dependent on the sequence of the drug exposure indicated by the CI (Fig. 1) and Dm values (Supplementary Table S2). A strong synergistic growth inhibitory effect of LNCaP-AI cells was achieved when the cancer cells were exposed to Natura-alpha and Taxol concurrently (Fig. 1A and B), in which case CI at each concentration points were well below 1, whereas only a moderate synergism was observed when the cells were treated with Natura-alpha first for 3 days followed by Taxol treatment for additional 3 days.

Figure 1. Effects of Natura-alpha (NTI) and Taxol on cell growth of androgen-independent PC (LNCaP-AI) with different treatment regimens. A, C, and E are dose–effect curves, and B, D, and F are CI–effect curves. Dash line in B, D, and F (CI = 1) is the divider of outcomes of 2-drug combination. CI value above the dash line indicates an antagonistic effect, below the line is a synergistic effect, and on the line implies an additive effect. A regression analysis of CI values versus effects in B, D, and F were done by Sigma plot 8, and the line direction indicates trend of a combination. Androgen-independent LNCaP-AI cells were treated simultaneously with NTI and Taxol (A and B); C and D (combination 2, NTI → Taxol); the cells were exposed to NTI first for 3 days followed by Taxol treatment for additional 3 days; E and F (combination 3, Taxol → NTI): the cells were treated with Taxol first for 3 days followed by NTI treatment for additional 3 days.
days. Notably, the trend of the combination became antagonistic when the cancer cells were exposed to Taxol for the first 3 days followed by exposure to Natura-alpha for an additional 3 days (Fig. 1E and F). Similar results were also obtained in LNCaP cells (data not shown).

Growth inhibition of Natura-alpha on PC cells was further supported by anchorage-independent assay. Although both LNCaP and LNCaP-AI cells could easily form colonies in soft agar in the absence of Natura-alpha (Fig. 2A), LNCaP-AI cells showed stronger capacity of colony formation. However, the colony formation of both LNCaP and LNCaP-AI cells was significantly inhibited by Natura-alpha (final concentration 2.5 μmol/L) as reflected by remarkable decrease in numbers and size of colonies under the same experimental conditions (Fig. 2B).

To examine whether Natura-alpha inhibits the invasive potential of PC cells, invasive activity of LNCaP and LNCaP-AI cells was determined via the BD Matrigel invasion assay. Results showed that invasive capacity of LNCaP cells was highly limited. Only a few cells migrated (data not shown). In contrast, LNCaP-AI cells showed strong invasive potential. More than 4,000 cells invaded per hpf during 48 hours culture in the presence of androgen. Interestingly, the invasive capacity of LNCaP-AI cells was strongly blocked by Natura-alpha in a concentration-dependent manner. Inhibition of invasive LNCaP-AI cells reached more than 87% and 99% at concentrations of 2.5 and 5.0 μmol/L of Natura-alpha, respectively (Fig. 2C).

**Inhibition of prostate tumor growth in vivo by Natura-alpha**

In an androgen-dependent (LNCaP) xenograft model, PC cells were injected s.c. into the flank region of male nude mice. When the prostate tumor grew for 4 to 5 weeks (20 to 30 mm$^3$), animals were randomly divided into 2 groups, 10 animals each, according to tumor size. A suspension of Natura-alpha was given at dose of 100 mg/kg by gavages once a day for 5 days a week. Mice fed with equal volume of solution of 0.05% Tween 20 in water (a solution used in preparing Natura-alpha suspension) served as vehicle controls. The tumor size was measured every 3 days, and tumor growth curves (tumor size vs. time) were plotted. As shown in Figure 3A and B, treating with Natura-alpha, starting at week 5, slowed tumor growth compared with the control group. By week 6, tumor growth in the Natura-alpha–treated group almost completely halted, whereas tumors in the vehicle-treated group continued to grow. Continued feeding with Natura-alpha not only completely halted tumor growth but significantly reduced the tumor volume. For example, on day 78, the average tumor volume of tumors in the Natura-alpha–treated group was reduced by 53% ($P = 0.035$). In addition, after dissection, tumor weight from the Natura-alpha–treated group was reduced about 6-fold as compared with the control group ($P = 0.001$) and HR is 0.168 (Fig. 3C).

To determine the effects of Natura-alpha on androgen-independent PC, we developed a xenograft model by using androgen-independent LNCaP-AI cells, with castration or sham castration. After 4 weeks of prostate tumor growth (20–30 mm$^3$ in size), animals were castrated or sham castrated and randomly divided into 4 groups, 10 animals each, on the basis of tumor size. Groups A and B consist of castrated mice fed with Natura-alpha or with equal volume of vehicle as control, respectively. Groups C and D consist of sham-castrated mice fed with Natura-alpha or with equal volume of vehicle as control, respectively. A suspension of Natura-alpha or equal volume of vehicle was given at dose of 100 mg/kg by gavage, once a day and 5 days a week starting on day 28.

As shown in Figure 3D–I, tumor volumes of castrated or sham-castrated mice from both vehicle groups showed constant growth. In contrast, the growth of tumors in

![Figure 2](https://example.com/figure2.jpg)
the Natura-alpha–treated group was much slower. The reduction of tumor volume between Natura-alpha and the vehicle-treated group was found to be statistically significant (Fig. 3E, \( P = 0.019 \) and Fig. 3H, \( P = 0.026 \)) starting at week 7. The tumor weight from the Natura-alpha–treated group was reduced approximately 2.33-fold (castrated group; Fig. 3F) and 2.6-fold (sham-castrated group; Fig. 3I) as compared with the control group. The HRs are 0.429 (castrated group) and 0.385 (sham-castrated group), respectively.

In an effort to determine whether Natura-alpha would prevent tumor growth, we fed mice with Natura-alpha 2 weeks before LNCaP-AI cell injection. After tumor cell injection, the mice were fed continuously with Natura-alpha until sacrifice. As shown in Supplementary Figure S1, tumor growth from the prefeeding group mice stopped by week 3 and did not grow any further. Tumor volume from the prefeeding group was reduced more than 3.5-fold as compared with that of the vehicle control group (\( P < 0.001 \)). In addition, prefeeding reduced tumor volume almost 2-fold when compared with that of mice fed with Natura-alpha beginning at week 5 postinjection (\( P < 0.001 \)).

**Natura-alpha reduces tumor burden in a patient with hormone-refractory metastatic PC**

An 86-year-old patient with advanced hormone refractory and metastatic PC (liver metastasis confirmed by...
biopsy) who had failed previous chemotherapy, was put on Natura-alpha therapy for his disease with permission from the FDA with 3 treatment cycles (4 weeks per cycle). During the 3 treatment cycles allowed by IRB, laboratory tests and imaging examinations have been done at the end of each treatment cycle (Supplementary Table S3).

**Biological response.** The value of alkaline phosphatase (APL) generally decreased during treatment period. For example, on December 28, 2008, it was 377 U/L, and it decreased to 123 U/L on March 30, 2009. The decrease of APL may reflect improvement of liver and bone metastases. There was, however, no significant improvement in his serum PSA after Natura-alpha treatment. Serum PSA initially was at 270 ng/mL on January 2, 2009, decreased to 160 ng/mL on January 20, 2009 and elevated to 294 ng/mL on March 20, 2009.

**Assessment of target response.** Target response was evaluated by anterior and posterior whole body images (bone scan) and a CT scan of the chest, abdomen, and pelvis at the end of each cycle. These studies showed that overall tumor burden was reduced. Using the "Guidelines to evaluate the RECIST" (17), 5 liver metastatic tumors at end of third cycle were compared with their baseline before Natura-alpha treatment. Multiple metastatic lesions within the liver were unchanged in number but decreased in size. As shown in Supplementary Figure S2, a 26% decrease in a sum of the longest diameters of 5 tumors was achieved, indicating Natura-alpha treatment stabilized the disease condition. A bone scan at the end of each cycle showed mostly unchanged as compared with the baseline before the study except for the following lesions in which the radiotracer uptake was slightly decreased; anterior left second rib, upper thoracic spine, and posterior upper ribs. Unfortunately, the patient expired 10 months after 3-cycle Natura-alpha treatment.

**Signal network proteins targeted by Natura-alpha by pathway array analysis on xenograft tumors**

To further explore the mechanism of tumor inhibition by Natura-alpha, we conducted proteomic pathway array analysis (PPAA) by using tumor samples from androgen-dependent LNCaP and androgen-independent LNCaP-AI xenografts with or without treatments of Natura-alpha.

PPAA showed that Natura-alpha significantly affects molecules involved in regulating cell proliferation and migration/invasion, or metastasis. Natura-alpha significantly (at least >2-fold) inhibited expression and activations of cdk, such as cdk2, cdk6, p-cdc2 Tyr15, and pRB Ser780, which confirmed our previous observations in vitro (16). As an inhibitor of cdkks, it seems that the inhibition of cdk activity of Natura-alpha (i.e., phosphorylation) was stronger than its reduction of protein expression. For example, only 2- to 3-fold decreases in levels of cdk2 and cdk6 were achieved, whereas almost complete inhibition of p-cdc2 Tyr15 was obtained by the compound. Natura-alpha showed little effects on expression of cyclin D1 and E. Another key cell-cycle regulator, FOXM1, however, was also significantly inhibited by Natura-alpha (Fig. 4).

Natura-alpha also significantly affected the expression of 2 important molecules, E-cadherin and Mesothelin, in LNCaP xenografts (Supplementary Fig. S3). These proteins are involved in adhesion, migration, and invasion/metastasis. Natura-alpha strongly upregulated expression of E-cadherin (<10-fold) while it considerably inhibited expression of Mesothelin (2-fold) in LNCaP xenograft tumors.

In addition, PPAA study also showed that Natura-alpha significantly (>2.5-fold) inhibited activations of various protein kinases, including p-PKCa, p-PKCδ, p-ERK, and p-p38. Because overactivation of these protein kinases has been shown to be involved in prostate tumor growth, progression, and drug resistance (27–29), inhibition of Natura-alpha on these protein kinases may also play an important role in suppressing tumor growth and metastasis. Moreover, p-ERK and p-p38 are also involved in lipo-polysaccharide-mediated inflammatory signaling (30), suggesting inhibition of activation of p-ERK and p-p38 may also play a role in the antiinflammatory activities of Natura-alpha.

**FOXM1 mediates inhibition of PC growth and invasion by Natura-alpha**

As mentioned above, the PPAA revealed that Natura-alpha significantly inhibited expression of cell-cycle regulator FOXM1. As shown in Figure 4A and B, expression of FOXM1 was reduced more than 3-fold by Natura-alpha in tumor samples from androgen-dependent LNCaP xenografts. Similarly, Natura-alpha also repressed expression of FOXM1 approximately 3-fold in tumor samples from androgen-independent LNCaP-AI xenografts (Fig. 4C and D). The PPAA results suggested that Natura-alpha could be an effective inhibitor of FOXM1 expression, resulting in repressing the FOXM1 pathway-mediated the tumor growth promotion.

Because repression of FOXM1 was seen in vitro from LNCaP and LNCaP-AI xenografts by Natura-alpha, we investigated in vitro expression of FOXM1 in LNCaP and LNCaP-AI cells. As shown in Figure 5A, endogenous FOXM1 was expressed in both LNCaP and LNCaP-AI cells, however, about 2-fold higher expression was seen in LNCaP-AI cells compared with LNCaP cells. Next, we examined the effects of Natura-alpha on FOXM1 expression in both LNCaP and LNCaP-AI cells by incubating these cells in media containing 5 μM/L Natura-alpha for 24 hours. FOXM1 expression was reduced more than 3-fold in both LNCaP and LNCaP-AI cells treated with Natura-alpha as compared with the control group (Fig. 5B panel 1 and 2). RT-PCR also revealed that Natura-alpha repressed FOXM1 expression at the transcriptional level (Fig. 5B panel 4 and 5).

To examine whether FOXM1 governs cell-cycle progression in both LNCaP and LNCaP-AI cells, we conducted FOXM1 knockdown by using siRNA and observed that cell cycle was arrested upon FOXM1 knockdown in both LNCaP and LNCaP-AI cells (Supplementary Fig. S4). This observation indicated that FOXM1 plays a key role in cell-cycle progression which is consistent with previous report (31).
To further explore whether Natura-alpha mediated repression of FOXM1 would cause cell-cycle arrest, stably transfected cell lines of LNCaP and LNCaP-AI with overexpression of FOXM1 were established by retrovirus system (Fig. 5C and E) and their proliferation rates were measured. Forced expression of FOXM1 was found to promote cell proliferation in both LNCaP and LNCaP-AI cell lines. Moreover, the overexpressed FOXM1 in both cell lines largely reversed the growth inhibition by Natura-alpha, indicating that repression of FOXM1 mediated by Natura-alpha was a primary cause of cell-cycle arrest by the compound (Fig. 5D and F).

Because invasion of LNCaP-AI cells was inhibited by Natura-alpha (see Fig. 1), we examined whether overexpression of FOXM1 played a role in the invasion of LNCaP-AI cells. As shown in Figure 5G and H, the overexpression of FOXM1 resulted in more than 2-fold increase in the invasion capability of LNCaP-AI cells as compared with the control (Fig. 5G column 1 and 3). Although Natura-alpha inhibited the invasion in both LNCaP-AI cells and FOXM1-overexpressed LNCaP-AI cells, the inhibitory effect of the compound on invasion, however, was diminished to some extent by the overexpression of FOXM1 (Fig. 5G column 2 and 4).

FOXM1 promotes cell-cycle progression at both G1/S and G2/M transitions, through regulating its direct target genes (cyclin B1, cyclin D1, cyclin E, and cdc23B) and indirectly regulated genes (cyclin A, cyclin F, cdc20, etc.; ref. 31). To further explore the mechanisms of Natura-alpha on inhibition of cell proliferation and invasion, we investigated expression of several downstream genes of FOXM1 in response to Natura-alpha treatment. We found that Natura-alpha slightly decreased the expression of cyclin D1 and cyclin E which is consistent with our PPAA results. Interestingly, Natura-alpha dramatically inhibited expression of FOXM1 direct targeted gene cyclin B1, indicating that Natura-alpha probably blocks cell-cycle progression through FOXM1-mediated downregulation of cyclin B1 (Fig. 5I).

Discussion

The development of new therapeutic drugs that simultaneously target both cell proliferation and inflammation would be ideal because it may not only suppress cancer growth but also prevent tumor metastasis. Natura-alpha has antiproliferative and antiinflammatory activities both in vitro and in vivo via inhibition of cdks and proinflammatory cytokines (16, 32). In this study, our data showed that Natura-alpha inhibited growth of PC cells LNCaP, LNCaP-AI, PC3, and DU145 by MTT and anchorage-independent assay. Matrigel assays also showed that Natura-alpha potentially inhibited invasive capability of cancer cells in vitro. In particular, a significant synergistic effect was also seen when Natura-alpha was concurrently combined with the antimicrotubule agent paclitaxel at a ratio of 1,000:1 (NTI-Onco2008-1:Paclitaxel) in androgen-dependent LNCaP and androgen-independent LNCaP-AI, indicating that Natura-alpha was able to enhance activity of a clinically available chemotherapeutic drug for PC.
Mechanisms by which different sequential combinations of Natura-alpha and Taxol produce different outcomes are currently not clear. It may depend on the timing of cell-cycle progression affected by the 2 drugs. Natura-alpha and taxol target 2 different cell proliferative pathways, the former inhibiting molecules in cell-cycle regulation (it may arrest cells at G1, S, and G2 + M phase) and the latter interfering with microtubule stability and mostly arresting cells at G2 + M phase. Thus, different sequential combinations may lead to different timing of cell-cycle progression dynamics by the 2 drugs, which may affect their ability to reduce growth. For example, if the cells are quickly arrested at G1, then the numbers of cells entering G2 + M phase will be reduced, and therefore the G2 + M phase target drug, the activity of Taxol will be reduced (less cells it can attack), and so, a less additive effect will occur. However, if cells in G1 and G2 + M are simultaneously attacked, theoretically, at least an additive effect or synergism will occur. Thus, our in vitro data clearly showed the activity of Natura-alpha against PC. The above in vitro results are substantiated by in vivo experiments by using mouse models with xenograft tumors from androgen-dependent (LNCaP) and androgen-independent (LNCaP-AI) PC cells. Continued treatment with the compound completely halted tumor growth from LNCaP cells and tumor volume was...
significantly reduced as compared with the control group. Most encouragingly, with the permission of FDA, 1 patient with advanced metastatic PC achieved stable disease condition after treatment with Natura-alpha for 3 months. All of his liver metastatic tumors reduced in size by approximately 26% as determined by using RECIST (17). Of note, although this patient showed response in liver metastases, PSA value remained the same. These phenomena need to be further studied. In general, the amount of PSA produced increases with the size of the tumor, thus PSA is widely used as a biomarker for PC and a biomarker for treatment response. However, there is a poor correlation between the PSA level and the actual size of the tumor (33). In addition, PC progression may occur in the presence of undetectable or low serum prostate-specific antigen level (34). A proof of concept clinical trial of Natura clinically.

PPA showed that 2 categories of molecules involved in regulating cell proliferation, and migration/invasion or metastasis were found to be significantly affected by Natura-alpha. Natura-alpha inhibited expression and activation of cdk, such as cdk2, cdk6, p-cdc2Tyr15, and p-RBSer780, as well as FOXM1, which confirmed some of our previous observations in vitro (16). E-cadherin and Mesothelin, 2 critical molecules involved in cell adhesion, migration, invasion and metastasis were also significantly impacted by Natura-alpha treatment, which provided strong molecular basis for Natura-alpha in suppression of tumor proliferation and metastasis observed both in vitro and in vivo, as well as in one human case. As tumors become more aggressive with the ability to metastasize, E-cadherin is commonly lost, as in PC (35–38). There is evidence that mesothelin may have potential as a new cancer diagnostic marker and a novel molecular target for gene therapy (39–41). Changes of these molecules in response to Natura-alpha treatments may not only help us to understand mechanisms of its activities on PC invasion/metastasis, but may also make E-cadherin and mesothelin valuable biological markers to monitor therapeutic activities of Natura clinically.

Our most important finding in this study is that Natura-alpha significantly inhibited FOXM1 in vivo as revealed by PPAA study showing that significant repression of FOXM1 by Natura-alpha may be the primary cause of cell-cycle arrest and inhibition of invasion by over-expression of siRNA to knockdown expression of FOXM1 in both LNCaP and LNCaP-AI cells. FOXM1 is a human proto-oncogene (42) and shown to regulate expression of a large array of G2/M-specific genes, such as Plk1, cyclin B2, Nek2, and CENPF and plays an important role in maintenance of chromosomal segregation and genomic stability (43). In addition, FOXM1 interacts with the cell-cycle–inhibitory pocket protein pRb and the cdk-activating phosphatase cdc25B in G1 and in G1/S, respectively (44). Because FOXM1 is essential for cancer cell viability, targeting FOXM1 has become a new strategy for developing novel anticancer drugs (45–46). Recent study showed that Iressa (Gefitinib) represses FOXM1 expression in breast cancer (47). Repression of FOXM1 by Natura-alpha primarily resulted in cell-cycle arrest and inhibition of invasion, thus, FOXM1 may be one of critical targets of Natura-alpha against PC. Our data together strongly suggest that Natura-alpha could become a novel and effective therapeutic agent in treating hormone-dependent and hormone-refractory PC with minimal side effects.

Disclosure of Potential Conflicts of Interest

L.G. Wang and S. Mencher are employee of Natrogen Therapeutics.

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