Emodin Down-regulates Androgen Receptor and Inhibits Prostate Cancer Cell Growth

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Abstract

Hormone-refractory relapse is an inevitable and lethal event for advanced prostate cancer patients after hormone deprivation. A growing body of evidence indicates that hormone deprivation may promote this aggressive prostate cancer phenotype. Notably, androgen receptor (AR) not only mediates the effect of androgen on the tumor initiation but also plays the major role in the relapse transition. This provides a strong rationale for searching new effective agents targeting the down-regulation of AR to treat or prevent advanced prostate cancer progression. Here, we show that emodin, a natural compound, can directly target AR to suppress prostate cancer cell growth *in vitro* and prolong the survival of C3(1)/SV40 transgenic mice *in vivo*. Emodin treatment resulted in repressing androgen-dependent transactivation of AR by inhibiting AR nuclear translocation. Emodin decreased the association of AR and heat shock protein 90 and increased the association of AR and MDM2, which in turn induced AR degradation through proteasome-mediated pathway in a ligand-independent manner. Our work indicates a new mechanism for the emodin-mediated anticancer effect and justifies further investigation of emodin as a therapeutic and preventive agent for prostate cancer. (Cancer Res 2005; 65(6): 2287-95)

Introduction

Prostate cancer is the most common malignant disease and the second leading cause of death in U.S. male cancer patients. Despite that diagnosis is earlier than in the past, the incidence and mortality rates of this cancer are still increasing steadily. About 220,900 cases diagnosed and 28,900 deaths were attributed to the disease in 2003 (1), and inevitably, 29,900 men are expected to have die of this disease in 2004 (2). On this devastating disease with tremendous impact on public health, unfortunately, the effective treatment options are limited and metastatic disease frequently develops even after potentially curative surgery or radiation therapy (3–5). Besides focusing on early diagnosis and treatment of this long-term and multistep malignant disease, prevention may be an alternative and more effective approach.

A recent and exciting prevention trial for prostate cancer has been done to show that finasteride, a 5-α reductase inhibitor that inhibits the conversion of testosterone to a more potent androgen, dihydrotestosterone, has the chemopreventive effect for prostate cancer development (6). Their results support that prevention could be a right direction and strategy while dealing with prostate cancer, but this study points out that finasteride also increases the risk of high-grade prostate cancer. One possible explanation for the outcome may have resulted from the fact that finasteride reduced the intraprostatic dihydrotestosterone level that created an environment more beneficial for those less androgen-dependent, high-grade cancers to grow (7, 8). Consistent to this notion, previous reports showed that men who developed prostate cancers with low testosterone levels have higher Gleason grades and worse outcomes than those with normal testosterone levels (9–11). Thus, although the clinical trial was successful, the results do not provide a clear resolution for patients and physicians to choose finasteride as a preventive agent due to the potential high risk for development of high-grade prostate cancer, which is associated with much higher mortality rate.

Prostate cancer depends on androgen receptor (AR) to mediate the effect of androgen on tumor initiation and progression (12). The standard hormone therapy for prostate cancer aims at inactivating AR transcriptional activity by androgen deprivation (through surgical or medical castration) or androgen blockade (with AR antagonists; refs. 13, 14). The same concept has been applied to prostate cancer prevention. However, for those advanced prostate cancer, this response is temporary; as disease progresses, almost all prostate cancers eventually become androgen independent. More and more evidence suggest that hormone therapies for prostate cancer may promote the phenotypic progression of those tumor cells that are able to survive the acute period of the therapy (15, 16). Although prostate cancer uses various schemes to subvert normal restraints on cell growth along with deprivation of androgen, a common feature among the diverse schemes is that the AR is still expressed and required for androgen-independent prostate cancer cell growth (17–20). These tumors are androgen independent but seem to remain AR dependent. Thus, it is important to develop new compounds that can inhibit AR function in an alternative, ligand-independent manner.

Many phytochemicals derived from plants, such as genistein and curcumin, have been shown to possess substantial anticancer activities in prostate cancer, and clinical trials using these phytochemicals to prevent prostate cancer are ongoing (21). In the present study, we found that emodin (1,2,8-trihydroxy-6-methylanthraquinone), a natural compound extracted from *Rheum palmatum*, has more potent anticancer activity of prostate cancer than genistein and curcumin. Our results showed that emodin inhibits AR transcriptional activity by preventing AR nuclear translocation. Emodin treatment results in decreased association of AR and heat shock protein 90 (hsp90) but increased association of AR and MDM2, thus inducing AR degradation in a ligand-independent manner. Most importantly, we showed that, through targeting AR, emodin could suppress prostate cancer cell growth

in vitro and prolong the survival of prostate cancer–producing C3(1)/SV40 transgenic mice *in vivo*. These results indicate a new mechanism for the emodin-mediated anticancer effect and justify further investigation of emodin, a natural compound as a therapeutic and preventive agent for prostate cancer.

Materials and Methods

Cell Culture. LNCaP, PC3, DU-145, 293, and COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in serum-containing medium as recommended by the supplier. PC3-AR cells are a clonal cell line derived by stable transfection of PC3 cells with a plasmid containing the coding region of the human AR. PC3-neo cells were stably transfected with the same vector lacking the AR cDNA sequence. Medium for PC3-AR cells also contained the selective antibiotic G418 (400 μg/mL, Life Technologies, Inc., Gaithersburg, MD). For experiments requiring an androgen-depleted condition, cells were incubated in phenol red–free RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum (c-FBS) for 1 day before initiation of the experiment.

Reagents and Plasmids. The synthetic androgen R1881 (Perkin-Elmer, Boston, MA) was dissolved in 100% ethanol and stored at $-20°C$ for up to 1 month. Emodin (Sigma Co., St. Louis, MO), MG-132, AG1478, LY294002, U0126, curcumin, and genistein (Calbiochem, San Diego, CA) were dissolved in DMSO. Anti-AR antibody (15061, 15071), anti-MDM2 antibody, and anti-hsp90 antibody were purchased from PharMingen (San Diego, CA), Oncogene (San Diego, CA), and Santa Cruz Biotechnology (Santa Cruz, CA) respectively. Expression plasmids used were pSG5-AR, pcDNA3-MDM2, and pcDNA3.1-green fluorescent protein (GFP)-AR. pSG5-AR was generated by inserting the human AR cDNA into the *Eco*RI and *Bam*HI sites near the start and termination codons of expression vector pSG5. pcDNA3-MDM2 was generated as described previously (22). pcDNA3.1-GFP-AR was kindly provided by Dr. Zhengxin Wang (University of Texas M.D. Anderson Cancer Center, Houston, TX).

Proliferation and [^{3}H]Thymidine Incorporation Assays. Cells were seeded in 96-well microtiter plate overnight. Thereafter, cells were treated with different concentrations of emodin (0, 10, 20, or 40 μmol/L) and equal volume of DMSO as the control and then incubated for additional 24, 48, or 72 hours. The proliferation rates of the cell lines were analyzed by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and [^{3}H]thymidine incorporation as described previously (23).

Reverse Transcription-PCR. Total RNA was extracted from cells and reverse transcription-PCR was done as per the manufacturer's instructions (SuperScript preamplification system, Life Technologies). The primers derived from the AR coding sequence (5′-CTCACCAAGCTCCTGGACTC-3′) and prostate-specific antigen (PSA) coding sequence (5′-GCAGCATT-GAACCAGAGGAG-3′) were used to amplify the AR and PSA transcripts, respectively. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control were 5′-AGGTGAAGGTCGGAGTCAAC-3′ and 5′-TCCATTGATGACAAGCTTCCC-3′. Amplification was done on a Perkin-Elmer DNA cycler 480 for 35 cycles with denaturing at 94°C for 30 seconds, annealing at 58°C for 1.5 minutes, and extension at 72°C for 1.5 minutes.

Transient Transfection and Luciferase Assay. LNCaP and PC3 cells were plated 1 day before transfection at a density of $2 × 10^5$ cells per well in six-well plates. LNCaP cells were cotransfected with a luciferase reporter plasmid (0.3 μg PSA-luc or probasin-luc), a β-galactosidase expression plasmid (0.2 μg CMV-β-gal), and expression plasmids with an empty vector (0.9 μg each) as indicated using liposomes. For PC3 cells, the procedure was the same, except that these cells were cotransfected with additional AR plasmid (0.3 μg pSG5-AR). After transfection, the cells were cultured in phenol red–free medium supplemented with 5% c-FBS in the absence or presence of the synthetic androgen R1881 (1 nmol/L) and various doses of emodin. Cell lysates were collected 48 hours after transfection, and the luciferase activity of each sample was measured using a luciferase assay kit (Promega, Madison, WI). β-galactosidase activity was determined to normalize variations in transfection efficiency.

Western Blot Analysis and Immunoprecipitation. LNCaP cells treated with emodin in presence or absence of 0.1 nmol/L R1881 were harvested, protein was extracted, and Western blots and immunoprecipitation were run as described previously (23). Primary antibodies against AR (1:500), MDM2 (1:1,000) were used to probe the blots. The intensity of the protein signal was quantitated by Bio-Rad PDQuest Image software (Hercules, CA).

Fluorescence Imaging and Fractionation. For immunodetection, treated cells were measured as described previously (22). Fluorescence imaging of living cells was done through an Axiovert 200 inverted fluorescence microscope (Zeiss, Germany). COS-1 cells were transiently transfected with chimeric GFP plasmid (pcDNA3.1-GFP-AR) and allowed to express chimeric protein for 24 hours. The cells were first analyzed without undergoing any treatment and then examined after the addition of different inhibitors for 30 minutes followed by the addition of 0.1 nmol/L R1881 into the same chamber. The same living cell was studied and recorded at different times. For cell fractionation, procedures were described previously.

Xenograft and Transgenic Mice Models. All animal experiments were done in accordance with institutional guidelines for animal welfare. PC3 ($4 × 10^6$) and PC3-AR ($4 × 10^6$) were injected s.c. into 5- to 6-week-old male athymic nude mice. One week after cell implantation, animals were randomized into two groups (n = 10 each). Each group was treated with i.p. bolus injections of either the drug vehicle (DMSO) or emodin (40 mg/kg) everyday. Tumors were measured with a caliper once a week, and their volumes were calculated using the formula: $\pi / 6 × a × b^2$, where a and b are the long and short diameters, respectively. Three pairs of C57BL/6J-TgN C3(1)/SV40 Tag-transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME) and the transgenic progeny was identified by PCR analysis of tail DNA isolated from 3-week-old litters using standard techniques. We treated 4-week-old mice (10 mice per group) with DMSO or emodin (40 mg/kg) everyday by i.p. injection. Each treated mouse received supplemental dosages at 140-day intervals, and the efficacy of the treatment was measured by body weight and survival. Additional groups of mice (six to seven mice per group) were given treatment as described above but sacrificed at age 21 weeks for histologic and Western blot analyses.

Immunohistochemical Staining. Immunohistochemical analysis for AR protein expression in tumor samples was done as described (24). Polyclonal antibody against AR was purchased from Upstate (Waltham, MA).

Statistics. All results from the *in vitro* experiments are presented as mean $\pm$ SE. Comparisons were made with Student's *t* test. SSPS software was used in all analyses.

Results

Emodin Inhibits Cell Proliferation of Androgen Receptor–Positive Prostate Cancer Cells. As an initial attempt to compare the effect of emodin on prostate cancer cells with other two well-characterized natural compounds, genistein and curcumin, we treated AR-positive LNCaP cells with low (10 μmol/L) and high (40 μmol/L) concentrations of emodin, genistein, and curcumin. Interestingly, the antiproliferative effect of emodin was more significant than genistein and curcumin even in low-dose range (Fig. 1*A* and *B*). Because AR-positive LNCaP cells respond and proliferate on androgen stimulation (25), we treated LNCaP cells with various concentrations of emodin in the presence of the synthetic androgen R1881. Emodin efficiently inhibited LNCaP cell proliferation and DNA synthesis stimulated by R1881 in a dose-dependent manner; meanwhile, the inhibition was also significant at low concentration (Fig. 1*C* and *D*). To further investigate the antiproliferative effect of emodin, we treated another prostate cancer cell line, DU-145, with various concentrations of emodin. DU-145 cells, which are derived from a brain metastasis, do not express the AR and grow independent of androgen. The AR-negative DU-145 cells are more resistant to the emodin-mediated antiproliferative effect than the AR-positive LNCaP cells (Fig. 1*E*). Although AR is not the only difference between LNCaP and DU-145

Figure 1. Effect of emodin on prostate cancer cells proliferation. *A* and *B,* LNCaP cells were treated with 10 or 40 μmol/L emodin, genistein, and curcumin for 24, 48, and 72 hours, respectively. Percentages of viable cells after treatment compared with control (DMSO treated defined as 100%) were determined by MTT assay. *C,* LNCaP cells were treated with DMSO and various concentrations [10 (*E10*), 20 (*E20*), and 40 (*E40*) μmol/L] of emodin in the presence of 1 nmol/L R1881 for 72 hours. Percentages of viable cells after treatment compared with control (DMSO treated defined as 100%) were determined by MTT assay. *, $P < 0.01$; **, $P < 0.001$, *t* test. *D,* LNCaP cells were treated with various concentrations of emodin in the presence of 1 nmol/L R1881. [^{3}H]Thymidine incorporation rates were done to measure DNA synthesis. *, $P < 0.01$; **, $P < 0.001$, *t* test. *E,* LNCaP and DU-145 cells were treated with DMSO or 40 μmol/L emodin for 24, 48, and 72 hours. Percentages of viable cells after treatment comparing to control were determined by MTT assay. *, $P < 0.01$; **, $P < 0.001$, *t* test. *F,* PC3 and PC3-AR cells were treated with DMSO and 10 or 40 μmol/L emodin for 72 hours. Percentages of viable cells after treatment were determined by MTT assay and compared with control. *, $P < 0.01$, *t* test.
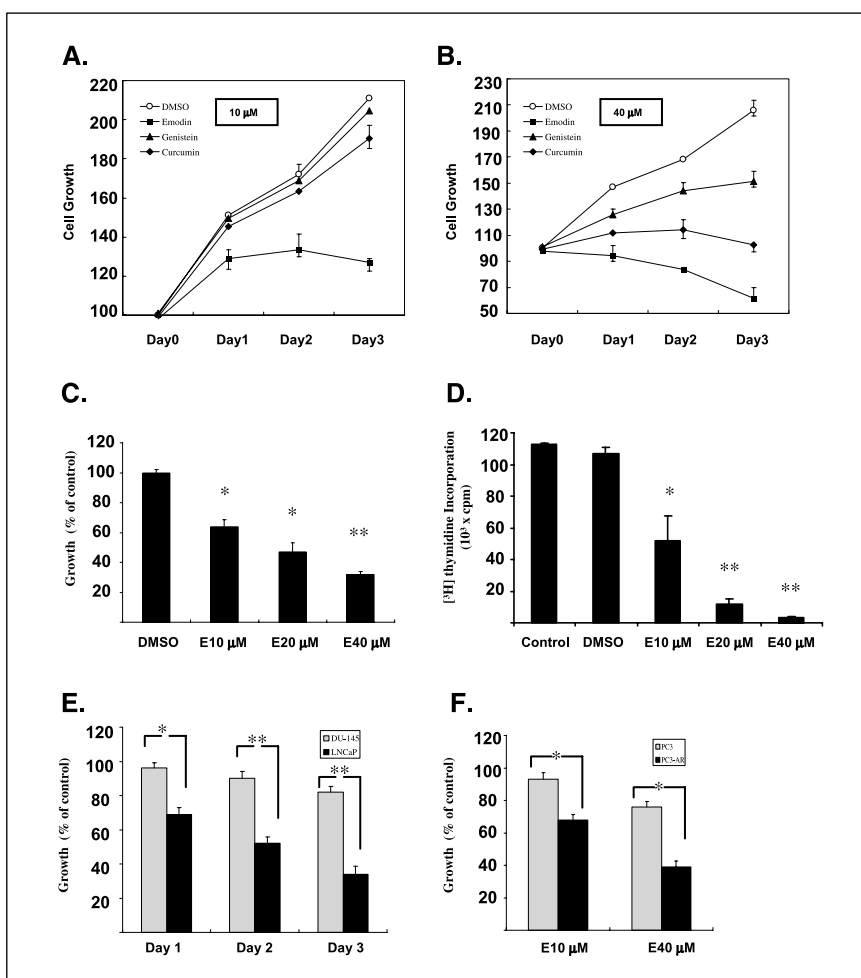
prostate cancer cells, this result raises an interesting possibility that AR-positive prostate cancer cells may be more sensitive to emodin treatment. To extend this observation, we tested the effect of emodin on a pair of AR-negative and AR-positive prostate cancer cell lines, PC3 and PC3-AR. PC3 is a well-defined AR-negative prostate cancer cell line and PC3-AR is a clonal PC3 cell line stably transfected with AR. Thus, they have identical genetic background, except for the AR status. Again, the AR-positive PC3-AR cells are much more sensitive to emodin treatment than the AR-negative PC3 cells (Fig. 1*F*). Treatment with low-dose (10 μmol/L) emodin yielded a significant inhibition of PC3-AR cell growth by 35% within 72 hours compared with parental PC3 cell growth by 7% ($P < 0.01$); high-dose (40 μmol/L) emodin showed some inhibition of PC3 cell growth by 22% but more significant inhibition of PC3-AR cell growth by 60% ($P < 0.001$). The antiproliferative effect of the PC3-AR cells was evident already 24 hours after emodin treatment but not of PC3 cells (data not shown). Thus, this result is consistent with the results showed in Fig. 1*E* and supports the notion that AR-positive prostate cancer cells are more sensitive to emodin treatment.

Emodin Inhibits Androgen Receptor Transcriptional Activity and Nuclear Translocation. Because AR mediates the effect of androgen on cell proliferation and survival in LNCaP cells, and AR-positive prostate cancer cells are more sensitive to emodin treatment, we hypothesize that emodin inhibits the function of AR. To test this hypothesis, we investigated the effect of emodin

on AR downstream target gene expression. Reverse transcription-PCR and Western blot analysis showed that expression of PSA, an AR-targeting gene, was down-regulated by emodin (Fig. 2*A* and *B*). To further test the effect of emodin on AR transcriptional activity, we transiently transfected LNCaP cells with PSA-luc and probasin-luc reporters, two well-characterized AR-targeting promoters. Both androgen-mediated PSA and probasin promoter activities were repressed in emodin-treated cells in a dose-dependent manner (Fig. 2*C*). The emodin-mediated repression of PSA and probasin reporter activities were also observed in PC3 cells with the cotransfection of AR-expressing vector, pSG5-AR plasmid and reporters (Fig. 2*D*). Without cotransfection of pSG5-AR, these two reporters will not respond to R1881 stimulation and emodin has no effect on their promoter activities in the AR-negative PC3 cells (data not shown). Thus, emodin inhibits the transcriptional activity of AR. The function of AR is closely related to its subcellular localization. Because emodin can inhibit AR transcriptional activity, we examined the effect of emodin on AR subcellular localization. The intracellular distribution of AR in LNCaP cells was assessed using immunofluorescence microscopy. AR was mostly localized in cytosol in the absence of androgen for 24 hours (Fig. 2*E*). After stimulation with 1 nmol/L R1881 for 2 hours, AR translocated into the nucleus as clearly indicated by the yellow staining in the nucleus merged from AR (green) and nuclear 4′,6-diamidino-2-phenylindole (red) staining. However, when cells were treated with both R1881 and emodin, AR was
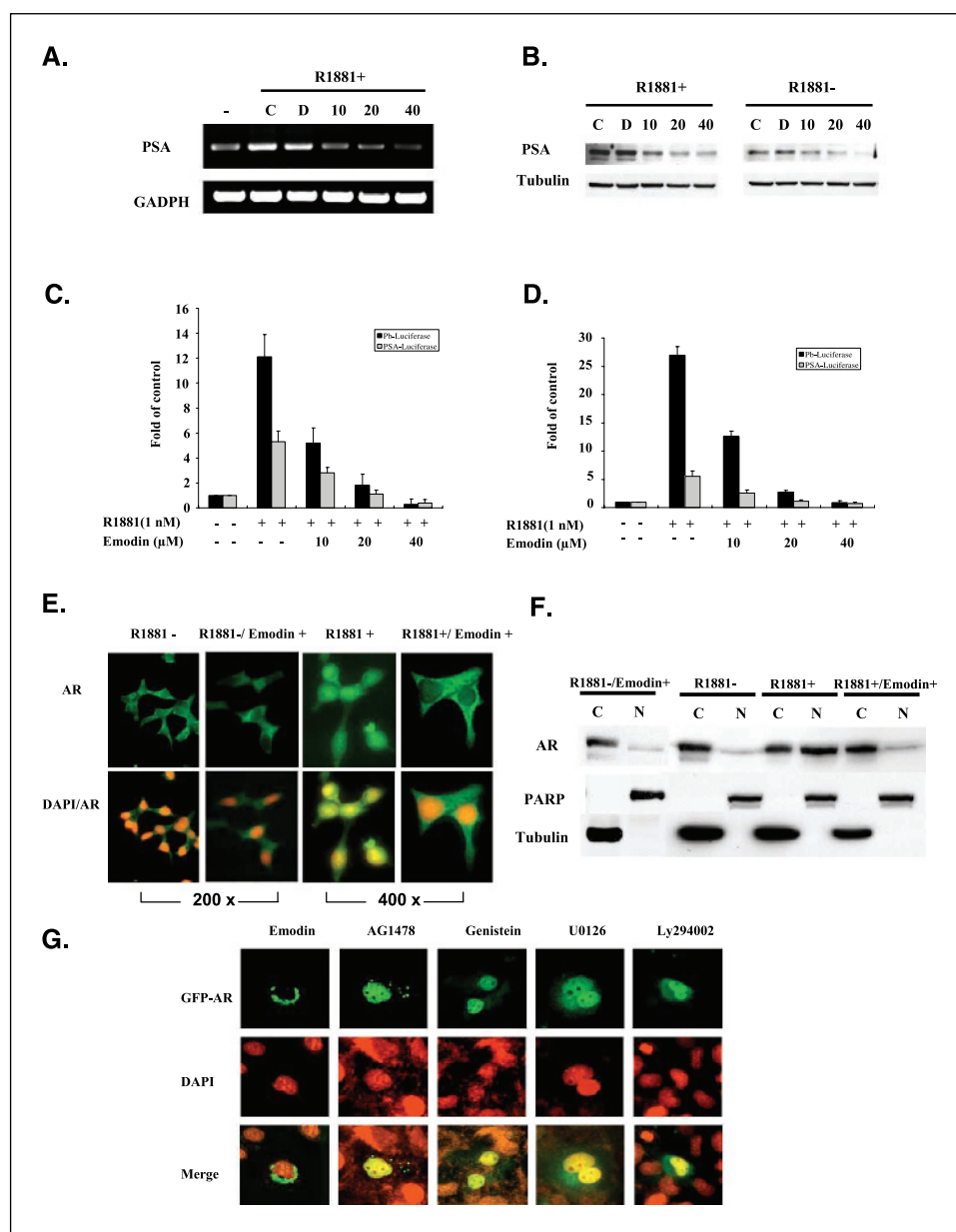
Figure 2. Effects of emodin on AR transcriptional activity and nuclear translocation. *A,* LNCaP cells were treated with solvent [DMSO (*D*)] and various concentrations of emodin (10-40 μmol/L) for 18 hours with (*C*) or without 1 nmol/L R1881. Expression of AR-targeting gene PSA was analyzed by reverse transcription-PCR. Expression of GAPDH was monitored as a control. *B,* PSA protein level was analyzed by Western blotting after emodin treatment and tubulin was measured to ensure consistent loading. *C,* PSA-luc and probasin-luc reporter genes was transiently transfected into LNCaP cells and luciferase activity was measured after emodin treatment. *D,* same as in *C,* except that PC3 cells were cotransfected with pSG5-AR plasmid and reporters. Corresponding β-galactosidase activity was used to normalize luciferase activity. *Columns,* mean of three independent experiments; *bars,* SD. *E,* LNCaP cells were treated with or without 40 μmol/L emodin for 30 minutes and then treated with or without 1 nmol/L R1881 for an additional 2 hours. Cells were fixed in cold methanol and immunostained with FITC-conjugated anti-AR antibody. *F,* results of fractionation experiments on LNCaP cells treated as described in *E.* AR in the cytosol (*C*) and nuclear extracts (*N*) was detected by anti-AR antibody. Anti–poly(ADP-ribose) polymerase (*PARP*) and anti-tubulin antibodies were run to distinguish between nuclear and cytosolic fractions, respectively. *G,* COS-1 cells were transiently transfected with GFP-AR and treated with emodin (40 μmol/L), AG1478 (10 μmol/L), genistein (50 μmol/L), LY294002 (20 μmol/L), or U0126 (20 μmol/L) in the presence of 1 nmol/L R1881 for 2 hours. GFP-AR fusion proteins were detected in living cells using an Axiovert 200 inverted fluorescence microscope. GFP-AR (*green*), 4′, 6-diamidino-2-phenylindole (*DAPI*; *red*), and merging of these two signals (*yellow*).

mostly retained in the cytosol as shown by the AR staining (green) in the cytosol and reduced yellow staining in the nucleus. The emodin-mediated cytosolic retention of AR was also supported by the biochemical approach from cellular fraction experiments (Fig. 2*F*). To further investigate the specific effect of emodin on AR nuclear localization, we did the time lapse experiment by using a fusion protein between AR and GFP to dynamically evaluate AR trafficking in a single living cell. We transfected the AR-GFP fusion protein to COS-1 cells and then tested the effects of different kinds of inhibitors—emodin, AG1478 (a tyrosine kinase inhibitor), genistein (a tyrosine kinase inhibitor), LY294002 (a phosphatidylinositol 3-kinase inhibitor), and U0126 (a mitogen-activated protein kinase inhibitor)—on AR nuclear localization under different times. Before proceeding with this experiment, all the inhibitors were tested and titrated to the optimal dosage for inhibition of their functional targets (data not shown). Under the stimulation by R1881 for 2 hours, only emodin,

but not the rest of the inhibitors, prevented AR nuclear translocation (Fig. 2*G*). This effect can be observed for up to 24 hours (data not shown).

Emodin Induces Androgen Receptor Degradation through Proteasome-Mediated Pathway. Because androgen stimulation induces AR nuclear translocation and emodin inhibits this activity resulting in AR accumulation in the cytosol, it becomes very interesting to further investigate the fate of AR under this condition. To this end, we treated LNCaP cells with various concentrations of emodin or for different lengths of time in the presence or absence of 1 nmol/L R1881 and measured AR protein expression. A time- and dose-dependent reduction of the AR protein level was observed (Fig. 3*A* and *B*). Although AR is more stable and has a higher basal level of expression in the presence of ligand, emodin can efficiently deplete AR even in cells undergoing synthetic androgen R1881 stimulation. Thus, emodin-induced depletion of AR is ligand independent. To investigate the molecular

mechanisms for AR depletion, we first investigate whether emodin may have effect on AR mRNA expression. We analyzed AR mRNA level under the treatment with various concentrations of emodin by reverse transcription-PCR and found that emodin did not influence AR mRNA level (Fig. 3C). We then further test the effect of emodin on AR protein stability. By using cycloheximide to inhibit protein synthesis, the AR protein stability was significantly reduced under emodin treatment (Fig. 3D). To test whether emodin induce AR degradation through the proteasome pathway, treatment with the proteasome inhibitor MG-132 resulted in a marked suppression of emodin-induced AR depletion (Fig. 3E). These phenomena were also observed in PC3-AR cells (Fig. 3F). Taken together, these findings indicate that emodin induces AR degradation through a proteasome-mediated pathway.

Emodin Disrupts Androgen Receptor-Heat Shock Protein 90 Association and Increases Androgen Receptor-MDM2 Association and Ubiquitination. AR is known to form a heteromeric complex with two molecules of hsp90, which has been shown to participate in regulating the protein stability of ligand-unbound AR. Previous reports showed that some tyrosine kinase inhibitors can reduce the AR protein level (26). We further tested the

efficacy of emodin and other tyrosine kinase inhibitors, such as genistein and AG1478, on the reduction of AR and the interaction between AR and hsp90. Due to different drugs having different kinetic and dynamic activities, we chose a relative high dose of AG1478 (10 μmol/L) and genistein (50 μmol/L) to ensure their efficacy (27, 28). Under this situation, emodin-induced reduction of AR is more potent than the other two tyrosine kinase inhibitors especially in the presence of R1881 (Fig. 4A). In addition, only emodin can significantly disrupt the association between AR and hsp90 whether treated for a short period (2 hours; Fig. 4B) or a longer period (8 hours; data not shown). This result indicates that emodin induces dissociation of AR and hsp90 involving a novel mechanism different from other tyrosine kinase inhibitors. The emodin-induced dissociation between AR and hsp90 may render the incomplete AR heterocomplex more vulnerable to degradation through the proteasome-mediated pathway. It has been shown that AR can be ubiquitinated by an E3 ligase, MDM2 (14). We hypothesized that dissociation of AR and hsp90 by emodin increases the interaction between AR and the E3 ligase MDM2 and therefore enhances degradation. To test this hypothesis, we measured the interaction between AR and MDM2 with or without

Figure 3. Effect of emodin on AR expression. *A,* LNCaP cells were treated with solvent (DMSO) and various concentrations of emodin [5 (*E5*), 10, 20, or 40 μmol/L] for 18 hours with or without 1 nmol/L R1881. AR protein level was analyzed by Western blot and quantitated by Bio-Rad PDQuest Image software and plotted as the percentage of the control (without emodin) after normalization with actin. *B,* LNCaP cells were treated with 40 μmol/L emodin for various lengths of time. AR protein level was measured by immunoblotting. *C,* expression of the AR gene in LNCaP cells was analyzed by reverse transcription-PCR after treatment with various concentrations of emodin for 18 hours. Expression of GAPDH was monitored as a control. *D,* LNCaP cells were treated with 40 μmol/L emodin and 10 μmol/L cycloheximide for various lengths of time. DMSO was added to the control. AR protein level was measured by Western blot analysis. *E,* LNCaP cells were treated with 40 μmol/L emodin and 5 μmol/L MG-132 with or without R1881 for 12 hours. DMSO was added to the control. AR protein level was measured by Western blot analysis. *F,* PC3-AR cells were treated with 40 μmol/L emodin and 5 μmol/L MG-132 for 12 hours. DMSO was added to the control. AR protein level was measured by Western blot analysis.
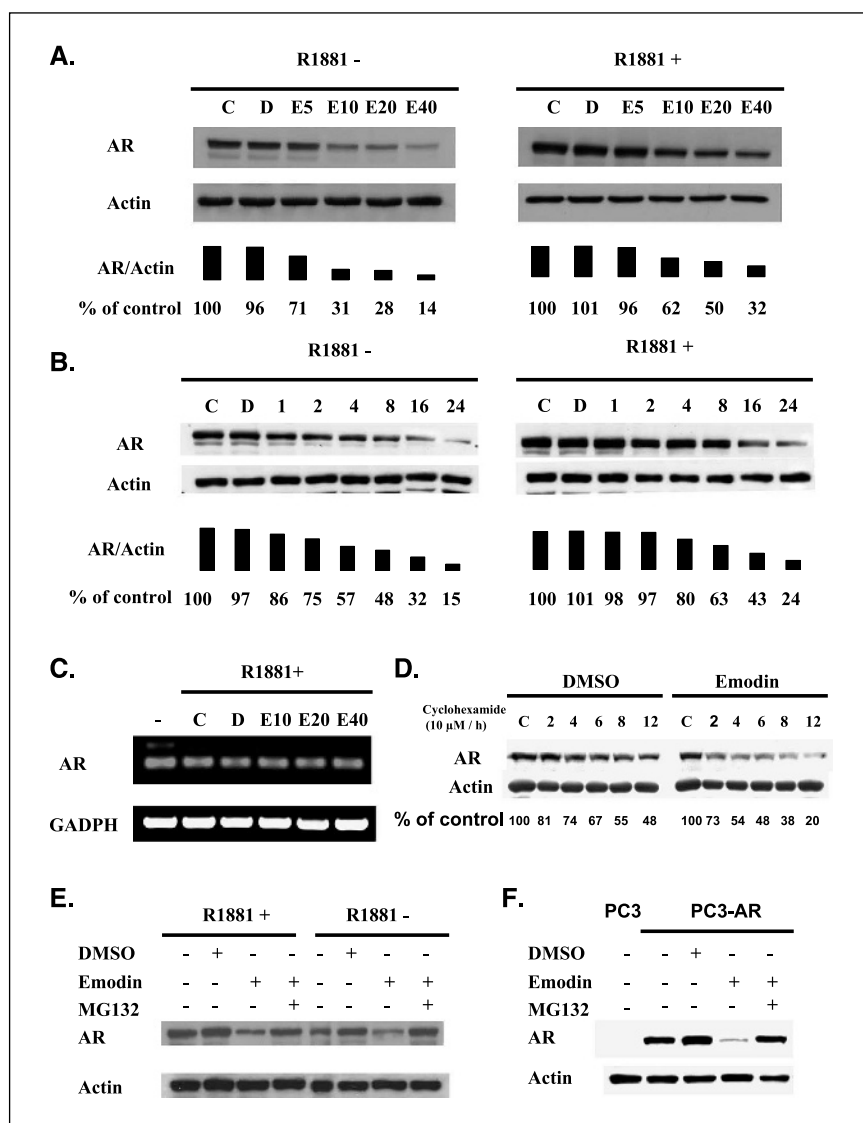
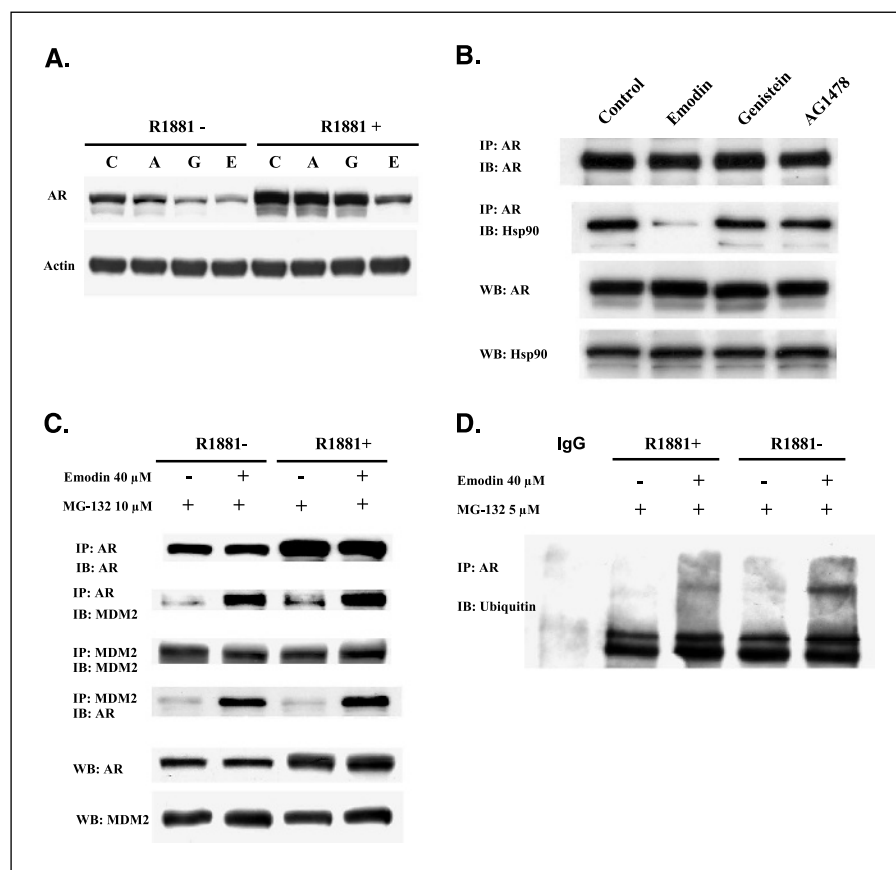**Figure 4.** Effect of emodin on the associations between AR and hsp90 and AR and MDM2. *A,* LNCaP cells were treated with emodin (40 μmol/L), AG1478 (10 μmol/L), and genistein (50 μmol/L) with or without R1881 for 18 hours. AR protein level was measured by Western blot analysis. *B,* LNCaP cells were incubated with c-FBS medium overnight and then treated with or without emodin (40 μmol/L), AG1478 (10 μmol/L), and genistein (50 μmol/L) for 2 hours. Immunoprecipitation and Western blot analysis were done with indicated antibodies. *WB,* Western blotting of whole cell lysates. *C,* LNCaP cells were incubated in c-FBS medium in the presence or absence of 1 nmol/L R1881 overnight and then treated with or without emodin (40 μmol/L) in the presence of MG-132 (10 μmol/L) for 2 hours. AR immunoprecipitation was done as in *B,* except that immunoblotting was done with anti-MDM2 antibody rather than anti-hsp90 antibody. *D,* LNCaP cells were incubated in c-FBS medium overnight and then treated with or without emodin (40 μmol/L) in the presence of MG-132 (5 μmol/L) and with or without 1 nmol/L R1881 stimulation for 8 hours. AR immunoprecipitation was done and immunoblotting was done with anti-ubiquitin antibody.

emodin treatment in LNCaP cells. Emodin treatment increased the association between the endogenous MDM2 and AR as evident from coimmunoprecipitation experiments using antibodies against MDM2 and AR (Fig. 4C). The emodin-induced AR-MDM2 association was also shown in PC3-AR cell line (data not shown), suggesting that this is a general phenomenon in different cell types. In addition, emodin treatment resulted in an increase of AR ubiquitination (Fig. 4D). Taken together, these results indicate that emodin dissociates hsp90 from AR and enhances AR and MDM2 association, which may lead to further ubiquitination and degradation.

Emodin Inhibits PC3-Androgen Receptor Tumor Growth and Down-regulates Androgen Receptor and Prolongs Survival of C3(1)/SV40 Transgenic Mice. The above results established a novel molecular mechanism to explain how emodin may down-regulate AR and inhibit prostate cancer cell growth in an *in vitro* cell culture system. To further investigate the *in vivo* antitumor activity of emodin, first we chose PC3 and PC3-AR xenografts as animal models. Low-dose (40 mg/kg) emodin showed significant antitumor activity in mice bearing PC3-AR xenograft ($P < 0.01$); however, the inhibitory effect was not effective in PC3 xenograft (Fig. 5A and B). Furthermore, we used C3(1)/SV40 transgenic mice as a second *in vivo* experimental model. It is known that the male mice of this strain will develop AR-positive prostate cancer and eventually die with prostate cancer because the SV40 large T antigen was driven by the promoter of rat prostatic steroid binding protein [C3(1)] gene in the transgenic mice (29). Because the carcinogenesis of C3(1)/SV40 transgenic mouse model is primarily driven by AR, this transgenic mouse model provides a clean background, and by using the tumor development as a readout, we can more specifically test the effect of

emodin on AR *in vivo*. We treated male C3(1)/SV40 transgenic mice with either emodin (40 mg/kg) or DMSO i.p. every other day while the mice were 4 weeks old and with no signs of tumors. Emodin-treated mice have significantly longer survival than the control group ($P < 0.001$; Fig. 5C). Emodin-treated mice maintained body weight gain; in contrast, DMSO-treated control mice significantly lost body weight gain after age 20 weeks ($P < 0.05$; Fig. 5D). We noticed that not only the size but also the hair grooming and cage activity were clearly different between emodin-treated and control groups. In general, emodin-treated mice looked much healthier, but the control mice seemed to be in distress with labored breathing, cachectic and lethargic patterns (Fig. 5E). This result showed that emodin not only has low drug toxicity but also maintains the physical activity of C3(1)/SV40 transgenic mice by preventing tumor progression. To further address whether the biological effect of emodin is related to its ability to down-regulate AR, we analyzed AR expression of tumor tissues from both emodin-treated and control groups. Immunohistochemical staining using an AR-specific antibody clearly indicated that prostate cancer tumor tissues from emodin-treated mice were much weaker than those from the control group (Fig. 5F and G). The same results were also obtained by using Western blot analysis of fresh tumor samples taken from other pairs of mice (Fig. 5H). In addition, when we analyzed the tumor progressive status by histopathologic investigation of mice at age 21 weeks, the emodin-treated mice clearly had a lower incidence of tumor invasion to the periurethral muscle structure (1/7) compared with the control group (7/7; Fig. 5I). Our results showed that emodin indeed can down-regulate AR in C3(1)/SV40 transgenic mice model. Considering the success of the recent finasteride prevention trial for prostate cancer and the dilemma it

created due to its androgen deprivation nature, emodin, which directly down-regulates AR in a ligand-independent manner, may have an advantage for further development as a therapeutic and chemopreventive agent for prostate cancer.

Discussion

Emodin, an active extract of *R. palmatum*, has been shown to have multiple biological activities, including anti-inflammatory, antibacterial, diuretic, immunosuppressive, vasorelaxant, and anticancer effects (30–33). We have shown previously that emodin inhibits HER-2/*neu* tyrosine kinase activity, and it preferentially suppresses growth and induces differentiation of the HER-2/*neu*-overexpressing breast cancer cells but has no effects on normal cells (34). Here, we found a novel emodin-mediated mechanism of inhibition of prostate cancer cell growth, especially AR-positive cells, through down-regulation of AR. AR mediates growth-promoting and survival effects through either genotropic action by transcriptional activation of target genes and nongenotropic action by activation of phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, and protein kinase C pathways (35–37). In PC3 cells stably expressing AR, androgens are able to activate these nongenotropic pathways (35, 37). Our results showed that both LNCaP cells with endogenous AR and PC3-AR stable transfectants are more sensitive to low-dose emodin. Emodin-mediated down-regulation of AR may inhibit both genotropic and nongenotropic pathways and make cells more vulnerable and result in growth inhibition.

AR is a nuclear transcription factor. Nuclear translocation is a key step for AR in response to androgen stimulation (38), and this process initiates the transactivation of downstream target genes to promote prostate cancer cell proliferation and survival. Our results showed that emodin inhibits androgen-mediated AR nuclear translocation and induces dissociation of AR and hsp90. However, this phenomenon did not occur with treatment of other kinase inhibitors, including receptor tyrosine kinase, mitogen-activated protein kinase, and Akt pathway inhibitors. Consistent with
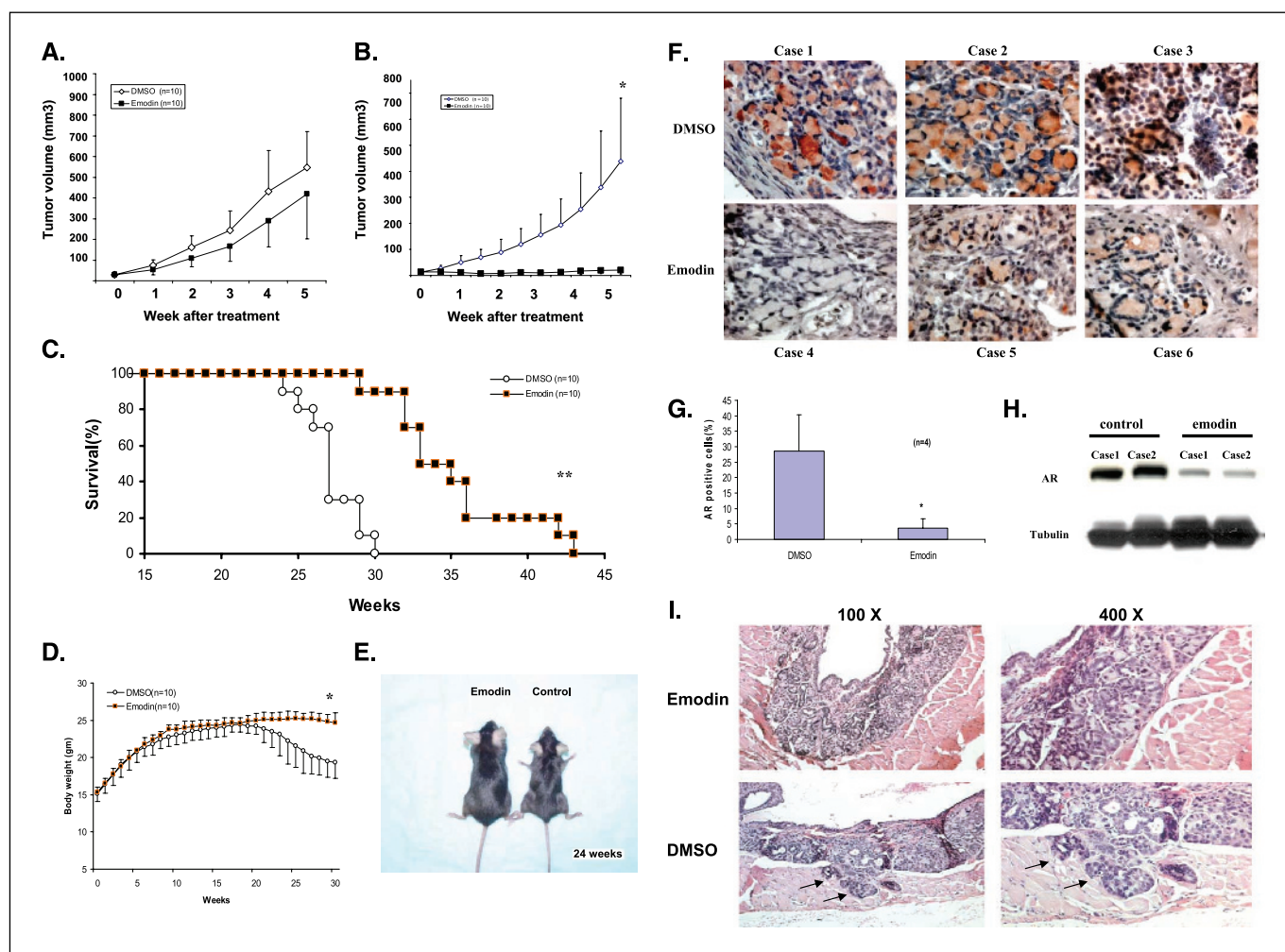

Figure 5. Effect of emodin on *in vivo* animal models. *A* and *B, in vivo* antitumor activity of emodin on nude mice bearing PC3 and PC3-AR xenografts. *, $P < 0.01$. *C*, 4-week-old mice were i.p. injected with 40 mg/kg emodin or DMSO every other day. Survival of mice treated with emodin or DMSO. **, $P < 0.001$. *D*, body weight gain profiles of emodin-treated and control transgenic mice from 0 to 30 weeks. *, $P < 0.05$. *E*, emodin-treated mouse maintained body weight gain and physical activity not seen in the control mouse (24 weeks old). *F*, tumor tissue sections from 21-week-old emodin-treated and control mice with identical treatment as survival experiment in *C* were stained with antibody specific to AR. *G*, quantitative analysis of AR expression between emodin-treated and control mice were assessed in 2,500 cells in several different views. *, $P < 0.05$. *H*, Western blot analysis of fresh tumor tissues obtained from two sets of 21-week-old emodin-treated and control mice with identical treatment as survival experiment in *C*. *I*, H&E-stained tumor tissue sections from the emodin-treated and control mice. Low magnification, ×100; high magnification, ×400. Control mice showed tumor invasion of periurethral muscle (*black arrow*) not seen in emodin-treated mice.

previous reports, the association between AR and hsp90 is not disrupted by other tyrosine kinase inhibitors (39). These results suggest that emodin-mediated inhibition of AR nuclear translocation is specific and involves a novel mechanism correlating with the dissociation of AR and hsp90. It has been shown that hsp90 function is required for AR nuclear translocation, which is consistent with our observation (40).

The steroid receptors interact with hsp90 and other co-chaperones to create a mature conformation for proper protein function (40–42). Without hsp90 binding, the misfolded or unfolded proteins will be recognized and degraded by the ubiquitin-proteasome system (43). Emodin treatment induces the dissociation between AR and hsp90 and increases the association of AR and MDM2, providing a plausible mechanism for the involvement of MDM2 as an E3 ligase for the emodin-mediated AR degradation. Previous studies have shown that the hsp90 inhibitors, such as geldanamycin and its derivatives, can directly bind to the ATP-binding pocket of hsp90 and inhibit its function and then further induce steroid receptor degradation (39, 41, 44–46). Although both emodin and geldanamycin share the similar feature of abrogating the interaction between AR and hsp90, their mechanisms are different. Emodin induces dissociation of AR and hsp90, but geldanamycin cannot. It will be interesting to see whether MDM2 is also responsible for the geldanamycin-induced AR degradation.

A recent study showed that overexpression of AR in hormone-refractory xenograft model is consistent with observations in human clinical specimens, and overexpression of AR promotes the transition of hormone-dependent xenograft into a hormone-independent xenograft (47, 48). These observations indicate that reducing AR expression to a critical level would contribute to preventing prostate cancer progression. Emodin-induced degradation of AR occurs in a ligand-independent manner. Thus, as long as AR is functional in prostate cancer regardless of androgen-dependent or androgen-independent status, emodin should inhibit cancer cell growth because of the induction of AR degradation. In addition to enhanced AR expression, other proposed mechanisms include cross-talk between AR and other signal transduction pathways also involved in development of the hormone-refractory state. Overexpression of receptor tyrosine kinases, such as HER-2/neu, also is known to contribute to prostate cancer development (49). In this regard, it should be mentioned that high-dose emodin also associates with the activity to inhibit tyrosine kinase and suppress HER-2/neu-mediated tumorigenecity in breast cancer

cells (30, 34). The dual functions of anti-AR and anti–tyrosine kinase may be an advantage for using emodin as a chemo-preventive agent to avoid the development of aggressive prostate cancer phenotype.

In addition to the observation of the effects of emodin in the *in vitro* cell culture system, the *in vivo* effects were also observed in emodin-treated C3(1)/SV40 transgenic mice. Our results showed that emodin induced degradation of AR in the tumor tissues, suppressed tumor development, and prolonged animal survival. Because the carcinogenesis of C3(1)/SV40 transgenic mice model is primarily driven by AR, emodin-induced degradation of AR may compromise the SV40 oncogene expression, which may not reflect the real chemopreventive effect on inhibition of tumor development in C3(1)/SV40 transgenic mice. However, AR is responsible for the initiation and progression of prostate cancer in a real situation. Our transgenic model showed that emodin efficiently down-regulated AR *in vivo*, which suggests that emodin have the potential as a chemopreventive agent for prostate cancer. Emodin-treated mice maintained their body weight gain and physical activity, suggesting that the effective dose of emodin, which suppresses tumor progression, is well tolerated and nontoxic. Tumor invasion resulting in distant metastases is the major cause of prostate cancer–related death (50). Emodin-treated transgenic mice had lower incidence of periurethral invasion, which represents the preventive effect of emodin contributing to prolong the survival of transgenic mice.

Considering the dilemma created by the recent Prostate Cancer Prevention Trial, this current study provides the evidence to support that directly targeting AR rather than its ligands could be a good strategy in the treatment or prevention of prostate cancer. Emodin may have the potential as a novel anti-AR therapeutic and preventive agent for prostate cancer.

Acknowledgments

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