Exisulind and Related Compounds Inhibit Expression and Function of the Androgen Receptor in Human Prostate Cancer Cells

Jin T. E. Lim, Gary A. Piazza, Rifat Pamukcu, W. Joseph Thompson, and I. Bernard Weinstein


ABSTRACT

In recent studies, we found that sulindac sulfide (SS), exisulind, CP248, and CP461 induce growth inhibition and apoptosis in a series of human prostate cancer cell lines, irrespective of cyclooxygenase expression, p53 mutations, or bcl-2 overexpression. Exisulind also inhibited the growth of the androgen-dependent LNCaP human prostate cancer cell line when grown as a xenograft in nude mice. This study demonstrates that doses of these compounds that induce growth inhibition and apoptosis in LNCaP cells also cause decreased prostate-specific antigen (PSA) secretion and decreased cellular levels of PSA. These effects appear to be a result, at least in part, of inhibition of the androgen receptor (AR) signaling pathway because the treated cells also display decreases in the level of the AR protein and mRNA and inhibition of transcription of an AR promoter luciferase reporter in transient transfection assays. SS and exisulind were more effective in inhibiting the expression of PSA and the AR than CP248 or CP461, apparently because of differential effects of these compounds on specific transcription factors. These findings suggest that the growth inhibition by these compounds in human prostate cancer cells may be mediated, in part, by inhibition of AR signaling. Thus, these compounds may provide a novel approach to the prevention and treatment of human prostate cancer.

INTRODUCTION

Prostate cancer is currently the most frequently diagnosed cancer and the second leading cause of cancer deaths in men in the United States. In 2002, ~189,000 new cases were diagnosed, and 30,200 men died from this disease (1). Although the precise mechanism by which steroid hormones affect the development of prostate cancer is not known, it is believed that prostate cancer develops only in the presence of androgens (2). The AR is the most important mediator of androgen action and functions as a ligand-dependent transcription factor (3). Mutations in the AR gene and amplification of this gene are thought to be two possible causes for the more aggressive hormone-independent forms of prostate cancer, but other unknown mechanisms also appear to play a role (4). It has been demonstrated that although castration-induced androgen deprivation causes a 95% loss in serum testosterone, the concentration of DHT, the active form of testosterone in prostate tissue is only reduced by 60% (5). Unfortunately, some clinically available antiandrogens have an increased affinity for mutant forms of the AR (6). Therefore, compounds that can down-regulate the expression of the AR gene, thereby bypassing AR gene mutations and AR gene amplification, might provide a more effective therapy for aggressive hormone-independent prostate cancers. Few studies have examined pharmaceutical compounds that act at this level.

Sulindac is a non-steroidal anti-inflammatory drug that has been used predominantly to treat patients with chronic inflammatory diseases and for its analgesic, antipyretic, and platelet inhibitory activities (7). The drug has been used for the treatment of patients with familial adenomatous polyposis of the colon. Sulindac sulfone (exisulind), a normal oxidative metabolite of sulindac, has been shown to also induce polypl regression and prevent polypl recurrence in familial adenomatous polyposis patients. In recent studies, we found that the sulindac metabolites SS and exisulind, as well as two more potent analogues of exisulind, CP248 and CP461 (8), induce growth inhibition and apoptosis in several human prostate cancer cell lines, irrespective of COX-1/2 expression, bcl-2 overexpression, or androgen dependence (9). Although the growth of normal prostate epithelial cells was also inhibited, these cells did not display apoptosis, even at high doses of these compounds (9). Related studies showed that oral administration of exisulind inhibited growth and induced apoptosis in xenografts of LNCaP cells in nude mice (10).

During the course of our cell culture studies (9), we noted that the LNCaP androgen-sensitive prostate cancer cell line was more sensitive to both growth inhibition and induction of apoptosis than the PC3 cells, an androgen-insensitive prostate cancer cell line. This study addresses whether this increased

Received 3/20/03; revised 6/6/03; accepted 6/11/03.

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.

This study was supported by Cell Pathways, Inc., and awards to I. B. W. from the T. J. Martell Foundation and the National Foundation for Cancer Research.

1 To whom requests for reprints should be addressed, at Herbert Irving Comprehensive Cancer Center, Columbia University College of Physicians and Surgeons, 701 West 168th Street, New York, New York 10032. Phone: (212) 305-6921; Fax: (212) 305-6889; E-mail: Weinstein@cuccfa.ccc.columbia.edu.

2 The abbreviations used are: AR, androgen receptor; DHT, dihydrotestosterone; ARE, androgen-response element; SS, sulindac sulfide; β-gal, β-galactosidase; IGFBP, insulin-like growth factor binding protein; CREB, cAMP-responsive element binding protein; PSA, prostate-specific antigen; Hsp, heat shock protein; INK-1, c-Jun NH2-terminal kinase 1; WCE, whole cell extract; COX, cyclooxygenase; CMV, cytomegalovirus; NF-1, nuclear factor-1; SP-1, sequence-specific.
sensitivity is related to the androgen-dependent nature of these cells. We found that treatment of LNCaP cells with concentrations of SS, exisulind, CP461, or CP248 that induce growth inhibition and apoptosis also inhibited expression of the androgen-responsive gene PSA. PSA is a secreted serine protease that augments cleavage of IGFBP3-IGF-1 and activates tumor growth factor β and other growth factors in the extracellular matrix (11). Blood levels of PSA provide a convenient diagnostic marker for prostate cancer (11). In this study, we obtained evidence that the down-regulation of PSA is attributable to the ability of these compounds to inhibit expression of the AR gene itself. These novel effects provide additional justification for the use of exisulind and CP461 in the chemoprevention and treatment of prostate cancer.

MATERIALS AND METHODS

Drugs and Plasmids. SS, exisulind, CP248 and CP461 were obtained from Cell Pathways, Inc. (Horsham, PA). We refer to these four agents as sulindac-related compounds. Exisulind, CP461, and CP248 have no analgesic or antiinflammatory properties, and the latter two drugs are clinically distinct from SS or sulindac sulfone. Aspirin (acetyl salicylic acid) was purchased from Sigma Chemical Co. (St. Louis, MO). The drugs were dissolved in 100% DMSO and added to the cell culture medium at a final vehicle concentration of 0.1% DMSO. The (ARR)4-luciferase, ARPN1-luciferase, and PSAP-luciferase reporter plasmids were gifts from Drs. Robert Matusik, Donald Tindall, and Marian Sadar, respectively. The pSVAR expression vector was generously provided by Dr. Albert O. Brinkmann. The (AP-1)-luciferase reporter plasmid was a gift from Dr. Zigang Dong.

Cell Culture. The LNCaP and PC3 human prostate cancer cell lines were purchased from the American Type Culture Collection. The cells were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.) and maintained at 37°C with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.). The cells were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.) and maintained at 37°C with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Inc.) and maintained at 37°C.

Intracellular Levels of PSA. The well-characterized LNCaP human prostate cancer cell line was used in these studies (13). LNCaP cells express the endogenous AR and various androgen-inducible genes, including human glandular kallikrein-2 and PSA. PSA, a biomarker for prostate cancer, is a secreted serine protease produced by both normal and malignant prostate epithelial cells. Several clinical studies have shown a direct correlation between increased serum levels of PSA and the progres-

RESULTS

Sulindac-Related Compounds Decrease Secreted and Intracellular Levels of PSA. The well-characterized LNCaP human prostate cancer cell line was used in these studies (13). LNCaP cells express the endogenous AR and various androgen-inducible genes, including human glandular kallikrein-2 and PSA. PSA, a biomarker for prostate cancer, is a secreted serine protease produced by both normal and malignant prostate epithelial cells. Several clinical studies have shown a direct correlation between increased serum levels of PSA and the progres-
sion of prostate cancer (14, 15). In cell culture, LNCaP cells secrete PSA into the medium, mimicking the secretion of PSA by prostate cancers into the circulation. Therefore, it was of interest to compare the levels of secreted PSA in the growth medium of LNCaP cells after treating the cells with sulindac-related compounds by performing Western blot analysis of the cell-free medium, with an anti-PSA antibody that detects a characteristic Mr 33,000–34,000 protein band. In these studies, we used concentrations of the sulindac-related compounds that induce growth inhibition and apoptosis (8, 9). Fig. 1A (left panel) indicates that the vehicle (0.1% DMSO)-treated cells showed a time-dependent increase of PSA in the growth medium. LNCaP cells treated with 0.2 mM SS or 0.5 mM exisulind showed decreases in the levels of secreted PSA, within 10–20 h, and the PSA levels remained low even after 48 h when compared with the vehicle (0.1% DMSO) control. The secreted levels of PSA in cultures of LNCaP cells treated with 0.5 mM CP248 or 5 mM CP461 were partially inhibited when compared with the vehicle control (0.1% DMSO)-treated cells. Treatment with a high concentration of aspirin (5 mM) did not inhibit the secreted level of PSA (Fig. 1A, left panel). LNCaP cells also secrete the protein IGFBP-2 into the growth medium (11), but none of these drugs caused an appreciable inhibition of the secretion of this protein (Fig. 1A, right panel). These results indicate that sulindac derivatives, but not aspirin, can decrease the levels of PSA secreted into the medium of LNCaP cells. It is curious that SS and exisulind were much more active than CP248 and CP461, although all four drugs were tested at concentrations that induce growth inhibition and apoptosis (8, 9).

To determine whether the decreased levels of secreted PSA seen in the above studies reflected a specific effect on intracellular levels of PSA, we next examined intracellular levels of PSA in the above described cells by Western blot analysis. The cells were washed repeatedly with PBS before lysis to reduce the secreted PSA adherent to the LNCaP cells. Within 10 h of treatment of LNCaP cells with 0.2 mM SS or 0.5 mM exisulind, there was a marked and persistent decrease in the intracellular level of PSA when compared with the DMSO control cells (Fig. 1B). LNCaP cells treated with 0.5 mM CP248 or 5 mM CP461 displayed decreased levels of PSA within 20–24 h. However, aspirin did not cause a decrease in the intracellular levels of PSA (Fig. 1B, left panel). In contrast to their inhibitory effects on PSA, SS, exisulind, CP248, and CP461 did not cause an appreciable inhibition of intracellular levels of the IGFBP-2 protein (Fig. 1B, right panel). These data suggest that the decrease in secreted levels of PSA, described in Fig. 1A, is secondary to a decrease in the intracellular levels of this protein.

**Sulindac-Related Compounds Inhibit the Transcriptional Activity of an Androgen-Responsive Promoter.**

The above results raised the possibility that these drugs might inhibit transcription of the PSA gene. The transcription of this gene is known to be controlled by an ARE. Therefore, we performed transient transfection luciferase reporter assays using a probasin (ARE)3-tk-luciferase reporter plasmid, composed of three consecutive cassettes of the probasin ARE region (nucleotides −244 to −96) adjacent to the thymidine kinase promoter (16). The probasin gene encodes a protein secreted by the rat dorsolateral prostate (17). The expression of this gene is specific to the prostate and is highly androgen responsive. LNCaP cells were transfected with the probasin reporter plasmid, together with a β-galplasmid as an internal control, and the cells were incubated in charcoal-stripped RPMI 1640 containing 20 mM DHT, together with DMSO (vehicle control) or each of the drugs, for 20 h. Cell extracts were then analyzed for luciferase activity. On the basis of previous studies (18–20), we used 20 nM DHT to maximize the activation of the ARE promoter. We found that 0.2 mM SS and 0.5 mM exisulind caused a dramatic decrease in luciferase activity when compared with the 0.1% DMSO-treated cells. However, treatment with 0.5 μM CP248 or 5 μM CP461 showed only a partial decrease in luciferase activity (Fig. 2A), and cells treated with 5 mM aspirin showed no inhibition of luciferase activity. Similar results were obtained in four independent studies. Negligible activity was obtained when the DMSO control studies were done in the absence of DHT, demonstrating that the activity of this reporter was androgen-dependent (Fig. 2A, third lane). The relatively low activity of CP248 and CP461, although they were tested at high concentrations with respect to growth inhibition and apoptosis, is consistent with our studies above on PSA secretion (Fig. 1).

Therefore, we also tested CP248 and CP461 in probasin-luciferase reporter assays using very high concentrations of CP248 and CP461, i.e., 10 and 50 μM, respectively. Again, we found only partial inhibition of CP248 and CP461, compared with the marked inhibition seen with exisulind and SS (Fig. 2B). We obtained qualitatively similar results with these sulindac-related compounds when we assayed for ARE activity using a PSA promoter-luciferase reporter (Fig. 2C). Results were statistically significant using student t test with P < 0.001 (SigmaPlot, SPSS United Kingdom).

**Sulindac-Related Compounds Do Not Directly Block Binding of the AR to an ARE.**

The above described inhibition of the transcriptional activity of an ARE by these compounds might be attributable to direct inhibition of the binding of the AR protein to the ARE DNA sequence. We tested this possibility by incubating LNCaP WCEs together with a radio-labelled ARE probe in the absence or presence of each of the sulindac derivatives and then carried out electrophoretic mobility gel shift assays (Fig. 3). Fig. 3, Lane 3, shows that binding of the LNCaP WCE to the ARE produced three major complexes. The addition of a 10-fold excess of the nonradioactive ARE sequence markedly inhibited the formation of these three bands (Fig. 3, Lane 2), indicating that the binding of the labeled oligonucleotide was saturable. We then added to the reaction mixture containing the labeled ARE DNA sequence and WCE the following compounds: 0.1% DMSO (Fig. 3, Lane 4); 0.2 mM SS (Fig. 3, Lane 5); 0.5 mM exisulind (Fig. 3, Lane 6); 0.5 μM CP248 (Fig. 3, Lane 7); 5 μM CP461 (Fig. 3, Lane 8); or 5 mM aspirin (Fig. 3, Lane 9). None of these drugs significantly inhibited the binding of the ARE to cellular proteins (compare with the control Fig. 3, Lanes 2 and 3). These results suggest that none of these compounds directly inhibit the formation of the AR-ARE complex.

**Sulindac-Related Compounds Decrease AR Protein Levels but Do Not Alter HSP70 Expression.**

Another possible explanation for the ability of these compounds to inhibit the transcriptional activity of the ARE is that they might cause a decrease in cellular levels of the AR protein. We investigated this possibility using Western blot analysis of the AR protein.
When compared with the 0.1% DMSO control cells, 0.2 mM SS or 0.5 mM exisulind caused a decrease in AR levels as early as 10 h after treatment (Fig. 4, left panel). LNCaP cells treated with 0.5 μM CP248 or 5 μM CP461 displayed a decrease in AR levels within 20–24 h. However, no inhibition was seen with 5 mM aspirin. Similar results were obtained in three independent experiments. These findings are consistent with our earlier results on the relative effects of these compounds in inhibiting secreted...
Fig. 2 A, effects of sulindac-related compounds on androgen-responsive probasin promoter-luciferase reporter activity. LNCaP cells were transiently transfected with a pCMV-β-gal and the probasin-luciferase reporter (left panel) or the PSA-luciferase reporter (right panel). The cells were grown in RPMI medium containing charcoal-stripped serum plus either 0.1% ethanol (vehicle control) or 20 nM DHT. The cells were treated with one of the following drugs: 0.1% DMSO; 0.2 mM SS; 0.5 mM exisulind; 5 μM CP461; 0.5 μM CP248; or 5 mM aspirin, for 20 h. Extracts were then assayed for luciferase activity and for β-gal activity as an internal control for efficiency of transfection, and the relative luciferase activity was calculated. Each sample was done in triplicate and the results as mean values. B, effects at higher concentrations of CP248 and CP461 on probasin promoter-luciferase reporter activity. Reporter assays were conducted as in Fig. 2A but with the following concentrations of drugs: 0.1% DMSO; 0.2 mM SS; 0.5 mM exisulind; 50 μM CP461; 10 μM CP248; or 5 mM aspirin. C, sulindac-related compounds inhibit androgen-responsive PSA promoter-luciferase reporter activity. Reporter assays were conducted with the same concentrations of drugs as in Fig. 2B but using the PSA promoter sequence linked to the luciferase gene reporter.
in AR mRNA levels, whereas 5 μM CP461 produced a moderate decrease. However, 0.5 μM CP248 caused no decrease at 10 h and only a slight decrease in AR mRNA at 20 h. There was no inhibition of AR mRNA levels in the cells treated with 5 mM Aspirin (Fig. 5). Agarose gel staining with ethidium bromide (data not shown) indicates equal loading and no mRNA degradation.

Sulindac-Related Compounds Do Not Decrease the Levels of an Exogenously Expressed AR Protein. We transiently transfected an exogenous AR expression vector driven by the CMV promoter into PC3 prostate cancer cells that do not otherwise express endogenous AR. We found that none of the sulindac-related compounds altered the level of this exogenously expressed AR protein (Fig. 6). These results suggest that the inhibitory effects of these compounds on the AR are specific to the promoter of the AR gene and do not affect the general transcriptional machinery or simply cause proteolytic degradation of the AR protein.

**Sulindac-Related Compounds Inhibit Transcriptional Activity of the Promoter of the AR Gene.** We next examined the possibility that the sulindac-related compounds might inhibit the transcriptional activity of the promoter of the AR gene. Therefore, we performed transient transfection luciferase reporter assays using two AR promoter-luciferase reporter plasmids, ARPN1 and ARPS1. ARPN1 contains a −1500 to +580 bp sequence of the AR gene linked to luciferase, and ARPS1 contains a −1250 to +580 bp sequence linked to luciferase. LNCaP cells were transfected with the respective plasmids, and the cells were then incubated for 20 h in charcoal-stripped RPMI 1640 containing 20 nM DHT, together with 0.1% DMSO (vehicle control) or each of the sulindac-related compounds, and cell extracts were analyzed for luciferase activity. We found that treatment of the ARPN1-transfected cells with 0.2 mM SS or 0.5 mM exisulind caused about a 90% inhibition of luciferase activity when compared with the 0.1% DMSO-treated cells. However, treatment with high doses of CP248 and CP461, 10 and 50 μM, respectively, caused only partial inhibition of luciferase activity. Cells treated with 5 mM aspirin showed no significant inhibition of luciferase activity (Fig. 5, left panel). Similar results were obtained with the ARPS1 plasmid (Fig. 5, right panel). These results were reproducible and statistically significant using the student t test with P < 0.001 (SigmaPlot, SPSS United Kingdom). They are consistent with the above-described ARE luciferase reporter assays (Fig. 2) and suggest that the decreased expression of PSA observed in Fig. 1, A and B, and the decrease in cellular levels of the AR mRNA and protein (Figs. 4 and 5), after treating LNCaP cells with these compounds may be due, at least in part, to inhibition of transcription of the AR gene. It is also apparent that CP248 and CP461 are relatively weak inhibitors of all of these effects when compared with cytotoxic doses of SS and exisulind.

**Sulindac-Related Compounds Down-Regulate the Transcription Factors SP-1, NF-1, and CREB but CP248 and CP461 Up-Regulate AP-1.** The AR gene promoter contains DNA binding sequences for several transcription factors (22). The above studies indicated that the sulindac compounds inhibited the transcriptional activity of a −1250 to +580 bp sequence of the AR gene (Fig. 5, right panel). To additionally explore the mechanism responsible for this effect, we examined the levels of expression of several proteins known to bind to

and intracellular levels of PSA (Fig. 1). Therefore, SS, exisulind, CP248, and CP461 cause a decrease in AR protein levels at concentrations similar to those that induce apoptosis (8, 9) and that cause a decrease in PSA levels in LNCaP cells (Fig. 1). Hsps are often bound to the inactive AR in the nucleus and Hsps and intracellular levels of PSA (Fig. 1). Therefore, SS, exisulind, CP248, and CP461 cause a decrease in AR protein levels at concentrations similar to those that induce apoptosis (8, 9) and that cause a decrease in PSA levels in LNCaP cells (Fig. 1). Hsps are often bound to the inactive AR in the nucleus and Hsp 90, 70, and 56 have been shown to coimmunoprecipitate with the AR protein (21). It has been previously reported that the promoter element of Hsp70B is activated in human monocytic cells treated with sodium salicylate and sulindac sulfoxide (7). However, we found that none of the sulindac-related compounds, nor aspirin, altered the level of the Hsp70 protein (Fig. 4, right panel). Thus, although the levels of the AR protein are decreased after treatment with these compounds, the levels of the Hsp70 protein, a partner of the AR within the nucleus, remain unaffected. These findings indicate that the sulindac-related compounds can preferentially down-regulate the AR protein and that the loss of the AR protein induced by these drugs is not simply because of nonspecific proteolysis.

**Sulindac-Related Compounds Down-Regulate the Level of AR mRNA.** We next determined the effects of these compounds on AR mRNA levels using Northern blot analyses. When compared with the DMSO control treated cells, 0.2 mM SS, 0.5 mM exisulind, and 5 μM CP461 caused a partial decrease in AR mRNA levels within 10 h of treatment (Fig. 5). After 24 h, 0.2 mM SS and 0.5 mM exisulind caused a marked decrease...
specific transcription control elements in this region of the AR
gene promoter (22). We found that when LNCaP cells were

treated with either SS, exisulind, CP248, or CP461 for 24 or

48 h, there was a marked decrease in the transcription factors

SP-1, NF-1, and CREB (Fig. 8). The transcription factor AP-1

contains the proteins c-jun and c-fos. Treatment with SS and

exisulind caused a slight increase in c-jun, but treatment with

CP248 or CP461 caused a greater increase in c-jun and also a

marked increase in c-fos (Fig. 8). In view of the above results, we transfected LNCaP cells with

a reporter plasmid containing four cassettes of the AP-1 promoter

sequence linked to a luciferase reporter (23). The cells were incu-

bated in charcoal-stripped RPMI 1640 containing 20 nM DHT,
together with DMSO (vehicle control) or each of the drugs, for

20 h. Cell extracts were then analyzed for luciferase activity. We

found that 0.2 mM SS and 0.5 mM exisulind caused no change in

luciferase activity when compared with the 0.1% DMSO-treated
cells. However, treatment with CP248 or CP461 caused a marked
increase in luciferase activity (Fig. 9). Similar results were obtained

in three independent studies. These results are consistent with the

ability of CP248 and CP461 to induce increased expression of both
c-Jun and c-Fos (Fig. 8) and previous evidence that these two
compounds are very potent activators of JNK-1, which phospho-

rylates c-jun, thereby activating its transcriptional activity (24).

**DISCUSSION**

In previous studies, we demonstrated that exisulind, SS,

CP248, and CP461 inhibit growth and induce apoptosis in a

series of human prostate cancer cell lines, irrespective of bcl-2

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>AR</th>
<th>HSP 70</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>20</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>24</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
<tr>
<td>48</td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
<td><img src="https://via.placeholder.com/150" alt="Image" /></td>
</tr>
</tbody>
</table>

**Fig. 4** The effects of sulindac-related compounds on cellular levels of the AR
and Hsp70 proteins. LNCaP cells were treated with 0.1% DMSO, 0.2 mM SS, 0.5 mM exisulind, 0.5 μM CP248, 5 μM CP461, or 5 mM aspirin, for the indicated time (h). Fifty μg of WCE were run on 10% SDS-PAGE and immunoblotted with an anti-AR antibody (left panel) or with anti Hsp70 antibody (right panel). Numerical values below each band indicate relative image density with respect to the 10-h DMSO control sample.

**Fig. 5** Sulindac-related compounds cause a decrease in AR mRNA. LNCaP cells were treated with 0.1% DMSO, 0.2 mM SS, 0.5 mM exisulind, 0.5 μM CP248, 5 μM CP461, or 5 mM aspirin for 10 or 24 h, and RNA extracts were prepared. Twenty μg of total mRNA was subjected to Northern blot analysis using a 32P-labeled AR cDNA probe encoding the 1549–2782-bp sequence of the AR gene.
overexpression, androgen dependence, or COX expression. However, exisulind did not induce apoptosis in prostate epithelial cells derived from normal prostate tissue (9). In this study, we found that when the androgen-dependent prostate cancer cell line LNCaP was treated with concentrations of SS, exisulind, CP248, or CP461 that induce growth inhibition and apoptosis, within 24 h there was a decrease in both secreted and intracellular levels of the PSA (Fig. 1). Furthermore, reverse transcription-PCR analysis indicated that the mRNA levels of PSA were also decreased in the drug-treated cells (data not shown). In transient transfection reporter assays using sequences derived from either the PSA or the probasin genes, which contain AREs, SS, exisulind, CP248, and CP461 inhibited the transcriptional activities of these promoters (Fig. 2). Treatment with SS, exisulind, CP248, and CP461 also led, within 24 h, to a decrease in cellular levels of the AR protein and AR mRNA, and these compounds also inhibited the transcriptional activity of an AR promoter sequence in transient transfection reporter assays (Fig. 7). However, treatment of LNCaP cells with 5 mM aspirin, which induces other biological effects in cell cultures (25), did not affect cellular levels of the PSA and AR proteins, AR mRNA, or the above-described reporter assays (Figs. 1–3, 7).

These results indicate that SS, exisulind, CP248, and CP461 can cause a decrease in cellular levels of the PSA and AR proteins in human prostate cancer cells by inhibiting transcription of both the PSA and AR genes. The down-regulation of PSA by sulindac derivatives may be due, at least in part, to the decrease in the AR caused by these drugs (Fig. 4). Both the PSA and the AR genes contain AREs in their upstream promoter regions (26, 27). The decreased levels of the PSA and AR proteins in the treated cells do not appear to be simply because of an increase in proteolytic activity, associated with the apoptotic response induced by these compounds, because we did not see a parallel decrease in the proteins IGFBP-2, HSP70 or actin (Figs. 1, A and B, and 3A; data not shown). The PC3 prostate cancer cell line does not express the AR protein (28). When we expressed an exogenous AR protein in PC3 cells by transfection with an AR cDNA under the control of a CMV promoter, treatment of the transfected cells with the above compounds did not cause a decrease in the cellular level of the AR protein (Fig. 3C). These results provide additional evidence that the sulindac-related compounds do not act simply by causing proteolysis of the AR protein.

In addition to inducing parallel decreases in the PSA and AR proteins and their respective mRNAs (Fig. 3B and data not shown), these compounds directly inhibited the transcriptional activity of the AR in transient transfection reporter assays using ARE-containing promoters (Fig. 2A) and also inhibited the transcriptional activity of the promoter region of the AR gene (Fig. 7). Electrophoretic mobility shift assays indicate that these drugs do not act by directly inhibiting binding of the AR protein to ARE DNA sequences (Fig. 2B). Additional studies are required to determine whether these drugs act by altering the state of phosphorylation of the AR, inhibiting the function of cofactors that bind to the AR, or altering the levels or activities of one or more transcription factors required for mediating the transcriptional activity of the AR. With respect to the latter possibility, we found that treatment of LNCaP cells with SS, exisulind, CP248, or CP461 led to a marked decrease in cellular levels of the transcription factors SP-1, NF-1, and CREB (Fig. 8), and all three of these factors play a role in transcriptional activation of the promoter sequence of the AR gene (22). However, CP248 and CP461, but not SS and exisulind, induced increased expression of c-jun and c-fos and caused marked activation of AP-1 reporter activity (Figs. 8 and 9). Therefore, we postulate that these compounds impair the transcriptional activity of the promoter of the AR gene by depleting cells of critical transcription factors. The significance of the induction of AP-1 activity by CP248 and CP461 is discussed below.

Recent studies indicate that SS, exisulind, CP248, and CP461 inhibit the activity of the cyclic GMP-specific phosphodiesterases of gene families, 2 and 1 (8), and that the subsequent activation of protein kinase G mediates the apoptosis induced by these compound via both its inhibitory effects on β-catenin (8) and activation of the JNK-1 pathway (24, 29). It is curious that on a molar basis, CP461 and CP248 are ∼100- and 1000-fold more potent, respectively, than exisulind with respect to cyclic GMP phosphodiesterase inhibition and induction of apoptosis (8, 9), and yet in the present study, we found that when CP461 and CP248 were tested at relatively high concentrations, these drugs were considerably less active than SS or exisulind in inhibiting PSA or AR expression or in inhibiting AR-related transcriptional activity (Figs. 2 and 7). As discussed above, we found that CP248 and CP461, but not SS or exisulind, caused marked activation of AP-1 activity (Fig. 9). This may be because these two compounds also induced increased expression of c-jun and c-fos (Fig. 8), and both compounds are highly potent activators of JNK-1, which phosphorylates c-jun, and thereby activates its transcriptional activity (24). Because the promoter sequence of the AR gene also contains an AP-1 element (22), we postulate that the increased AP-1 activity induced by CP248 and CP461 may partially compensate for the decrease in other transcription factors, thus blunting the inhibitory effects of these two compounds on AR-related transcription. Additional studies are required to determine how the activation of protein kinase G might lead to differential effects on the expression of specific transcription factors. Additional studies are also required to exclude other possible mechanisms by which these compounds inhibit AR-mediated functions.

Finally, it is of interest to discuss possible clinical signif-
Significance of this study. Normal prostate development and maintenance depend on androgens, and at least 75% of the tumors in men with metastatic prostate cancer are androgen dependent at the initial time of diagnosis (30). Several therapeutic drugs target the steroid-AR interaction, whereas other drugs such as finasteride directly inhibit 5α-reductase, the enzyme that converts testosterone to its biologically active form DHT. Unfortunately, agents like flutamide can also act as agonists when they bind to mutant forms of the AR, which are frequently observed in prostate cancers (9). Thus, flutamide binds to the 868 (Thr<sup>a</sup> Ala) mutant of the AR, and this induces dissociation of the AR-Hsp complex, and thereby activates the transcription of androgen responsive genes (11). Moreover, there is increasing evidence that the overexpression of the AR gene might be a precursor to androgen independence (30). Therefore, it would be desirable to have antiprostate cancer drugs that bypass the function of mutant or amplified forms of the AR gene by directly inhibiting transcription of the AR gene in prostate cancer cells. In this context, the results obtained with sulindac-related compounds in this study may have considerable clinical significance.

Other investigators reported that the nonsteroidal antiinflammatory drug flufenamic acid, but not piroxican or sulindac sulfoxide, also inhibits expression of the AR in LNCaP cells (31). Our results using exisulind (sulindac sulfoxide) and SS, which are metabolites of sulindac sulfoxide, indicate that these compounds inhibit expression of the AR in LNCaP cells. Furthermore, in contrast to flufenamic acid, exisulind lacks COX inhibitory activity (9). In addition, although aspirin inhibits COX activity, we found that it did
not inhibit AR-related activity in LNCaP cells. Therefore, our results provide evidence that sulindac-related compounds inhibit AR-related activity by a COX-independent mechanism. We should emphasize that exisulind and related compounds also induce apoptosis in androgen-independent prostate cancer cell lines (9). Therefore, the clinical use of these compounds would not be limited to patients with androgen-dependent prostate tumors. At the same time, we hypothesize that inhibition of the AR could augment the antiproliferative effects of these compounds in androgen-dependent tumors and precursor lesions. This may be of particular importance in the chemoprevention of prostate cancer.

In contrast to the drug suramin, which can decrease PSA secretion in the absence of growth inhibition (32), exisulind, CP248, and CP461 inhibit PSA and AR expression in LNCaP cells at concentrations similar to or higher than those needed for growth inhibition and apoptosis induction. Thus, it seems unlikely that a decrease in PSA levels in a prostate cancer patient treated with exisulind or related compounds will represent a false positive for tumor antiproliferative activity. Therefore, PSA levels should remain useful as biomarker for the efficacy of these compounds in clinical trials on prostate cancer therapy or chemoprevention. Although as emphasized above, the inhibitory effects on transcription of the AR may potentiate antitumor activity.

We should emphasize that in this study, SS and exisulind were used at relatively high concentrations, 0.2 and 0.5 mM, respectively. These concentrations are in the range used in previous cell culture studies with these compounds (8, 9, 33–36). However, they probably exceed maximum in vivo blood levels. On the other hand, during chronic administration to patients, these drugs may be concentrated in tumor tissue and also exert cumulative effects. Indeed, despite relative low blood levels sulindac, indomethacin, and exisulind do exert antitumor effects in rodents (10, 35, 37). Furthermore, the administration of exisulind to postprostectomy prostate cancer patients with rising levels of serum PSA caused a significant decrease in serum levels of PSA when compared with placebo controls (38).

ACKNOWLEDGMENTS
We thank Dr. Robert Matusik, Donald Tindall, A. O. Brinkmann, and Marian Sadar for providing the (ARR)3-luciferase, ARPN1-luciferase, pSVAR, and PSAP-luciferase reporter constructs, respectively. We also thank Drs. Masumi Suzui, Muneyuki Masuda, and Gerhard Sperl for their very helpful advice.

REFERENCES


Exisulind and Related Compounds Inhibit Expression and Function of the Androgen Receptor in Human Prostate Cancer Cells
