A Novel Sulindac Derivative Lacking Cyclooxygenase-Inhibitory Activities Suppresses Carcinogenesis in the Transgenic Adenocarcinoma of Mouse Prostate Model

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Abstract

Nonsteroidal anti-inflammatory drugs including sulindac are well documented to be highly effective for cancer chemoprevention. However, their cyclooxygenase (COX)-inhibitory activities cause severe gastrointestinal, renal, and cardiovascular toxicities, limiting their chronic use. Recent studies suggest that COX-independent mechanisms may be responsible for the chemopreventive benefits of nonsteroidal anti-inflammatory drugs and support the potential for the development of a novel generation of sulindac derivatives lacking COX inhibition for cancer chemoprevention. A prototypic sulindac derivative with a N,N-dimethylammonium substitution called sulindac sulfide amide (SSA) was recently identified to be devoid of COX-inhibitory activity yet displays much more potent tumor cell growth-inhibitory activity in vitro compared with sulindac sulfide. In this study, we investigated the androgen receptor (AR) signaling pathway as a potential target for its COX-independent antineoplastic mechanism and evaluated its chemopreventive efficacy against prostate carcinogenesis using the transgenic adenocarcinoma of mouse prostate model. The results showed that SSA significantly suppressed the growth of human and mouse prostate cancer cells expressing AR in strong association with G1 arrest, and decreased AR level and AR-dependent transactivation. Dietary SSA consumption dramatically attenuated prostatic growth and suppressed AR-dependent glandular epithelial lesion progression through repressing cell proliferation in the transgenic adenocarcinoma of mouse prostate mice, whereas it did not significantly affect neuroendocrine carcinoma growth. Overall, the results suggest that SSA may be a chemopreventive candidate against prostate glandular epithelial carcinogenesis.

Introduction

One in six American men will be diagnosed in their lifetime with prostate carcinoma (PCa), which is the second leading cause of male cancer death in the United States (1). Available treatment options including surgery, hormone ablation, radiation, and chemotherapy are not typically curative but only offer a temporary delay in the progression to hormone-refractory disease (2). Because prostate cancer has a long latency and progresses slowly, chemoprevention is considered as a highly plausible approach to reduce the incidence of this lethal disease through early intervention (3, 4).

Epidemiologic, laboratory, and clinical studies have shown that the nonsteroidal anti-inflammatory drugs (NSAID) including sulindac have promising chemopreventive efficacy against several types of carcinomas such as colon, breast, esophagus, bladder, stomach, lung, ovary, and prostate (5–11). These nonselective NSAIDs possess cyclooxygenase (COX)-inhibitory activities (including COX-1 and COX-2; 12), which are generally considered to account for their chemopreventive efficacy (13–15). Long-term use of NSAIDs, however, including the selective COX-2 inhibitors (celecoxib and rofecoxib) has been linked to serious gastrointestinal, renal, and/or cardiovascular side effects due to COX-1 and/or COX-2 inhibition, limiting their clinical use for chemoprevention (16–19). The pharmacologic effects of NSAIDs are undoubtedly complex and may involve both COX-dependent and COX-independent mechanisms. Recent studies suggest that COX inhibition is ancillary for the antineoplastic activities of NSAID (20–23), and numerous mechanisms have been suggested to explain their chemopreventive benefits (24–27), although the precise molecular target

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(s) and mechanisms have not been well defined. Thus, it may be feasible to develop a novel generation of NSAID derivatives lacking COX inhibition for cancer chemoprevention. Further studies to define their mechanism of action will help delineate the crucial activities and targets of this important drug class.

Sulindac is considered to be one of the most promising NSAIDs because of its ability to dramatically regress the colorectal adenomatous polyps in patients (28–31). Sulindac sulfide amide (SSA; Fig. 1A) is a novel prototypic sulindac derivative and was specifically designed to replace the carboxylic acid with a positively charged N,N-dimethylethyl ammonium moiety to disrupt COX-1 and COX-2 binding (32). SSA displayed ∼40-fold higher potency to inhibit the growth of human HT-29 colon tumor cells compared with the parent drug, sulindac sulfide (32). In addition, gastric gavage administration of SSA inhibited the growth of colon cancer xenografts in athymic mice (32). Earlier work with sulindac sulfone, an irreversible metabolite of sulindac, has shown a suppression of androgen receptor (AR) and its signaling in LNCaP prostate cancer cells (33). These results suggested the potential that SSA might prevent prostate carcinogenesis through novel COX-independent mechanism involving the suppression of androgen signaling.

The transgenic adenocarcinoma of mouse prostate (TRAMP) model, originally developed by Greenberg and

![Fig. 1.](image_url)

A, the chemical structure of sulindac sulfide and its N,N dimethylaminoethylamide derivative SSA. B, the growth-inhibitory response curves of prostate cancer cells exposed to SSA for 48 h by crystal violet staining. Points, mean (n = 3); bars, SEM. C, cell cycle distribution of LNCaP, LNCaP C4-2, and TRAMP-C2 cells after 24 h of SSA treatment. D, suppression of cyclin D1 and cyclin E1 abundance after 24 h of SSA treatment and induction of cleaved poly ADP ribose polymerase (PARP) and apoptotic nucleosomal fragmentation (Death ELISA) after 6 or 24 h of SSA treatment in LNCaP cells. Flow cytometry experiments and ELISA were done in triplicate flasks. Statistical significance from control was \( P < 0.05 \) (*) and \( P < 0.01 \) (**).
colleagues (34), is driven by the rat probasin promoter–driven SV40 T-antigen (T-Ag) expression. The TRAMP model is advantageous for chemoprevention studies compared with the commonly used prostate cancer cell xenograft models because carcinogenesis occurs in situ from initiated cells in the transgenic mice, affording the opportunity to examine the action of chemopreventive agents on early-stage lesions, whereas the xenograft models deal with frank cancer cells. Recent studies including our own have shown that C57Bl/6 TRAMP mice represent two distinct models of prostate carcinogenesis: one involving AR-dependent glandular epithelial lesions and the other involving AR-independent neuroendocrine (NE)-like poorly differentiated (PD) carcinomas (35–37). The epithelial lesions, driven by AR-transactivated T-Ag expression, usually arise from the dorsal-lateral prostate (DLP) lobes and progress in a stepwise manner from prostatic intraepithelial neoplasia (PIN) to more advanced stage lesions. The epithelial lesions are characterized by the expression of AR and epithelial markers such as E-cadherin. In contrast, the NE-like lesions are driven by Foxa1- and/or Foxa2-transactivated (but not AR transactivated) T-Ag, and usually originate from the ventral prostate (VP) lobe, expanding rapidly to large PD carcinomas. The NE-like carcinomas are characterized by the expression of NE markers such as synaptophysin, but AR and E-cadherin are absent (37). In the C57Bl/6 background, the lifetime incidence of the NE-like PD carcinomas has been estimated to be 20%. The TRAMP mouse model is by far the most widely available and practical transgenic model for prostate cancer chemoprevention studies.

In the present study, we examined potential COX-independent antineoplastic targets of SSA, especially AR signaling pathway and mechanisms of action in prostate cancer cell lines, and evaluated its in vivo chemopreventive efficacy in TRAMP mice.

Materials and Methods

Compounds

SSA was synthesized by Drs. R.C. Reynolds and G.A. Piazza (Southern Research Institute, Birmingham, AL) as reported recently (32). For the cell culture studies, SSA stock solution was prepared in DMSO and stored at −20°C. For the animal study, SSA was mixed with Teklad 2918 powder diet at Southern Research Institute and shipped overnight delivery to The Hormel Institute. The test diet was stored at 4°C.

Cell culture

The androgen-dependent human LNCaP cells were obtained from the American Type Culture Collection and were cultured under the recommended conditions. The androgen-independent variant LNCaP C4-2 cells were a generous gift from Dr. Donald Tindall (Mayo Clinic, Rochester, MN) and were cultured in the same condition as LNCaP cells. Both cell lines retain functional mutant AR. The mouse TRAMP-C2 cells were obtained from Dr. Michael Grossmann (The Hormel Institute, Austin, MN) and was cultured in American Type Culture Collection–recommended conditions. The TRAMP-C2 cells retain AR expression and highly proliferative as indicated by S-phase and G2 combined fractions of over 60%. All cells were maintained in their appropriate medium supplemented with 10% fetal bovine serum, and were treated in the same medium supplemented with 1% fetal bovine serum except when indicated otherwise. All the cells were maintained in the standard 37°C and 5% CO2 humidified environment.

Growth assay

Crystal violet staining of cellular proteins was used to evaluate the overall growth-inhibitory effect of SSA on PCa cells as previously described (38). Briefly, the PCa cells were treated with SSA and vehicle control for 48 hours, and then the culture medium was removed and the cells remaining attached were fixed in 1% glutaraldehyde in PBS for 15 minutes followed by staining with 0.02% crystal violet solution. After extensive washing with distilled water, the plates were air dried. The retained crystal violet dye was dissolved in 70% ethanol, and the optical absorbance was measured at 570 nm with the reference 405 nm using a microplate reader (Beckman Coulter).

Cell cycle distribution

Cells were exposed to SSA at indicated doses for 24 hours, collected by trypsinization, and then the cell cycle distribution were analyzed by flowcytometry and propidium iodide staining as we previously reported (39).

Western blotting

The whole-cell lysate and mouse tissue lysate were prepared, and the Western blots were done as previously described (37). Anti–E-cadherin and cleaved poly ADP ribose polymerase were obtained from Cell Signaling Technology. Anti-AR, anti–cyclin D1 anti–cyclin E1, and anti–proliferating cell nuclear antigen were obtained from Santa Cruz Biotechnology, Inc. Anti-synaptophysin was obtained from BD Transduction Laboratories. Anti–SV40 T-Ag was from Calbiochem.

Death ELISA for apoptotic DNA nucleosomal fragmentation

After exposure to SSA for 6 or 24 hours, the floating and attached cells were collected. The released oligonucleosomes after gentle lysis of the cells were quantified by Cell Death ELISA System double sandwich kit (Roche Diagnostics GmbH) as we previously described (38).

RNA isolation and real-time PCR

Total RNA was isolated from LNCaP cells exposed to SSA or vehicle (0.1% DMSO) for 24 hours by using the RNeasy Mini kit (Qiagen). Total RNA (2 μg) from each sample was reverse transcribed by using Oligo-dT primers according to a reverse transcription Super Script II RT kit manual (Invitrogen). Real-time PCR was done on an
Applied Biosystems 7500 machine by using FastStart Universal SYBR Green Master (Roxy) system (Roche Diagnostics GmbH) and the primers of AR and prostate-specific antigen (PSA; Sigma-Genosys). β-Actin was used as a normalizing control. The PCR condition was 1 cycle of 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C, 1 minute at 60°C, then followed by 1 cycle of 15 seconds at 95°C, 1 minute at 60°C, 15′ at 95°C, and 15′ at 60°C referred to previously described (40). AR primers were forward 5′-CAG GAA GGA GGA GAG GCT TC-3′ and reverse 5′-AGG AAG GCT GCA AAG GAG TC-3′. PSA primers were forward 5′-CCC ACT GCA TCA GGA ACA AA-3′ and reverse 5′-GAG CCG GTG TGG GAA GCT-3′.

Stable transfection and luciferase reporter assay
LNCaP cells were seeded and allowed to grow to 80% to 90% confluence. Transfection of PSA-luc was carried out with the Lipofectamine 2000 reagent (Invitrogen) as previously described (41). PSA-luc is a luciferase reporter driven by a 6-kb PSA promoter (kindly provided by Dr. Charles Young, Mayo Clinic, Rochester, MN). After 2 weeks of selection with G418 (Invitrogen), the cells stably expressing PSA-luc were amplified and referred to as LN-PSA-luc. Luciferase reporter assay was carried out as previously described (42). Briefly, the cells were seeded in complete growth medium and were allowed to grow to 40% to 50% confluence, and then were fed phenol red-free RPMI1640 medium supplemented with 10% charcoal-stripped serum for 24 hours. The cells were treated with 0.5 nmol/L mibolerone (kindly provided by Dr. Charles Young, Mayo Clinic, Rochester, MN) alone or combined with indicated doses of SSA in phenol red-free RPMI1640 medium supplemented with 10% charcoal-stripped serum for another 24 hours. Decursin at 10 μmol/L was used as a positive control as previously described (43, 44). The whole-cell lysate was processed according to the Promega Luciferase System protocol (Promega Corp.) and was analyzed using a Luminoskan (Thermo Electron Corp.). The luciferase activity was normalized to protein concentration.

Chemoprevention study of spontaneous carcinogenesis in TRAMP mice
The animal study was approved by the Institutional Animal Care and Use Committee of the University of Minnesota and was carried out at the Hormel Institute animal facility. The C57BL/6 TRAMP mice were bred and genotyped as previously described (37). Forty-one (ages 6 wk) male C57BL/6 TRAMP mice were randomly divided into two groups and fed Teklad 2918 powder diet without supplement (n = 21) or that diet supplemented with 2,000 ppm SSA (n = 20), as were 17 wild-type (WT) male mice (n = 8 or n = 9, respectively). The choice of a 2,000-ppm dosage was based on a feeding experiment in which this dosage was effective against colonic carcinogenesis in Fox Chase Cancer Center Min transgenic mice.4 The male mice were housed individually to prevent fighting and aggression-related complications. All the mice were monitored daily and weighed weekly, and euthanized at 24 weeks of age except for two that had to be killed early (21 and 23 wk of age) because of large tumor burden. Blood was collected before euthanasia for serum preparation. The lower genital urinary (GU) tract, including seminal vesicle, prostate, testes, and bladder, was dissected and weighed. The DLP lobes, VP lobes, large tumors, and swollen lymph nodes were carefully dissected and weighed. A portion of the specimen was fixed for 24 hours in 10% (v/v) neutral-buffered formalin (Fisher Scientific) and stored in 70% ethanol until processing for histology and immunohistochemistry (IHC). The other portion was frozen on dry ice and stored at -70°C for later molecular biomarker analyses.

Histology and IHC
H&E and IHC staining were routinely done as previously described (37). Briefly, the prostate tissues and tumors were processed, and the paraffin-embedded sections (4-μm thickness) were used for H&E and IHC staining. The anti-AR (Upstate), anti–E-cadherin (Cell Signaling Technology), and anti-synaptophysin (BD Transduction Laboratories) were diluted in PBS supplemented with 1% bovine serum albumin at 1:400, 1:400, and 1:150, respectively. The lesions were quantified based on the percentage of different stages of lesions within each slide based on the published classifying criteria (35, 36, 45).

Measurement of SSA and sulindac sulfide levels in serum
Mice were bled on the day of sacrifice within 4 hours of the onset of the light cycle in the morning. Serum was processed and analyzed for SSA and sulindac sulfide levels using reverse-phase chromatography (Perkin-Elmer Series 200 autosampler and micropumps) with tandem mass spectrometry (Perkin-Elmer Sciex API 3000) detection operating in the positive ion mode using multiple reaction monitoring as previously described (32).

Statistical analyses
Results were analyzed by ANOVA or t test to determine the statistical significance among or between the specific groups.

Results
SSA inhibited the growth of human and mouse prostate cancer cells in vitro with enhanced potency compared with sulindac
Because prostate adenocarcinomas arise from AR-expressing cells, we determined the potency of SSA to inhibit the growth of prostate cancer cells expressing AR. These included human LNCaP and its variant LNCaP C4-2 cells and mouse TRAMP-C2 (derived from a prostate tumor of a TRAMP mouse). After exposure to SSA for 48 hours, the number of cells remaining attached was

measured by crystal violet staining of cellular proteins. As shown in Fig. 1B, SSA decreased the number of human PCa cells in a dose-dependent manner with a GI50 of ∼6 μmol/L. The mouse TRAMP-C2 cells were suppressed to even a greater extent, with GI50 of ∼3 μmol/L, probably due to much faster growth than the human cells. The growth-suppressing potency of SSA on prostate cancer cells was comparable with that of colon tumor cells as previously reported and was at least an order of magnitude higher than sulindac sulfide (32).

**SSA inhibited the proliferation of prostate cancer cells associated with an induction of G1 arrest**

Because SSA displays potent PCa cell growth-inhibitory activity, but is devoid of COX-1– or COX-2–inhibitory activity (32), we sought to investigate the cellular mechanism for its COX-independent antineoplastic activity by determining its effects on cell cycle progression and apoptosis. As shown in Fig. 1C, SSA caused a concentration-dependent increase in the percentage of cells in G0-G1 phase with a reduction of cells in S phase in the LNCaP and LNCaP C4-2 cell lines, which is indicative of an induction of G1 arrest. The TRAMP-C2 cells contained much higher S and G2 fractions compared with the human cells and showed G1 arrest upon SSA exposure (Fig. 1C). Furthermore, SSA treatment decreased cyclin D1, one of the crucial cyclins needed to activate CDK4/6 to promote G1-S transition (Fig. 1D). At higher exposure levels, SSA induced caspase-mediated apoptosis of prostate cancer cells after a treatment of 6 and 24 hours as indicated by increased poly ADP ribose polymerase cleavage and increased DNA oligonucleosomal fragmentation detected by Death ELISA (Fig. 1D). Taken together, these data suggested that SSA inhibited the in vitro growth of prostate cancer cells by G1 arrest, as well as the induction of apoptosis.

**SSA downregulated AR and suppressed AR-dependent transactivation in prostate cancer cells**

Because AR is indispensable for the G1-S transition and proliferation of the androgen-dependent prostate cancer cells (46, 47) and sulindac sulfone, an irreversible metabolite of sulindac, has been shown to inhibit AR signaling (33), we were interested in whether AR signaling was affected by SSA treatment. As shown in Fig. 2A, both AR mRNA and protein levels were decreased by SSA in LNCaP C4-2 cells after 24 hours of treatment. SSA treatment decreased the mRNA level of PSA, which is an endogenous target of AR-dependent transactivation (Fig. 2B). SSA also inhibited PSA expression in LNCaP cells (data not shown). Furthermore, a luciferase reporter driven by a 6-kb human PSA promoter was dose-dependently inhibited by SSA in LNCaP cells (Fig. 2C), providing direct evidence of the inhibitory activity of SSA on AR-dependent transactivation. Taken together, these data showed that SSA suppressed AR signaling, as manifested by PSA, at least in part through a downregulation of AR level in prostate cancer cells.

**SSA treatment suppressed dorsolateral prostatic growth (weight) in the TRAMP mice**

Encouraged by the in vitro results, we evaluated the in vivo chemopreventive efficacy of SSA using the TRAMP

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**Fig. 2.** SSA suppresses AR expression and antagonizes AR signaling in human prostate cancer cells. A, downregulation by 24 h of SSA treatment of both AR mRNA (top, real-time PCR) and AR protein (bottom, Western blot) levels in LNCaP C4-2 cells. The mRNA levels were normalized to those of the vehicle controls. B, suppression by 24 h of SSA treatment of PSA mRNA level (real-time PCR) in LNCaP C4-2 cells. The mRNA levels were normalized to those of the vehicle controls. C, dose-dependent inhibition of PSA-luc reporter in LNCaP cells by SSA treatment for 24 h. The luciferase activities were normalized to protein concentration and then were converted into the relative folds to the mibolerone-stimulated control. Mibolerone is a stable analogue of dihydrotestosterone. Decursin is an inhibitor of AR signaling used as a positive control (44). PSA-luc is a luciferase reporter driven by a 6-kb human PSA promoter. The assays of real-time PCR and luciferase reporter were done in triplicate. Statistical significance from control were P < 0.05 (*) and P < 0.01 (**).
The weights of GU tracts and DLP lobes are commonly used as gross parameters of prostatic carcinogenesis in TRAMP mice (34, 45). Consumption of SSA from 6-24 weeks of age decreased the body weights of TRAMP and WT mice by 5.6% and 16.2%, respectively (Fig. 3A). To correct for the difference due to body weight, the GU tract, DLP, and VP weights were normalized against the end point body weights (Fig. 3B-D). Consumption of SSA at 2,000 ppm in the diet significantly repressed both the actual and relative weight increments of the GU tracts of TRAMP mice (Fig. 3B) by 64.1% and 63.7% ($P < 0.001$ and $P < 0.001$), respectively, in reference to their baseline weight in the age-matched WT mice. SSA also significantly decreased those of the DLP lobes of TRAMP mice by 64.0% and 61.9% ($P < 0.001$ and $P < 0.001$), respectively (Fig. 3C). SSA numerically reversed the increased VP weights (20-30% greater than those of the WT mice) of TRAMP mice to the level of WT mice (not statistically significant; Fig. 3D).

It is noteworthy that three mice with large tumors, one weighing 7.11 g from the control group, and the other two...
weighing 1.59 and 2.59 g from the SSA-fed group, were excluded from the above analyses. These tumors were much larger compared with the whole prostates, and they arose from a lineage different (confirmed later as NE-like carcinomas (see Figs. 4 and 5) from the DL prostate glandular epithelial lesions.

Dietary consumption of SSA did not affect the actual and relative weights of the GU tracts (P > 0.05), DLP lobes (P > 0.05), and VP lobes (P > 0.05) of WT mice (Fig. 3B-D). Taken together, these data show that dietary SSA consumption repressed the T-Ag–driven dorsolateral prostatic growth in the TRAMP mice without an evident inhibition of the rapid growing, poorly differentiated carcinomas, which are characterized as NE carcinomas by synaptophysin expression (see below).

SSA treatment retarded the AR-expressing glandular epithelial lesions but not AR-negative NE carcinomas

To further verify the chemopreventive efficacy of SSA in vivo, the DLP lobes, VP lobes and tumors were processed for histopathologic analyses. We distinguished both AR-dependent glandular epithelial lesions and AR-independent PD NE carcinomas/lesions by the differential expression patterns of AR and E-cadherin versus synaptophysin (Figs. 4A versus B and 5). In addition to the three large NE-like carcinomas, four microscopic NE-like lesions (three from the control group and one from the SSA supplement group) were found in the VP lobes (Fig. 4D). The overall NE lesion incidence (17.1%, 7 of 41 mice) was similar to the previous reports (~20%) of C57BL/6 TRAMP mice (35, 36). We excluded these mice from the histologic analyses of the DLP and VP epithelial lesions to eliminate the potential confounding effect of NE carcinoma on these epithelial lesions in the same mice. One lymph node metastasis was found in each group (Fig. 4D), verified by the presence of T-Ag and the absence of AR staining by IHC (data not shown).

Dietary SSA consumption increased the percentage of normal and/or lower grade lesions (low-grade and high-grade PINs), but decreased that of the well-differentiated and moderately differentiated adenocarcinomatous lesions in the DLP lobes (Fig. 4A and C). SSA did not significantly affect the lesion distribution patterns in the VP lobes (Fig. 4A and C). SSA did not affect the incidence of NE-like lesions (Fig. 4B and D). The histology of the normal DLP and VP lobes of the WT mice was not affected by SSA (Fig. 4A and C). Taken together, these histopathologic data suggest that SSA delayed the progression of the AR-expressing glandular epithelial lesions but did not affect the AR-negative NE lesions/carcinomas in the TRAMP mice.

Dietary SSA downregulated biomarkers of cell proliferation in the TRAMP mice

To delineate the molecular effectors of SSA in vivo, we randomly selected several DLP samples and NE carcinomas for Western blot analyses. Consistent with the IHC results, E-cadherin and AR were detected in the DLP of the TRAMP mice and also in the WT mice, albeit much lower levels, but were undetectable in the NE carcinomas. In contrast, synaptophysin was detected in the NE carcinomas, which expressed massive amount of T-Ag, but was absent in the DLP (Fig. 5). Compared with TRAMP mice receiving the control diet, dietary SSA consumption suppressed AR and T-Ag signals in the DLP lobes of TRAMP mice. In addition, SSA decreased proliferating cell nuclear antigen (PCNA, a proliferative marker) and cyclin E1 (a crucial cyclin for G1-S transition and proliferation in TRAMP mice) in the DLP of TRAMP mice. Interestingly, the cyclin D1 level was significantly reduced in the TRAMP DLP when compared with the WT DLP, and this loss was attenuated in the SSA-treated TRAMP DLP. SSA did not significantly affect those in the NE carcinomas, which lacked the expression of AR. Furthermore, we did not detect increased cleavage of poly ADP ribose polymerase, an apoptosis marker of caspase activation, in the DLP of SSA-supplemented TRAMP mice compared with the DLP from control TRAMP mice (data not shown). The lack of in vivo proapoptosis activity emphasized the primary role of the antiproliferative activity of SSA in retarding the prostatic glandular epithelial lesion progression. Taken together, these data suggest that SSA suppressed epithelial cell proliferation and lesion progression in TRAMP mice associated with AR signaling inhibition.

Dietary consumption led to elevated serum SSA level and prostate tissue accumulation

Measurement of serum SSA and sulindac sulfide of five mice randomly picked from each group showed that feeding from 6 to 24 weeks of age resulted in average serum SSA levels of 3.3 and 4.9 μmol/L in the WT mice and TRAMP mice, respectively (Fig. 6A). Only a low level of sulindac sulfide at 0.4 and 0.7 μmol/L (Fig. 6A) was detected in WT and TRAMP mice, respectively. This indicates that SSA was not appreciably converted to the parent compound. Measurement of the anterior prostate lobe of TRAMP mice for SSA indicated approximately 250 ± 5 (SEM, n = 5 mice) nmol/g wet tissue, whereas sulindac sulfide was not detectable (Fig. 6B). These results therefore support the attainment of lower micromolar levels of serum SSA in mice fed with SSA with minimal back conversion to sulindac sulfide and the accumulation of SSA in prostate tumors.

Discussion

The lackluster performance of chemotherapy for advanced recurrent prostate cancer that has failed hormone ablation, surgery, and radiation calls for new approaches to combat this disease at its early stages. The long latency period and high prevalence of prostate cancer in men make chemoprevention of prostate carcinogenesis an attractive strategy to manage this disease by attacking it at its root.

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Fig. 4. Histopathologic evaluation of the effects of dietary SSA consumption on the TRAMP epithelial lesions and NE-like carcinomas. A, typical AR-dependent glandular epithelial lesions in the DLP and VP lobes of the TRAMP versus histology of the WT mice (magnification, ×200). IHC staining: E-cad, E-cadherin; Syn, synaptophysin. B, typical AR-negative NE carcinomas. All the NE-like lesions were PD lesions, synaptophysin-positive (arrow), but absent of E-cadherin and AR. The trapped glands (*) were E-cadherin and AR positive (arrowhead), but absent of synaptophysin. C, distribution patterns of the AR-dependent glandular epithelial lesions among the specific groups. WT−, WT mice without SSA supplement (n = 8); WT+, WT mice with 2,000 ppm SSA (n = 9); TRAMP−, TRAMP mice without SSA supplement (n = 17); TRAMP+, TRAMP mice with 2,000 ppm SSA (n = 17). N, normal; LP, low-grade PIN; HP, high-grade PIN; WD, well-differentiated carcinoma; MD, moderate differentiated carcinoma. Mean ± SEM. Statistical significance from the TRAMP− group was calculated by two-tailed t test. D, summary of the AR-independent NE carcinomas and metastases. PD, poorly differentiated carcinoma.
In the present study, we showed that SSA, a novel non-COX-inhibitory derivative of sulindac, significantly suppressed the growth of human and mouse prostate cancer cells expressing AR in vitro in strong association with G1 arrest at the lower micromolar levels of exposure and caspase-mediated apoptosis at higher concentrations (≥10 μmol/L). In vivo, dietary consumption of SSA led to the achievement of 4 to 5 μmol/L SSA with little conversion to sulindac sulfide (Fig. 6) and the attainment of 250 nmol SSA/g wet prostate tissue (∼250 μmol/L). Interestingly, SSA feeding retarded the AR-expressing epithelial spontaneous carcinogenesis in the TRAMP mice without evident efficacy against AR-negative NE carcinoma development (Figs. 4 and 5). There was no increased apoptosis in the SSA-supplemented DLP compared with the control DLP. Given that NE carcinomas arise de novo from the VP, not from trans-differentiation and progression of the AR-expressing DLP lesions (35, 36), our data suggest that antiproliferative effect might play a primary role in mediating the in vivo efficacy of SSA against the susceptible target AR-expressing epithelial cells in the TRAMP model.

Concerning molecular targets and mechanisms of action, we showed in vitro that SSA decreased AR and suppressed AR-dependent transactivation in prostate cancer cell culture models. It has been firmly established that AR plays an essential and critical role in the cell cycle progression and proliferation of androgen-dependent prostate cancer cells (46, 47). Some cell cycle regulators are the direct or indirect targets of AR-dependent transactivation, and AR silencing and/or androgen deprivation primarily block G1-S transition and progression (46, 47). Consistent with the cell culture results on AR suppression and signaling inhibition, we detected decreased AR abundance in the DLP of SSA-fed TRAMP mice by Western blot analyses. We also detected by Western blot the decreased expression of the T-Ag, which is mediated by AR transactivating the rat probasin promoter. We observed decreased cyclin E1 and proliferating cell nuclear antigen in the SSA-DLP samples, but no decrease of cyclin D1. Although the in vivo data do not allow a definitive establishment of cause-effect among the affected proteins, one scenario is that an inhibition of AR expression and signaling by SSA may result in a decreased expression of T-Ag because the probasin promoter/TA-g transgene is AR driven, and this in turn decreased the bound form of Rb to T-Ag, freeing up Rb to suppress E2F transcription of cyclin E1, leading to a blockage at the G1-S transition. Corroborating this scenario, in the NE cells in which high-level T-Ag expression is not driven by AR, SSA was not active to suppress their in vivo growth. These data suggest an antiproliferative action of SSA through cyclin E1-related cyclin-dependent kinases in vivo to inhibit AR-dependent glandular epithelial lesion progression. Alternatively, SSA consumption could inhibit epithelial cell proliferation, decreasing the proportion of AR- and T-Ag-expressing cells in the DLP gland. Consistent with this alternative scenario, IHC detection of AR expression at the cellular level did not show less staining intensity on a per cell basis for SSA-treated DLP versus control DLP (Fig. 4). Clearly, more detailed analyses of the relevant molecular targets and cellular pathways are crucial to resolve these issues.
Although we found cyclin D1 suppression by SSA in cell culture, this cyclin is lost over the course of lesion progression in the TRAMP model as evident in Fig. S5 when compared with WT DLP, consistent with the "cyclin switch" reported by Greenberg's laboratory (48). The upregulation of cyclin D1 in the SSA-treated TRAMP DLP in comparison with control TRAMP is therefore consistent with retarded epithelial lesion progression and highlights a difference between the TRAMP model and human cancer cells.

We should note that SSA exerted some adverse effect on body weight in TRAMP and WT mice (Fig. 3A), implying a possible narrow window between chemoprevention and safety. The athymic nude mice receiving gavage of SSA (250 mg/kg, bid, p.o.) displayed good tolerance and efficacy against colon cancer xenografts (32). We could not exclude the potential effect of different genetic backgrounds of the mice on their tolerance to SSA, nor dosing regimens. More studies with different strains of mice and good laboratory practice grade product will be helpful to address this issue.

Overall, this study suggests the feasibility of developing a novel generation of sulindac derivatives lacking COX inhibition for prostate cancer chemoprevention. We showed that SSA inhibited prostate glandular epithelial lesion growth and progression, at least in part through the suppression of cell proliferation. SSA did not display efficacy against AR-negative NE carcinomas in this model, suggesting a potential selectivity of the compound on AR signaling. Additional studies using preclinical models of prostate adenocarcinogenesis without the complication of NE carcinogenesis of the TRAMP model are necessary and essential to validate the efficacy and survival benefit of this novel sulindac derivative, as well as other analogues that may have potency and/or bioavailability advantages to provide compelling rationales for future translational consideration.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Yong Zhang, Jinhui Zhang, Lei Wang, et al.