The R-enantiomer of the nonsteroidal antiinflammatory drug etodolac binds retinoid X receptor and induces tumor-selective apoptosis

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Prostate cancer is often slowly progressive, and it can be difficult to treat with conventional cytotoxic drugs. Nonsteroidal antiinflammatory drugs inhibit the development of prostate cancer, but the mechanism of chemoprevention is unknown. Here, we show that the R-enantiomer of the nonsteroidal antiinflammatory drug etodolac inhibited tumor development and metastasis in the transgenic mouse adenocarcinoma of the prostate (TRAMP) model, by selective induction of apoptosis in the tumor cells. This proapoptotic effect was associated with loss of the retinoid X receptor (RXRα) protein in the adenocarcinoma cells, but not in normal prostatic epithelium. R-etodolac specifically bound recombinant RXRα, inhibited RXRα transcriptional activity, and induced its degradation by a ubiquitin and proteasome-dependent pathway. The apoptotic effect of R-etodolac could be controlled by manipulating cellular RXRα levels. These results document that pharmacologic antagonism of RXRα transactivation is achievable and can have profound inhibitory effects in cancer development.

Materials and Methods

Drug Preparation. R-etodolac was prepared from pharmaceutical-grade tablets of racemic etodolac to a purity of >97% as described (see Supporting Materials and Methods, which is published as supporting information on the PNAS web site) (20). The drug was titrated by Sibtech (Newington, CT) and purified with HPLC. The resulting material had a specific activity of 20–25 Ci (1 Ci = 37 GBq)/mmol, and it was stored in acetone at a concentration of 0.45 mCi/ml at −20°C. The RXR-selective retinoids SR11237, SR11345, and SR11246 were described in ref. 21 and provided by M. Dawson (The Burnham Institute). Staurosporine, MG-132 (Calbiochem) and 9-cis-RA (Sigma) were purchased commercially.

Cell Lines. LNCaP, PrEC, CV-1, ZR-75–1, and HEK 293T cells were maintained in standard media. The F9 murine embryonal carcinoma cell line with both alleles of RXRα maintained in standard media. The F9 murine embryonal carcinoma cell line with both alleles of RXRα disrupted was provided by P. Chambon (Institut de Genetique et de Biologie Moleculaire)

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Abbreviations: HA, hemagglutinin; NSAID, nonsteroidal antiinflammatory drug; COX, cyclooxygenase; RXR, retinoid X receptor; TRAMP, transgenic mouse adenocarcinoma of the prostate; LBD, ligand-binding domain; PPARγ, peroxisome proliferator-activated receptor γ; RA, retinoic acid; CAT, chloramphenicol transferase; siRNA, small interfering RNA; RAR, RA receptor; RARE, RA-response element.

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et Cellulaire, College de France, Illkirch, France) (22). See Supporting Materials and Methods for details.

**Murine Studies.** Transgenic mouse adenocarcinoma of the prostate (TRAMP) and C57BL/6 mice were purchased from The Jackson Laboratory and bred at UCSD. All animal protocols received prior approval by the institutional review board. Plasma R-etodolac levels were measured on a group of seven 15-week-old C57BL/6 male mice who were fed R- etodolac (1.25 mg/kg) chow for 2 weeks by a bioanalytical LC/MS-based method developed by Maxxam Analytics (Mississauga, ON, Canada). Chiral HPLC (23) was used to confirm the lack of R- to S-etodolac in vivo interconversion.

We started 46 male TRAMP mice at 9–12 weeks of age on chow with R- etodolac 1.25 mg/kg or control food (prepared by Dyets, Bethlehem, PA) randomized by cage. At 30 weeks, or after appearance of a gross palpable tumor mass, the animals were sacrificed and necropsies were performed. The urogenital system, the periaortic lymph nodes, and the major organs were removed and weighed. Tissues were fixed and stained with hematoxylin and eosin. The prostate sections were dissected and separated into individual lobes and weighed. Tissues were fixed in 10% formalin embedded in paraffin, sectioned in step sections at 50-μm intervals, and stained with hematoxylin and eosin. The prostate sections were scored for carcinoma grade on a 1–6 scale (see Supporting Materials and Methods). The liver, lung, and lymph node sections were scored for the presence or absence of tumor. The weights of the different tissues, and the frequencies of metastases, in the drug treated and control animals were compared by the Mann–Whitney test or Fisher’s exact test, with $P < 0.05$ considered significant.

**Ligand Binding.** The human RXRα LBD (223–462), prepared as a polyhistidine-tagged fusion protein in pET15b (Novagen) (1 μg), was incubated with radiolabeled ligand in the presence of different concentrations of unlabeled 9-cis RA or R- etodolac at 4°C for 14 h. The RXRα LBD was captured by nickel-coated beads. Bound radiolabeled ligand was determined in a scintillation counter.

**Immunohistochemistry and Apoptosis Assays.** For 2 weeks, we fed 6- to 7-month-old TRAMP mice R- etodolac supplemented or control chow. The prostates were removed, and serial frozen sections were assayed for terminal deoxynucleotidyl transferase (TdT; TUNEL; Chemicon) or stained with anti-human RXRα (D20; Santa Cruz Biotechnology) followed by staining with DAPI (50 μg/ml; Sigma) containing DNase-free RNase A (100 μg/ml; Boehringer Mannheim) to visualize the nuclei and examined by fluorescence microscopy (18, 19, 24). Single-cell apoptosis was detected in vitro by removing adherent cells from the plate with 5 mM EDTA, incubating them with annexin-V–phycoerythrin (BD PharMingen) and analyzing by flow cytometry.

**Transient-Transfection Assay.** Expression vectors for RXRα, RA receptor β (RARβ), hemagglutinin (HA)-ubiquitin, and reporter gene βRARE-tk-chloramphenicol transferase (CAT) were prepared and transfected as described (25, 26). We mixed ~300 ng of reporter plasmid, 50 ng of β-gal expression vector (pCH 110; Pharmacia), and vector expressing RXRα with carrier DNA (pBluescript) to give 1.0 μg of total DNA per well. CAT activity was normalized for transfection efficiency on the basis of cotransfected β-gal gene activity. Transfected cell lysates were separated by SDS/PAGE and immunoblotted (see Supporting Materials and Methods).

**RXRα Small Interfering RNA (siRNA) Transfections.** A SMARTpool of siRNAs specific for RXRα and GFP control siRNA were purchased from Dharmacon (Lafayette, CO). We identified the cell line with the highest response to androgen ablation using flow cytometry analysis of annexin-V– phycoerythrin positivity. We transfected LNCaP cells with siRNA specific for RXRα and GFP control siRNA for 2 days before determination of annexin-V–phycoerythrin positivity and apoptosis.

**Table 1. Incidence of primary tumors and metastases**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Treated</th>
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<tbody>
<tr>
<td>Primary tumor incidence*</td>
<td>24/24 (100%)</td>
<td>16/17 (94%)</td>
</tr>
<tr>
<td>Metastasis incidence†</td>
<td>14/24 (58%)</td>
<td>5/17 (29%)</td>
</tr>
<tr>
<td>Animals with gross masses‡</td>
<td>6/24 (25%)</td>
<td>2/17 (12%)</td>
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Percentages of mice are given in parentheses.

*No. of mice with histologic evidence of carcinoma (grade ≥4).
†No. of mice found to have histologic evidence of metastasis to lymph node, lung, or liver tissues. **P < 0.05** by Fisher’s exact test.
‡No. of mice found to have a gross urogenital mass at postmortem laparotomy.

![Fig. 1. Inhibition of prostate cancer progression in the TRAMP model. Male TRAMP mice were fed control chow or chow with R- etodolac (1.25 mg/kg). (a) At 30 weeks of age, or after development of a gross palpable mass, the mice were sacrificed, and the urogenital systems were removed and weighed. (b–f) The prostate lobes were separated from the other organs and weighed separately; anterior (c), ventral (d), lateral (e), and posterior (f). The prostates were not dissectible in mice that had gross tumor masses (two in the treatment group and six in the control group). The weights of the control urogenital tracts, lateral, and dorsal prostates were significantly higher than those of the treated group, as determined by the Mann–Whitney test.]
Activity of R-Etodolac in the TRAMP Model. Preliminary dose-ranging pharmacokinetic data showed that plasma concentrations of 370 ± 30 μM could be achieved by supplementing a standard mouse chow diet with 1.25 mg/kg R-etodolac for 2 weeks. Chiral HPLC revealed no detectable conversion of R-etodolac to the S-stereoisomer. Therefore, in vivo experiments in the TRAMP mouse model were undertaken under these conditions. Male TRAMP mice develop histological intraepithelial neoplasia of the prostate by 8–12 weeks of age that progresses to adenocarcinoma with distant site metastases by 24–28 weeks of age (27, 28). Control chow or diets supplemented with R-etodolac were initiated at 9–12 weeks. By 30 weeks, nearly all of the prostates in the untreated groups had macroscopic evidence of tumor (Table 1). However, both the average tumor mass (Fig. 1) and the frequencies of metastases (Table 1) were significantly lower in the R-etodolac-treated animals. Histological evaluation of the excised tissues confirmed the anti-metastatic effects of the drug and did not show evidence of drug toxicity (Fig. 2 a–l). Collectively, these data indicated that R-etodolac retarded the progression and metastasis of prostate cancer in the TRAMP system.

R-Etodolac SelectivelyInduces Apoptosis in Cancerous Prostates. To determine whether R-etodolac treatment resulted in apoptosis in vivo, 6- to 7-month-old male TRAMP and nontransgenic littermates were fed with R-etodolac or control chow for 2 weeks and
Similarly RXRα binding to RXRα and R-etodolac displacement of [3H]9-cis-RA bound to RXRα LBD (Fig. 4a), with an IC_{50} value of ~200 μM for R-etodolac. Furthermore, [3H]R-etodolac directly bound the RXRα LBD, and this binding was competitively inhibited by both unlabeled R-etodolac and 9-cis-RA (Fig. 4b and c).

**R-Etodolac Binding Induces Conformational Change in RXRα.** Binding of ligands to their receptors often induces changes in susceptibility to proteolysis (13, 14). Digestion of the RXRα LBD with a low concentration of trypsin (3 μg/ml) yielded a proteolytic fragment of ~20 kDa, whereas higher concentrations of trypsin (10 or 30 μg/ml) completely digested the LBD (see Fig. 8, which is published as supporting information on the PNAS web site). Preincubation of the RXRα LBD with 9-cis-RA did not alter its sensitivity to trypsin digestion, consistent with previous studies (29). However, incuba-
R-Etodolac inhibited transcriptional activity of endogenous RXRα. An RA-response element (βRARE) in the RARβ promoter, which binds various RXR-containing heterodimers including RXR/RAR and RXR/PPARγ (26, 30, 31), was transfected into ZR-75–1 breast cancer cells. Reporter activity was induced by all-trans-RA, presumably because of binding of endogenous RXR/RAR heterodimer. Cotreatment with R-etodolac suppressed all-trans-RA-induced reporter activity in a R-etodolac concentration-dependent manner, suggesting that R-etodolac inhibited transcriptional activity of endogenous RXR/RAR heterodimers (Fig. 5b). The transcriptional effect of R-etodolac on RXRα was confirmed by analyzing its effect on the expression of RARβ. Treatment of ZR-75–1 cells with all-trans-RA strongly induced the endogenous expression of RARβ (Fig. 5c), which was completely inhibited by R-etodolac, consistent with the inhibitory effect of R-etodolac on the βRARE reporter gene (Fig. 5b). To determine the effect of R-etodolac on RXR/PPARγ heterodimer activity, ZR-75–1 cells were stimulated with the PPARγ ligand ciglitazone and the RXRα ligand SR11237 in the presence or absence of R-etodolac (Fig. 5d and e) (30). The induction of endogenous RARβ expression by the two drugs in combination was abolished by R-etodolac cotreatment (Fig. 5e).

**Loss of RXRα Expression After R-Etodolac Treatment.** Subcellular localization of RXRα plays a role in the regulation of apoptosis (17). To analyze whether R-etodolac treatment altered RXRα subcellular localization, in vivo immunostaining of RXRα was performed on the prostate tissues from male TRAMP and nontransgenic littermates fed with R-etodolac or control chow. RXRα was predominantly localized in the nucleus in the prostates of the nontransgenic mice fed with or without R-etodolac chow (Fig. 6a). TRAMP mice fed with control chow displayed similar RXRα nuclear staining. However, RXRα staining was greatly reduced in prostates of TRAMP mice fed with R-etodolac.

RXRα protein levels in LNCaP cells were also markedly reduced after treatment with R-etodolac (Fig. 6b). R-etodolac-induced degradation of RXRα levels was completely prevented by the proteosome inhibitor MG132 (Fig. 6b). Proteins are often ubiquitinated before degradation by proteasomes (32). Thus, we determined whether R-etodolac induced ubiquitination of RXRα. Myc-tagged RXRα was cotransfected into HEK 293T cells with or without an expression vector for HA-tagged ubiquitin, followed by treatment with R-etodolac in the presence of MG132. Immunoprecipitation with anti-myc antibody, followed by immunoblotting with an anti-HA antibody, revealed that RXRα was extensively ubiquitinated after R-etodolac treatment (Fig. 6c) but not after treatment with the synthetic RXRα ligands SR112345 and SR11246 (Fig. 6d). Instead, these ligands abrogated R-etodolac-induced RXRα ubiquitination (Fig. 6e), probably because of their competition for binding to RXRα. Collectively, these results demonstrate that R-etodolac binds RXRα and induces its degradation in a proteasome-dependent manner.

**Discussion**

The standard therapy for progressive prostate cancer is androgen ablation. However, many patients become unresponsive and develop metastatic disease (33). Thus, there is a compelling need for the development of unconventional agents that can delay the progression of prostate cancer. In this article, we report that chronic oral administration of the COX-inactive R-steroisomer of the common NSAID etodolac inhibited tumor expansion and metab-
tasis in the TRAMP model. By analogy, R-etodolac could be a prospective agent for the treatment of human prostate cancer.

In the TRAMP model, treatment with the COX-2 selective agent celecoxib or the R-enantiomer of the NSAID flurbiprofen resulted in a significantly lower primary-tumor incidence and a reduced incidence of metastases (34, 35). However, both of these drugs may have exerted their effect by active COX inhibition because 15% of the R-flurbiprofen was converted to the active COX inhibitor S-flurbiprofen by 2–4 h after administration. In contrast, the stereoisomers of the conformationally rigid etodolac molecule, S-etodolac, was undetectable in the plasmas of the mice given diets supplemented with the R-stereoisomer. Hence, the cytostatic and antimitotic effects of R-etodolac in the TRAMP model must be attributed to the drug or to a metabolite.

The results presented here reveal an unexpected function of RXRα as a mediator of the apoptotic effect of R-etodolac. A recent study (12) demonstrated that inhibition of prostate tumor growth by R-etodolac was associated with initial enhancement of PPARγ transcriptional activity, followed by degradation of the receptor (12). However, ligand competition and direct binding assays using PPARγ recombinant protein failed to demonstrate any direct binding of R-etodolac to PPARγ (data not shown). Because PPARγ activity depends on heterodimerization with RXRα, it is possible that modulation of PPARγ and degradation by R-etodolac is mediated by its binding to RXRα. Antagonists of RXR homo- or heterodimers are known to function as agonists of RXR/PPARγ heterodimers (36, 37).

Exactly how RXRα mediates the apoptotic effects of R-etodolac remains unknown. It is unlikely that R-etodolac exerts its anticancer effect through its inhibition of RXRα transactivation (Fig. 5) because many RXR agonists potently inhibit the growth of prostate cancer cells. However, the binding of RXRα by R-etodolac could affect the function and stability of several nuclear receptors that dimerize with RXRα, including PPARγ and Nur77.

Our results demonstrate that R-etodolac induced apoptosis of prostate cancer, but not normal epithelium (Fig. 2 and 7). The contrasting effects might be attributable to differences in RXRα posttranslational processing in cancer and normal cells (38). In this regard, it was reported recently that RXRα was phosphorylated by MAP kinase in surgically resected hepatocellular carcinoma samples but not in the corresponding noncancerous surrounding tissues (39).

A recent population-based study of NSAID use and prostate cancer revealed that the relative odds of prostate cancer among the drug users was 0.2 (95% confidence interval 0.1–0.5) in men during the eighth decade of life but only 0.9 in men during the sixth decade, compared with similarly aged men who did not use NSAIDs (3). The stronger effect among older men raised the possibility that NSAIDs may prevent the progression of prostate cancer from latent to clinical disease, rather than reduce the frequency of primary lesions. The experimental results with R-etodolac in the TRAMP model of prostate cancer display many parallels with the human population data. Thus, it is possible that R-etodolac could represent a potential approach toward preventing the progression of hormone refractory prostate cancer, especially in the very elderly patients who are not candidates for cytotoxic therapy.

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