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Gene expression profiling in R-flurbiprofen-treated prostate cancer: R-Flurbiprofen regulates prostate stem cell antigen through activation of AKT kinase

Marina Zemskova [a], *William Wechter* [b], *Svetlana Bashkirova* [a], *Chien-Shing Chen* [c], *Robert Reiter* [d], *Michael B. Lilly* [a,*]

[a] *Center for Molecular Biology and Gene Therapy, Loma Linda University, Loma Linda, CA, United States*
[b] *Encore Pharmaceuticals, Ojai, CA, United States*
[c] *Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore*
[d] *Division of Urology, Department of Surgery, University of California at Los Angeles, Los Angeles, CA, United States*

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ABSTRACT

We have used gene expression profiling to characterize genes regulated by the anti-tumor non-steroidal anti-inflammatory drug (NSAID)-like agent *R*-flurbiprofen (RFB) in murine TRAMP prostate cancer. Mice with spontaneous, palpable tumors were treated with RFB 25 mg/(kg d) × 7d orally, or vehicle only. RNA was then extracted from tumor tissue and used for microarray analysis with Affymetrix chips. Fifty-eight genes were reproducibly regulated by RFB treatment. One of the most highly up-regulated genes was prostate stem cell antigen (*psca*). We used TRAMP C1 murine prostate cancer cells to examine potential mechanisms through which RFB could regulate *psca*. RFB induced dose-dependent expression of PSCA protein, and activity of the *psca* promoter, in TRAMP C1 cells in culture. Increased *psca* promoter activity was also seen following treatment of cells with sulindac sulfone, another NSAID-like agent, but not with celecoxib treatment. RFB activation of the *psca* promoter could be attenuated by co-transfection of dominant-negative *akt* and *h-ras* constructs, but not by dominant-negative *mek1* plasmids. Immunoblotting revealed that RFB increased expression of phosphorylated AKT at concentrations that stimulated *psca* promoter activity, and that increased PSCA protein expression. In addition, RFB-dependent up-regulation of PSCA protein expression could be blocked by AKT inhibitors. These data demonstrate that RFB, and possibly other NSAID-like analogs, can increase expression of the *psca* gene both *in vivo* and in culture. They further suggest the utility of combining RFB with AKT inhibitors or with monoclonal antibodies targeting PSCA protein, for treatment or prevention of prostate cancer.

1. Introduction

New therapeutic approaches to the prevention and treatment of prostate cancer are of great interest, due to the high prevalence the disease and its poor response to conventional anti-tumor agents. Many data support the development of non-steroidal anti-inflammatory drugs (NSAIDs) as agents that could effect both the prevention and the treatment of

* *Corresponding author at*: Center for Molecular Biology and Gene Therapy, Loma Linda University School of Medicine, 11234 Anderson Street, Loma Linda, CA 92354, United States. Tel.: +1 909 558 8777; fax: +1 909 558 0177.
E-mail address: mlilly@som.llu.edu (M.B. Lilly).
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prostate cancer [1–3]. Clinical development of NSAIDs has been limited, however, by the toxicities of the available drugs, such as aspirin, ibuprofen, and indomethacin. The recent development of less toxic NSAID-like drugs, sulindac sulfone (exisulind) and the cyclooxygenase (COX)-2 selective inhibitors such as celelcoxib, and exisulind, has renewed interest in these agents for chemoprevention and treatment of prostate cancer and other epithelial malignancies [4].

While the anti-tumor activity of such NSAID-like compounds is clear, the molecular mechanisms through which these drugs act are not. Inhibition of cyclooxygenase enzymes does not appear to be the primary mechanism [5–7]. Alternate molecular targets for NSAIDs have also been proposed. These include transcription factors such as NFκB [8], AP-1 [9], and PPARγ [10]; the AKT [11] and RSK2 [12] kinases; apoptosis-inducing proteins such as BAX [13] and PAR-4 [14]; and alternative arachidonic acid enzymes such as 15-LOX-1 [15]. Few studies compare various NSAIDs in the same assay system, preventing clear distinctions among the various agents. Furthermore, the observed effects are usually described from tissue culture systems, and occur at drug concentrations significantly higher than can be achieved in animals or patients. Not surprisingly, there are discrepancies between *in vitro* and *in vivo* anti-tumor effects of NSAIDs [16]. The rare studies that have identified NSAID-regulated targets in intact animals have focused on a small number of proteins. Thus, NS398, celecoxib, and rofecoxib have also been shown to decrease COX-2 protein in various tissues [17–19]. Rofecoxib can also decrease several proteins implicated in cancer development in a murine xenograft model, such as cyclin D1, β-catenin, and metalloproteinases [20]. However, there are no publications describing genome-wide searches for NSAID targets in animal models or patients.

Recently, we have investigated another NSAID-like compound, *R*-flurbiprofen (RFB, E-7869, MPC-7869), for potential use in the treatment or prevention of cancer. As with other drugs in the aryl propionic acid class, flurbiprofen can exist as *R*- and *S*-enantiomers that differ significantly in their biologic properties. The *S*-enantiomer is many times more active than RFB at inhibiting both COX-1 and COX-2. This may account for the near-complete lack of ulcerogenic activity of RFB, when tested in an animal model [21]. In spite of its lack of gastrointestinal toxicity, RFB demonstrates marked anti-proliferative activity [22]. In the APC^{min}/+ model of intestinal polyposis, RFB was able to both prevent the appearance of adenomas, and block the growth of established neoplasms, leading to a dramatic increase in lifespan of the treated animals [23,24]. RFB also strongly suppressed the development of both primary and metastatic adenocarcinomas in transgenic adenocarcinoma of murine prostate (TRAMP) mice [25].

Because of the lack of molecular data derived from clinically relevant models, we have performed gene expression profiling of TRAMP murine prostate cancers excised from mice treated for 7 days with RFB, to investigate early gene expression responses. We identified 58 genes that were reproducibly regulated by RFB. Several of these genes encoded tumor suppressors, differentiation markers and genes whose expression is lost during transformation, but some of up-regulated genes were associated with cell growth and survival, including the gene for prostate stem cell antigen, *psca*.

2. Materials and methods

2.1. Mice

TRAMP mice were derived from our breeding colony. The original breeding stock was kindly provided by Dr. Norm Greenberg (Fred Hutchinson Cancer Research Center) [26]. Breeding and use of the mice were under the auspices of an IACUC-approved protocol. Mice were used for study when they had palpable suprapubic tumor masses. RFB powder was suspended in 1% carboxymethylcellulose (CMC), then administered by gavage at 25 mg/(kg d) × 7d. Vehicle-treated mice received a similar volume of 1% CMC only.

2.2. Reagents

Pharmaceutical grade RFB (99% chirally pure) was synthesized as described [21].

2.3. RNA extraction and microarray analysis

Tumor tissue was excised and rinsed briefly with PBS. It was then cut into 3–4 mm cubes and immediately immersed in RNALater preservative solution (Ambion). Samples were stored in RNALater at −20 C for up to 1 week before RNA extraction. For RNA extraction, the RNALater was aspirated from the preserved tissues, and Trizol reagent was added. Tissues were homogenized in Trizol (Invitrogen) with a Tissue-Tearor instrument. RNA extraction then proceeded as per manufacturer's instruction, with the addition of a final, additional acid phenol–chloroform extraction, followed by ethanol precipitation. Satisfactory RNA preparations had an OD_{260}/OD_{280} ratio of 1.8–2.0, and showed intact 18S and 28S bands by agarose gel electrophoresis. Four biological replicates and two technical replicates were performed for each treatment condition. Two drug-treated animals and two vehicle-treated animals were used for each of two independent experiments, performed approximately 4 months apart. In each experiment, equal amounts of RNA from the two similarly treated animals were combined to create a drug-treated pool or vehicle-treated pool of RNAs.

First and second strand cDNA were synthesized from 5–15 μg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Gibco Life Technologies) and oligo-dT_{24}-T7 (5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-3′) primer according to the manufacturer's instructions. cRNA was synthesized labeled with biotinylated UTP and CTP by *in vitro* transcription using the T7 promoter-coupled, double-stranded cDNA as template and the T7 RNA Transcript Labeling Kit (ENZO Diagnostics Inc.). Briefly, double-stranded cDNA synthesized from the previous steps were washed twice with 80% ethanol and resuspended in 22 μL Rnase-free H_2O. The cDNA was incubated with 4 μL of 10× each Reaction Buffer, Biotin Labeled Ribonucleotides, DTT, Rnase Inhibitor Mix and 2 μL 20× T7 RNA Polymerase for 5 h at 37 °C. The labeled cRNA was separated from unincorporated ribonucleotides and precipitated at −20 °C for 1 h to overnight.

The cRNA pellet was resuspended in 40 μL Rnase-free H_2O and 10.0 μg was fragmented by heat and ion-mediated hydrolysis at 95 °C for 35 min in 200 mM Tris–acetate,