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Involvement of FLIP in 2-methoxyestradiol induced tumor regression in transgenic adenocarcinoma of mouse prostate (TRAMP) model

Manonmani Ganapathy, Rita Ghosh, Xie Jianping, Xiaoping Zhang¹, Roble Bedolla², John Schoolfield³, I-Tien Yeh², Dean A Troyer², Aria F Olumi¹, and Addanki P Kumar^{*}

¹Department of Urology, Bertucci Center for Genitourinary Cancers, Massachusetts General Hospital, Harvard Medical School Boston, MA 02114

²Department of Pathology, School of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900

³Department of Medicine, School of Medicine, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900

Abstract

Purpose—The purpose of this study is to investigate whether Fas-associated death domain interleukin-1 converting enzyme like inhibitory protein (FLIP) inhibition is a therapeutic target associated 2-methoxyestradiol (2-ME₂) mediated tumor regression.

Experimental Design—Expression and levels of FLIP was analyzed using (i) Real-Time PCR and immunoblot analysis in androgen independent PC-3 cells treated with the newly formulated 2-methoxyestradiol (2-ME₂) and (ii) immunohistochemistry in different Gleason pattern human prostate tumors. Transient transfections and Chromatin Immunoprecipitation (ChIP) assays were used to identify the transcription factors that regulate FLIP. Involvement of FLIP in 2-ME₂-induced tumor regression was evaluated in transgenic adenocarcinoma mouse prostate (TRAMP) mice.

Results—High Gleason pattern (5+5) human prostate tumors exhibit significant increase in FLIP compared to low Gleason pattern 3+3 ($p < 0.04$). 2-ME₂ reduced the levels and promoter activity of FLIP ($p = 0.001$) in PC-3 cells. Transient expression assays show sequences between –503/+242 being sufficient for 2-ME₂ induced inhibition of FLIP promoter activity. Co-transfection experiments show that overexpression of Sp1 activated, while Sp3 inhibited Sp1 transactivation of FLIP promoter activity ($p = 0.0001$). 2-ME₂ treatment reduced binding of Sp1 to the FLIP promoter as evidenced by ChIP. Further, levels of FLIP associated with Fas or FADD decreased, while cleavage of caspase-8,

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*Reprint requests: Addanki P Kumar, Department of Urology, School of Medicine, University of Texas Health Science Center San Antonio, TX 78229, Tel: 210-567-5647, Fax: 210-567-6868, e-mail: E-mail: kumara3@uthscsa.edu.

Statement of Clinical Relevance

Many anticancer drugs currently in use or under development work through induction of apoptosis some specifically in tumor cells. However tumors develop resistance to apoptosis through overexpression of a variety of signaling molecules including FLIP. Further, development of agents that target not only FLIP but genes involved in its regulation that lead to its activation or overexpression may be an ideal approach for successful management of apoptosis-resistant cancer. The study presented here demonstrates (i) transcription factor Sp1 as a potential activator and Sp3 as an inhibitor of Sp1 transactivation of FLIP; (ii) high grade human prostate tumors express significantly elevated levels of FLIP; and (iii) orally bioavailable 2-ME₂ that was developed using nanocrystal colloidal dispersion technology regresses prostate tumors in a preclinical animal model; (iv) regression of tumor development is associated with reduced levels of Sp1 and FLIP and increased levels of Sp3. These results taken together demonstrate the potential of developing 2-ME₂ as a therapeutic modality tailored towards prostate cancer patients who have developed apoptosis resistance. Further, this study sets the stage for the need to undertake in-depth investigations into the regulation of FLIP so that it may be manipulated for developing therapeutic benefit for prostate cancer patients.

levels of Bid and apoptosis increased in response to 2-ME₂ treatment in PC-3 cells. Administration of 2-ME₂ regressed established prostate tumors in TRAMP mice that was associated with reduced expression of FLIP and Sp1.

Conclusion—Targeting Sp1 mediated FLIP signaling pathway may provide a novel approach for prostate cancer management.

Keywords

FLIP promoter; apoptosis; 2-methoxyestradiol; Fas-FADD interaction; Sp1; Sp3

Introduction

Cells undergo apoptosis either through mitochondrial or death receptor signaling pathways (1–2). Fas/APO-1/CD95 (36 kDa) is a member of the tumor necrosis factor (TNF) receptor superfamily which binds Fas ligand (Fas-L) and recruits Fas-associated protein with death domain (FADD; 1–3). An anti-apoptotic protein, Fas-associated death domain interleukin-1 converting enzyme like inhibitory protein (FLIP) regulates death receptor signaling by inhibiting caspase-8 activation (4–5). FLIP is a key inhibitor of Fas-induced apoptosis and has homology to caspase-8 and 10. However unlike caspase-8 and 10, it lacks protease activity. At least three splice variants of FLIP, a long (FLIP_L), short (FLIP_S) and FLIP_R have been reported (6). Binding of pro-caspase 8 to Fas-associated protein with death domain FADD initiates the caspase cascade through activation of caspase-8 leading to cell death. Alternatively, caspase-8 can activate Bid which then promotes leakage of Cytochrome C. In the presence of dATP, Cytochrome C complexes with and activates Apaf-1 that can begin caspase cascade leading to induction of apoptosis (7). FLIP can block apoptosis either by competing with caspase-8 for binding to DED domains of FADD or by preventing the recruitment of caspase 8 to FADD (4–5). FLIP thus regulates death receptor signaling by inhibiting caspase-8 activation through death inducing signaling complex (DISC). Down regulation of FLIP confers not only sensitivity to TRAIL and Fas induced apoptosis but also to chemotherapy induced apoptosis in various tumor models (8–10). In addition down regulation of FLIP has been shown to sensitize breast cancer cells to doxorubicin induced apoptosis (11). Overexpression of FLIP has been observed in a variety of tumors including bladder, colorectal and gastric (12–18) suggesting prognostic significance. Ectopic expression of FLIP promoted androgen independent growth of LNCaP tumors in nude mice (19). These studies suggest that FLIP may be an ideal molecular target in inducing apoptotic cell death in cancer cells. However the role of FLIP as a target for prostate cancer prevention/treatment has not been studied. In order to develop FLIP as a therapeutic target, it is important to understand its regulation.

Regulation of FLIP is complex occurring at multiple levels including transcriptional, translational and posttranslational. Expression of FLIP has been shown to be modulated by transcription factors including AP-1 (20), NFκB (21–22), c-Myc (23) and p53 (24) in addition to cell survival kinase Akt (25) and E3 ubiquitin ligase (26). Studies have shown that treatment with synthetic triterpenoids, flavopiridol and Silibinin can down regulate FLIP and subsequently sensitize TRAIL induced apoptosis in breast cancer and glioma cells (27–29). Down regulation of FLIP by Silibinin is due partly to proteasome-mediated degradation of FLIP (29). Further JNK activation by TNFα can reduce stability of FLIP via JNK-mediated phosphorylation and activation of E3 ubiquitin ligase Itch, which ubiquitinates FLIP and induces FLIP degradation (26). Despite these data implicating FLIP as a potential therapeutic target, to the best of our knowledge no studies have explored targeting FLIP as an approach for tumor growth inhibition *in vivo* (30).

2-methoxyestradiol (2-ME₂) is an endogenous non-toxic metabolic by-product of estradiol that is present in human urine and blood. 2-ME₂ has been shown to (i) inhibit endothelial cell proliferation implicating it in angiogenesis; (ii) inhibit the growth of lung, breast, pancreatic, hepatocellular, neuroblastoma, medulloblastoma, melanoma and gastric cancer (31–35). We have shown that 2-ME₂ prevents early stage prostate cancer development using the transgenic adenocarcinoma of mouse prostate (TRAMP) model (33). Further, 2 ME₂ was found to be safe, well tolerated, reduce or stabilize PSA levels when given to hormone refractory prostate cancer (HRPC) patients who had failed other treatments including hormone therapy (36). However that study concluded that bioavailability of 2 ME₂ was low due to its inactivation and a new formulation of 2-ME₂ with improved bioavailability should be developed. Accordingly second generation 2 ME₂ was developed using nanocrystal colloidal dispersion (NCD) technology and has increased its bioavailability by 5–10 fold (EntreMed, Inc, Rockville, MD). Several mechanisms have been put forth to explain 2-ME₂-induced apoptosis including phosphorylation of Bcl-2, JNK activation, NFκB inhibition (31–35). It has been reported that 2-ME₂ down regulates FLIP through inhibition of Akt/NFκB mediated signaling in prostate cancer cells (37). However the factors involved in the upregulation of FLIP and whether its inhibition is associated with prevention or regression of prostate tumor development *in vivo* is unexplored.

In the current study we report the ability of this newly formulated 2-ME₂ with enhanced bioavailability (obtained from EntreMed, Inc; Rockville, MD) to regress established tumors in TRAMP mice. Further we show here that levels of FLIP increase in high grade human prostate tumors compared with low grade tumors. Treatment of PC-3 cells with 2 ME₂ showed (i) reduced levels of FLIP in Fas or FADD immunoprecipitated complexes; (ii) cleavage of Caspase-8 and Bid, and (iii) induction of apoptosis. Treatment of PC-3 cells with 2-ME₂ also reduces levels of FLIP expression and promoter activity. Transient expression assays using deletion constructs of FLIP promoter identified transcription factor Sp1 as an activator of FLIP. Sp3 was found to inhibit Sp1 transactivation. Transcription factor Sp1 belongs to a multigene family comprising of Sp1, Sp2, Sp3, Sp4 and Sp5 that bind and act through GC boxes to regulate gene expression (38–42). Within this family, Sp1 and Sp3 are ubiquitously expressed in mammalian cells and participate in regulating expression of genes involved in various cellular processes including cell growth, apoptosis, angiogenesis and invasion. Unlike Sp1 which is an activator of transcription, Sp3 can function as an activator or a repressor depending on the cell and promoter context (38–43). Regression of tumor development in TRAMP mice is associated with reduced levels of FLIP and Sp1. In addition ChIP assays indicate binding of Sp1 to FLIP promoter region that is reduced in response to 2-ME₂ treatment. All of these results taken together suggest that FLIP plays an important role in apoptosis induction in androgen independent disease and inhibition of FLIP signaling may provide a means to manage prostate cancer.

Materials and Methods

Reagents and Plasmids

c-FLIP (L) promoter (–1700 to +300) was cloned by PCR amplification from Bac-IP11–536118 (Children's Hospital, Oakland Research Institute, Oakland, CA) using primers: sense-5'-CTC GAG TGA ACC TGG GAG GTT AAG GC 3' and antisense-5'-AGA TCT GAG GCA AAG AAA CCG AAA GC-3' containing XhoI and Hind III sites. The PCR products were inserted into pGEMT-Easy vector (Promega, Madison, WI). After confirming the sequence it was sub cloned into the PGL3-enhancer vector (Promega, Madison, WI) at XhoI and Hind III sites. Expression vectors pCMV-Sp1 and pPac-Sp3 were obtained from Drs Sophia Y. Tsai (Baylor College of Medicine, Houston, TX) and Guntram Suske (Institut für Molekularbiologie und Tumorforschung, Marburg, Germany; 43). Newly formulated second generation 2-ME₂ with

enhanced bioavailability was obtained from EntreMed, Inc (Rockville, MD). This orally administered suspension was developed using nanocrystal colloidal dispersion (NCD) technology and has increased its bioavailability by 5–10 fold (EntreMed, Inc, Rockville, MD; www.entremed.com).

Cell Culture

Androgen independent PC3 and androgen responsive LNCaP cells were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% FBS and 1% Pens/Strep in a humidified incubator supplied with 5% CO₂ and at 37°C as previously described (32–33).

Animal experiments

Transgenic adenocarcinomas of mouse prostate (TRAMP) mice were obtained from Jackson Laboratories (Maine, FL). A group of 22–25 week old TRAMP mice was given 50 mg/kg body weight 2-ME₂ in drinking water and a control group received water without 2-ME₂. The dose was chosen from our published study showing the efficacy of 2-ME₂ through dietary administration (33). The ability of 2-ME₂ to regress progression of well differentiated carcinoma was assessed after 25 weeks of intervention by determining (i) number of animals showing palpable tumors, (ii) volume of the prostate seminal vesicle complex (PSVC) and (iii) histological evaluation of the prostate tumor as described earlier (33). Body weight and water consumption were measured weekly. At the time of necropsy, all other organs including lungs, liver, and kidney were collected for histopathological evaluation. Animal studies were conducted in compliance with University of Texas Health Science Center at San Antonio IACUC (UTHSCSA IACUC).

Transient Transfections

To examine FLIP promoter activity, actively growing PC3 and LNCaP cells were plated at 5×10^4 cells per well in the 6 well plates as described (32–33;44–45). Briefly, full length FLIP promoter and all deletion constructs (1µg/well) and pRL TK plasmid (50 ng/well; Renilla luciferase for normalization) were transfected using Lipofectamine™2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Cells were treated with or without 2-ME₂ (3µM) for 2 hours and Luciferase activity was assayed using Dual Glo™ Luciferase assay system (Promega Corporation, Madison, WI). For cotransfection experiments 1µg/well of Sp1, Sp3 or both Sp1 and Sp3 were used along with FLIP reporter plasmid. Results are expressed as ratio of firefly luciferase/Renilla luciferase at equal amounts of protein. Each experiment represents the mean of three replicates and was repeated at least 5 times using three independent plasmid DNA preparations.

Preparation of cell extracts, immunoblotting and immunoprecipitation

Whole cell or nuclear extracts from PC-3 cells treated with 2-ME₂ (3µM) were prepared and immunoblotting was performed essentially as described previously (32–33;44–45). Bound antibody was detected by enhanced chemiluminescence using Western lightning western chemiluminescence reagent plus (enhanced luminol) following the manufacturer's directions (PerkinElmer Life and Analytical Sciences, Shelton, CT). All the blots were stripped and re-probed with β-actin to ensure equal loading of protein. Images were captured and analyzed using Genesnap software (Syngene, Frederick, MD). Quantification was carried out using Genetool software (Syngene, Frederick, MD). For immunoprecipitation experiments 50 µg of the pre-cleared lysate was immunoprecipitated with either normal rabbit serum, anti-Fas or anti-FADD (2-µg) at 4°C over night by gentle rocking using protein A/G agarose beads (Santa Cruz Biotechnology, CA). Immunoprecipitated proteins were washed thrice with PBS by spinning at 3000 rpm for 1min and separated on SDS-PAGE.

Real-Time PCR analysis

RNA was prepared from LNCaP or PC-3 cells treated with 2 ME₂ (3 μM) or solvent control for 2h and expression of FLIP was measured. Expression of FLIP was measured by Real-Time PCR using Taqman 1-step expression assay on an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA). Primers for FLIP and GAPDH were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). Levels of FLIP were normalized to GAPDH. Expression in the absence of 2-ME₂ was set to 1 after normalization to GAPDH. Data shown is normalized FLIP RNA expression. Data presented here is an average ± s.d of two independent experiments.

Chromatin Immunoprecipitation Assays

ChIP was performed using ChIP-it express kit essentially as described by the manufacturer (Active Motif, Carlsbad, CA). Briefly logarithmically growing PC-3 cells were treated with 2-ME₂ (3 μM) for 0.5, 2 and 6h. Following this incubation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature and nuclei were isolated. Isolated nuclei were sonicated on ice to break chromatin DNA to an average length of ~300 bp. Soluble chromatin was used in immunoprecipitation with Sp1 antibody and IgG (as negative control) and immune complexes were absorbed with protein G magnetic beads. Following reversing the crosslinks and Proteinase K digestion, immunoprecipitated DNA was amplified by a primer pair corresponding to a 230 bp fragment using -503/+242 of the FLIP promoter by PCR. Primers used were aaaattagtcgggcatggc (-636F) and tgggagtcataagcaaactg (-406 R). PCR products were resolved on 3% agarose gel. Densitometry was used to quantify the PCR products and the results were normalized to respective input values.

Immunohistochemistry

Sections from formalin fixed, paraffin embedded prostate tissue blocks were cut and stained for FLIP (SC-8347, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Sections were deparaffinized at 60°C, cleared and rehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01M citrate buffer at pH6 for 20 minutes at 121°C in pressure chamber. Sections were blocked successively with; 3% Hydrogen peroxide- TBS buffer and 10% BSA-TBS buffer. Tissues were incubated with FLIP rabbit polyclonal antibody or Rabbit Ig fraction as negative control for one hour at 25°C and secondary antibody and peroxidase-labeled streptavidin for 15 minutes each (LSAB-2 System Dako Cytomation Corp, Carpinteria CA) and diaminobenzidine substrate for peroxidase-based IHC (Dako Cytomation Corp, Carpinteria, CA). Slides were counter-stained with Hematoxylin and mounted.

Double staining

In order to determine the detection of both FLIP and p63 (basal cell marker) in the same specimen, double immuno-staining was performed using Envision TM G2 Double stain System (DAKO Cytomation Carpinteria, CA). This is highly sensitive peroxidase (detected as brown staining using DAB as chromogen) and Alkaline phosphatase-based (AP detected as red staining using permanent red as chromogen) visualization kit for simultaneous detection of two different antigens within the same specimen. FLIP antibody was developed with HRP-DAB and p63 with AP-Permanent Red.

Human Tissue

Formalin fixed paraffin embedded samples of human prostate cancers were obtained from an IRB approved tissue repository at University of Texas Health Science Center at San Antonio (UTHSCSA).

Semi-quantitative Analysis

The degree of staining was evaluated blindly by a pathologist (DAT). Total staining was scored as the product of the staining intensity (on a scale of 0–3) and the percentage of cells stained, resulting in a scale of 0–300. Staining intensity was scored as follows: 0, none of the cells stained positively; 1, weak staining; 2, moderate staining intensity; and 3, strong staining intensity. Positive and negative controls were used with each staining run to identify problems with immunohistochemistry. All blocks were stained at the same time with the same reagents.

Statistical analysis

Percentage of animals showing palpable tumors was compared across the treatment groups using Fisher's Exact Tests. PSVC weight and scores for histological evaluation of the prostate were compared across the treatment groups using Kruskal-Wallis tests. Weekly weights across the intervention duration were analyzed using two-way (treatment group and week) repeated measures ANOVAs. If the F-test for the treatment by week interaction term was significant, then post-hoc treatment group comparisons were performed for each week to identify the weeks that had significant treatment group weight mean differences.

Spearman rank correlation was performed to identify any linear association between Gleason scores and FLIP total scores for tissue samples. Kruskal-Wallis ANOVA was performed to determine if mean ranks of FLIP total scores differed among tissues grouped by the observed Gleason scores (4,6,7,8,9, and 10). Mann-Whitney U test was performed to determine if mean ranks of FLIP total scores differed among tissues grouped by Gleason of 4 or 6 vs. Gleason of 7 to 10. Box plots were produced to depict the distribution of FLIP total scores within Gleason score groups. For all statistical tests, $p < 0.05$ was considered significant. SPSS 15.0 and Stata 10.0 were used to perform the statistical analyses.

Results and Discussion

FLIP expression was evaluated using low (Gleason score $\leq 3 + 3 = 6/10$; $n=41$) and high grade ($\geq 3 + 4 = 7/10$; $n=92$) and adjacent normal human prostate cancer biopsy specimens from prostatectomy patients. As shown in figure 1a low grade Gleason pattern $3+3 = 6/10$ tumor glands show no FLIP staining (indicated by an asterisk in a). However high grade Gleason pattern $4+3 = 7/10$ (cribriform malignant gland shown by asterisk in b) and Gleason pattern $5+5 = 10/10$ (indicated by arrows in c) tumors show FLIP staining.

Staining in tumor cells was granular, cytoplasmic and membranous. Interestingly basal cells in the normal prostate gland showed FLIP staining (shown by an arrow in a) while stromal cells showed no staining (shown by an arrow in b). In order to show that the observed FLIP staining is in basal cells, we performed double immunostaining using p63 as basal cell marker. As shown in figure 1d, the double staining shows co-localization of FLIP and p63 in the same cell and such double stained cells are basally situated within the glands. These data provide evidence that these are basal cells and FLIP is expressed in these cells. Negative controls were included by omitting the primary antibody showed no staining (data not shown).

These findings were analyzed for statistical significance by determining the percentage of cells stained (0–100) and the degree of staining on a scale of 0 to 3 (0 being no staining and 3 being highest intensity of FLIP staining). Total staining was scored as the product of the staining intensity and the percentage of cells stained, resulting in a scale of 0–300. For the correlation between Gleason scores and FLIP total scores, Spearman's $\rho = 0.081$ with 95% CI = (-0.091, 0.248), so there was no significant linear association between the measures for the 133 tissue samples. For the Kruskal-Wallis ANOVA, Chi-square = 11.27 ($p = 0.046$), indicating significant differences for FLIP total score ranks among the Gleason score groups. For the

Mann-Whitney U test, $z = 2.04$ ($p = 0.042$), indicating the group of 92 tissues with Gleason scores of 7 to 10 had significantly higher FLIP total score ranks than the group of 41 tissues with Gleason scores of 4 or 6. These data show good correlation between FLIP expression and tumor grade in human prostate cancer and are consistent with published reports showing higher expression of FLIP in bladder, colorectal, gastric and hematological malignancies (12–18).

Previously we have shown that 2-ME₂ inhibits proliferation of prostate cancer cells through induction of apoptosis (32). However the mechanism through which it occurs is incompletely understood. We tested whether FLIP plays a potential role in 2-ME₂-induced apoptosis in prostate cancer cells. As shown in figure 2a, expression of FLIP was reduced significantly in both LNCaP and PC-3 cells upon treatment with 2-ME₂. Protein levels also decreased significantly following 2-ME₂ treatment for 2h (figure 2b; $p=0.001$). Previously we have published that under these experimental conditions 2-ME₂ induces apoptosis in PC-3 cells as evidenced by morphological and TUNEL analysis (32). This is also consistent with published studies showing inhibition of proliferation and induction of apoptosis in a variety of cancer cells including prostate (31–35). These data suggest that FLIP has a role in 2-ME₂-induced apoptosis. However the mechanism through which FLIP inhibition leads to induction of apoptosis is unclear. It is possible that 2-ME₂ treatment lowers the amount of FLIP available for DISC formation which leads to cleavage of Caspase-8. We analyzed the expression of FLIP in Fas and FADD immunoprecipitated extracts from cells treated with 2-ME₂. As shown in figure 2c, FLIP was reduced in Fas or FADD immunoprecipitated samples compared to untreated cells. It is known that activation of caspase-8 can lead to cleavage of the proapoptotic protein Bid. Translocation of truncated Bid to mitochondria initiates the mitochondrial cascade of events leading to cell death. We measured the levels of Bcl-2, caspase-8 and truncated Bid under experimental conditions where FLIP is inhibited with 2-ME₂. As shown in figure 2d, treatment with 2-ME₂ resulted in the cleavage of Bid, caspase-8 and reduction in the level of Bcl-2. These data show that treatment of PC-3 cells with 2-ME₂ leads to inhibition of FLIP, activation of caspase-8, cleavage of Bid and subsequently to apoptosis. To demonstrate the direct role of FLIP in apoptosis, we generated stable cells of LNCaP overexpressing FLIP. These cells showed resistance to induction of apoptosis by 2-ME₂ (data not shown). Although the data discussed above shows that 2-ME₂ treatment induces apoptosis in PC-3 cells that is associated with FLIP inhibition, the mechanism of FLIP regulation is unknown.

Since FLIP RNA is reduced with 2-ME₂, we explored whether transcriptional regulation of FLIP is a mechanism in 2-ME₂-induced biological effect. We first investigated whether 2-ME₂ modulates constitutive levels of FLIP transcriptional activity in PC-3 cells. Transient transfections were performed using a full length FLIP promoter sequence (–1300/+300) in PC-3 cells. As shown in figure 3a, 3 μ M 2-ME₂ treatment for 2h reduced FLIP promoter activity by more than 50% ($p<0.001$). To identify factors that regulate FLIP expression in response to 2-ME₂, we performed transient transfections using a series of deletion constructs of FLIP promoter and identified sequence elements between –503/+242 to be sufficient to mediate 2-ME₂ responsiveness (figure 3b). Examination of this 745 bp sequence using the transcription factor binding site search algorithms with TRANSFAC 7.0 as described by Matys et al identified 15 potential sites for transcription factor Sp1 (–502, –478, –359, –334, –290, –282, –250, –230, –204, –162, –130, +20, +123, +131, +202; 46).

Transcription factor Sp1 belongs to a multigene family comprising of Sp1, Sp2, Sp3, Sp4 and Sp5 that bind and act through GC boxes to regulate gene expression (38–42). Within this family, Sp1 and Sp3 are ubiquitously expressed in mammalian cells and participate in regulating expression of genes involved in various cellular processes including cell growth, apoptosis, angiogenesis and invasion. Unlike Sp1 which is an activator of transcription, Sp3 can function as an activator or a repressor depending on the cell and promoter context (38–43). We investigated the role of Sp1 and Sp3 in the regulation of FLIP in PC-3 cells. Cotransfection

experiments were carried out with -503/+242 fragment of FLIP promoter in the presence of Sp1 and Sp3 separately and in combination. As shown in figure 3c, Sp1 but not Sp3 trans-activated FLIP promoter which is consistent with published reports on the role of Sp3 (38–43). Interestingly overexpression of Sp1 and Sp3 reduced FLIP transcriptional activity to the basal level. These data suggest that while Sp1 transactivates FLIP, Sp3 inhibits Sp1-mediated transactivation. In order to directly demonstrate that Sp1 transactivates FLIP by binding to FLIP promoter sequence and that 2-ME₂ treatment inhibits this binding, ChIP assays were performed using PC-3 cells treated in the absence and presence of 2-ME₂ (3 μM for 0.5, 2 and 6h). As shown in figure 3d, FLIP specific primers amplified Sp1-immunoprecipitated DNA from untreated PC-3 cells. Whereas when Sp1-immunoprecipitated DNA from 2-ME₂ treated PC-3 cells was used, the level of product formed was significantly reduced by 6h. These results illustrate that under normal conditions Sp1 is bound to the FLIP promoter (making it active) and 2-ME₂ treatment reduces binding of Sp1 to the FLIP promoter (making it transcriptionally inactive). We do not know the importance of specific Sp1 sites in the FLIP promoter at this time; on-going mutational analysis studies are examining the importance of specific Sp1 binding sites in the regulation of FLIP. Data presented here shows that 2-ME₂ can reduce FLIP level through transcriptional regulation involving Sp1 and Sp3.

Although Sp1 is considered to be an ubiquitous transcription factor, recent reports suggest a potential role for Sp1 and family members in tumor growth and development (38–42;47–48). Interference with Sp1 activity has been shown to suppress tumor cell growth, block cell cycle progression and form tumors in nude mice (47–48). It is noteworthy to mention that expression of Sp1 has been shown to increase during epidermal and thyroid carcinogenesis (39,48). Our results showing regulation of FLIP by Sp1 and its family members are consistent with published studies demonstrating a potential role for Sp1 and its family members in tumor growth and development through regulation of genes involved in cell growth, apoptosis resistance, invasion and angiogenesis (38–43;47–48).

Previously we showed that dietary administration of 2-ME₂ prevented tumor progression using the TRAMP model (33). In the current study we examined whether 2-ME₂ could be used in a therapeutic setting. To examine this question we used TRAMP animals with preformed tumors as described in methods. Our results show that 25 week intervention with 2-ME₂ caused tumor regression in 90% of the animals. Only 10% of the animals in the intervention group (n=10) had palpable tumors compared to 80% animals with palpable tumors in the control group (n=15). Analysis of these data show that intervention with 2 ME₂ significantly suppressed occurrence of palpable tumors relative to controls (p=0.008; data not shown). Further PSVC weight for the intervention group also decreased significantly compared to the control animals (p<0.001; figure 4a). These data demonstrate that 2-ME₂ caused tumor regression and significantly reduced weight of the prostate gland compared to animals receiving normal drinking water. It is noteworthy to mention here that unpublished studies from our laboratory indicate that 2-ME₂ administration for 18 weeks produced no significant difference in the pathological score of the prostate tumors compared to control (p=0.204) although there was a reduction in the prostate seminal vesicle complex volume (p=0.006).

Prostatic lesions were graded as described earlier on a scale of 1 to 6 as described previously (33). Briefly non-cancerous lesions graded as 1, 2 or 3 were indicative of normal tissue, low PIN and high PIN respectively. Grades 4, 5 and 6 were indicative of well, moderately and poorly differentiated cancerous lesions respectively. Analysis of the data show that tumors from control group animals were poorly-differentiated adenocarcinoma characterized by variable nuclear shape with little or no gland formation consistent with published results using this model. In contrast, prostate tissue from animals receiving 2-ME₂ exhibited pathological features consistent with the appearance of normal prostatic epithelium, resulting in significantly lower scores than controls (p<0.001). Although these data suggest complete

regression of tumors in TRAMP mice, since we performed pathological evaluation at the time of termination of the experiment, we do not know if this is due to slower rate of tumor progression in this group of animals. Graphical representation of pathological score is shown in figure 4b. Histo-pathological evaluation of the prostate tumor/tissue from TRAMP mice on normal drinking water and 2-ME₂ is shown in figure 4c and d. Taken together these data (reduction in the palpable tumors, PSVC weight and histological evaluation) confirm our interpretation that 2-ME₂ intervention led to regression of prostate tumors in these animals.

Previously, we showed that 2-ME₂ inhibited proliferation of prostate cancer cells by inducing apoptosis (32). Here we investigated if intervention with 2-ME₂ inhibited tumor cell proliferation and induction of apoptosis *in vivo* using Ki67, as a proliferation marker and TUNEL staining to determine apoptosis. Immunohistochemical analysis shows that prostates from TRAMP mice receiving normal drinking water are highly positive for Ki 67 immunostaining and therefore highly proliferative. The number of Ki67-positive cells decreased significantly in animals receiving 2-ME₂ (figure 4e, f and g). These *in vivo* data are consistent with our published work in cells showing inhibition of proliferation in prostate cancer cells in response to 2-ME₂ (32). Prostate tumors from TRAMP mice receiving normal drinking water had apoptotic cells compared with 2-ME₂-receiving animals as indicated by TUNEL and cleaved caspase 3 staining (figure 4e and data not shown). At first glance this result was surprising since in cultured cells we found an increase in apoptosis with 2-ME₂ treatment. However when the *in vivo* apoptosis data was looked at in the context of the near normal prostate architecture it is expected that the level of apoptosis be lower in the tissues obtained from animals on 2-ME₂ treatment. To confirm apoptosis and proliferation in non-cancerous prostate tissue, we assessed Ki 67 and TUNEL staining in prostate from non-transgenic animals. Our results show that in non pathological prostate tissue the level of Ki67 and TUNEL staining is undetectable and comparable to our results seen in 2-ME₂ treated animals (data not shown). Therefore results presented in figure 4e, f and g suggest that 2-ME₂ treatment restored normal tissue architecture.

Since TRAMP mice were generated with SV40 large T antigen coupled with probasin promoter we examined whether 2-ME₂ mediated effects are a consequence of down regulation of the PB-Tag transgene using western blot analysis. As shown in figure 4h, PB Tag & β -actin expression was detected both from control and treated tumor/tissue obtained from age-matched TRAMP mice with no significant changes in levels. These results demonstrate that 2-ME₂ - induced biological effects are due to direct suppression of carcinogenesis and not due to the down regulation of PB-Tag.

We determined the expression of Sp1, Sp3 and FLIP in tumors and prostate tissue obtained from animals in the control and 2-ME₂ treated groups (from three individual animals from each group) respectively using immunohistochemistry and immunoblotting. Prostate tumors from TRAMP mice receiving normal drinking water showed higher levels (approximately 3-fold) of Sp1 and FLIP (figure 5). Prostate tissues from 2-ME₂ treated animals showed significant reduction in the levels of both Sp1 and FLIP (> 50% p=0.008 and 0.03 respectively). When we used Sp3 antibody, we detected a band around 100 kDa and a doublet around 60 kDa (indicated by thick and thin arrows in figure 5) consistent with the published reports (43). The protein level of 100 kDa Sp3 band was 3-fold higher in the extracts from 2-ME₂ treated animals (p=0.01). Quantitative analysis of the data is also shown in figure 5 right panel. Similar results were obtained using immunohistochemistry (data not shown). These results show there is a correlation between the levels of Sp1/ Sp3 and FLIP *in vivo*. Although we have not yet established that these transcription factors regulate FLIP *in vivo* all the data taken together suggest that this may be the case.

Although the data presented so far including increased expression in human prostate tumors show the potential involvement of FLIP in prostate carcinogenesis, it is not known if overexpression of FLIP enhances tumorigenic potential of prostate cancer cells. To address this we investigated the effect of overexpression of FLIP on anchorage independent growth of PC-3 cells. As shown in figure 6 FLIP transfected cells formed significantly higher number of colonies compared with vector transfected control cells suggesting that overexpression of FLIP may have growth promoting effects on prostate cancer cells. Similar results were obtained using stable PC-3 cells expressing FLIP (data not shown). In addition 2-ME treatment completely inhibited the formation of colonies in vector transfected cells. However treatment of FLIP transfected cells showed only 88% inhibition indicating resistance of these cells. We are characterizing the stable transfectants overexpressing FLIP to understand the mechanism involved in the development of resistance.

In the current study we report the ability of this newly formulated 2-ME₂ with enhanced bioavailability obtained from EntreMed, Inc (Rockville, MD) to regress the established tumors in TRAMP mice. Additionally regression of tumors is accompanied by inhibition of Sp1 and FLIP expression. Although we have not shown that inactivation of Sp1 or FLIP prevents tumor development, it is tempting to speculate that 2-ME₂ regresses prostate tumors in TRAMP mice through down regulation of Sp1-mediated FLIP transcriptional activity. It is noteworthy to mention that dietary administration of 2-ME₂ reduced serum concentrations of testosterone without affecting estradiol in TRAMP mice (33). Therefore 2-ME₂-induced reduction in the serum concentration of testosterone may inhibit FLIP with consequent regression of tumor development. Studies are in progress to test this hypothesis. In addition work from many laboratories including our own shows that the growth inhibitory effect of 2-ME₂ is specific for tumor cells and does not affect normal cells. Therefore results presented here that show the inhibition of FLIP using a non toxic, tumor-specific compound is noteworthy. We have developed LNCaP cells that overexpress FLIP stably to determine whether IC₅₀ values for 2-ME₂ increase in these cells with increased levels of FLIP. These cells are resistant to 2-ME₂ induced proliferation inhibition (data not shown). Previously we and others have demonstrated that PC-3 cells are differentially sensitive to 2-ME₂ induced proliferation inhibition (IC₅₀ of 3 μ M compared to 1 μ M for LNCaP cells). We speculate that this differential sensitivity could be due to higher constitutive levels and promoter activity of FLIP in PC-3 cells. These observations are also consistent with results demonstrating sensitization of PC-3 cells by 2-ME₂ through down regulation of FLIP (37). These authors have shown that 2-ME₂ down regulates FLIP through inhibition of Akt/NF κ B mediated signaling using dominant negative approaches. However that study did not identify the transcription factors involved in its regulation. Results presented in the current study show that transcription factor Sp1 activates FLIP and that Sp3 represses Sp1-mediated trans-activation (figure 3c). These findings are consistent with studies showing involvement of Sp1 during tumorigenesis through regulation of genes involved in cell growth, apoptosis resistance, invasion and angiogenesis. Our data also show higher expression of FLIP in high Gleason grade tumors compared to low Gleason grade (figure 1). In addition 2-ME₂ regresses prostate tumor development in TRAMP mice that is associated with down regulation of FLIP (figure 4 and figure 5). Recently it has been shown that FLIP expression is stronger in hormone-resistant cases than in hormone-naïve or neoadjuvant treated patients (49). These authors have also demonstrated FLIP as a target of androgen receptor co-activator protein Par-4 (Prostate apoptosis response factor-4) indicating a potential role for FLIP during the development of hormone refractory prostate cancer. Although we have not examined expression of Sp1 in prostate cancer biopsy samples we speculate that higher expression of FLIP in high grade tumors could be due to higher level of Sp1. Other studies have shown increased expression of Sp1 in epidermal and gastric tumors. However it is not known whether overexpression of Sp1 in prostate epithelial cells is sufficient to elevate FLIP expression with consequent inhibition of apoptosis. Studies are currently in progress to determine whether modulation of Sp1/Sp3 FLIP signaling pathway plays a role in

the development and progression of androgen independent prostate cancer. Understanding the mechanism involved in Sp1/Sp3-FLIP in prostate carcinogenesis may contribute to the ongoing efforts to overcome development of resistance to apoptosis in prostate cancer patients.

Abbreviations

2-ME₂, 2-methoxyestradiol; FADD, Fas-associated death domain; FLIP, Fas-associated death domain interleukin-1 converting enzyme like inhibitory protein; Sp1, specificity protein 1; TRAMP, transgenic adenocarcinoma of mouse prostate.

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Figure 1e

Figure 1. FLIP Expression in human prostate tumors

FLIP expression was assessed in prostate adenocarcinomas by immunohistochemical staining of histological formalin fixed paraffin embedded sections from prostatectomy samples. Both low (Gleason score $\leq 3 + 3 = 6/10$; $n=41$) and high grade ($\geq 3 + 4 = 7/10$; $n=92$) tumors were evaluated. Representative staining for Gleason pattern 3, pattern 4 (asterisk), and pattern 5 adenocarcinomas (arrows). Normal basal epithelial cells stain for FLIP (arrow in a) while stromal cells do not stain (arrow in b). Benign prostate gland dual stained for FLIP (brown) and p63 (red). Arrows point to representative red-stained nuclei situated along the basal aspect of benign prostatic glandular epithelium. Brown stained cytoplasm can be observed surrounding the p63 positive nuclei (d). Box plots were produced to depict the distribution of FLIP total scores within Gleason score groups and presented in e.

Figure 2. Expression of FLIP and its response to 2-ME₂

A. LNCaP and PC-3 cells were treated or not with 3 μ M 2-ME₂ for 2h. RNA was extracted and expression of FLIP was determined using Real Time PCR using FLIP and GAPDH primers (obtained from Applied Biosystems, Inc., Foster City, CA). Data presented here is an average expression of FLIP from two independent experiments normalized to GAPDH.

B. Whole cell extracts were prepared from PC-3 cells treated without or with 3 μ M 2-ME₂ for different times as indicated in the figure and levels of FLIP were determined by immunoblot analysis. The blot was stripped and re-probed with β -actin. Experiment was repeated three times. Quantitative analysis of the data is shown in the graph.

C. Whole cell extracts prepared from PC-3 cells treated with 2-ME₂ for 30 and 120 minutes were immunoprecipitated with either Fas or FADD antibodies as described in methods. These immunoprecipitated complexes were resolved on SDS-PAGE and immunoblotted with FLIP antibody.

D. Representative image of immunoblot analysis of extracts prepared from PC-3 cells treated with 2-ME₂ as a function of time using antibodies against Bcl-2, Bid and Caspase 8. The blot was stripped and re-probed with β -actin for loading control.

Figure 3a

Figure 3 c

Figure 3. Modulation of FLIP promoter activity by 2-ME₂ treatment

A. Actively growing PC3 cells were plated at 5×10^4 cells per well in the 6 well plates. Following their attachment, full length FLIP promoter (1 μ g/well) and pRL-TK plasmid (50 ng/well; Renilla luciferase for normalization) were transfected using Lipofectamine^{TM}2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Cells were treated with or without 2-ME₂ (3 μ M) for 2 hours and Luciferase activity was assayed using Dual Glo^{TM} Luciferase assay system (Promega Corporation, Madison, WI).

B. Transfections were performed using indicated deletion constructs of FLIP essentially as described in A.

C. For cotransfection experiments 1 μ g/well of Sp1, Sp3 or both Sp1 and Sp3 were used along with FLIP reporter plasmid (–503/+242). These transfections were also performed as described in A.

Results are expressed as ratio of firefly luciferase/Renilla luciferase at equal amounts of protein (avg $\pm$ sd). Each experiment represents the mean of three replicates and was repeated at least 5 times using three independent plasmid DNA preparations. Statistical significance of the data was determined using students t-test and p values less than 0.05 was considered significant.

D. Analysis of Sp1 binding to FLIP promoter *in vivo* using Chromatin

immunoprecipitation: ChIP was performed using ChIP-it express kit essentially as described by the manufacturer's recommendations (Active Motif, Carlsbad, CA) with Sp1 antibody (ChIP-specific from Active Motif, Carlsbad, CA), water (W) and IgG (negative control).

Primers used were aaaattagtcgggcatggtc (–636F) and tgggagtcataagcaaactg (–406 R). PCR products were resolved on 3% agarose gel. Representative image of three independent experiments is shown.

Figure 4. Effect of 2-ME₂ on established prostate tumors in TRAMP animals

22 week old TRAMP mice were randomized into two groups of 15 animals. A group of 15 animals were given 50 mg/kg body weight of 2-ME₂ in drinking water. A second group of 15 animals received drinking water without 2-ME₂ for a total of 25 weeks as described in methods. Animals were observed for tumors by abdominal palpation and body weight changes throughout the study. Weight of PSVC (mean weight in grams $\pm$ s.d) of TRAMP mice on control and 2-ME₂ treatment at 47 weeks is shown in panel **a**. Prostatic lesions were graded as described previously on a scale of 1 to 6. Briefly non-cancerous lesions were graded as 1, 2 or 3 indicating normal tissue, low PIN and high PIN respectively. Grades 4, 5 and 6 indicated well differentiated, moderately and poorly differentiated cancerous lesions respectively. Power analysis was performed using PASS 6.0 software as described in methods. For all statistical tests, p-values less than 0.05 were considered significant and quantitative analysis of the data is shown in **b**. Sections of prostate tissue/tumor excised from TRAMP mice (control or 2-ME₂ treated) were stained with H&E. Prostate from control animals showed poorly-differentiated adenocarcinoma characterized by variable nuclear shape with little or no gland

formation (**panel c**) whereas prostate tissue from mice fed the high-dose of 2-ME₂ exhibited pathological features consistent with the appearance of normal prostatic epithelium (**panel d**). **Proliferation and TUNEL staining in prostate tumor tissues:** Paraffin-embedded tissues sections were stained with Ki-67 (SP6) antibody (Lab vision, Fremont, CA) to assess proliferation. The secondary and tertiary antibodies were a biotinylated link and streptavidin HRP (Biocare 4 Plus Kit, Biocare Medical, Concord, CA). Apoptosis was assessed in prostate tumor or tissues using the *in situ* terminal transferase dUTP nick end-labeling (TUNEL) assay, with biotin-16-dUTP (Roche Applied Science, Indianapolis, IN) and terminal deoxynucleotidyl transferase (TdT) (Invitrogen, Carlsbad, CA; figure 4e). Positively stained cells were counted in 5 different fields from three different samples to calculate % proliferation and apoptosis that is shown as graph (figure 4f and g). Negative controls were included by omitting the primary antibody or TdT (for TUNEL; data not shown).

Immunohistochemical analysis of SV40 T Ag expression in representative tumors or prostate tissue from age-matched control and treated TRAMP mice: Whole-cell extracts were prepared separately from TRAMP prostate tumors (three individual animals on control diet) or prostate tissue (treatment) was used in immunoblot analysis. Equal loading of protein was confirmed with β -actin antibody. Bound antibody was detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent Substrate, following the manufacturer's directions (Pierce, Rockford, IL). The blots were imaged using Syngene G Box Fredrick MD and quantified using Gene tools software. Graphical representation of the quantified data is shown. Ns indicates non-significant difference.

Figure 5. Modulation in the levels of Sp1, Sp3 and FLIP in TRAMP tissues following 2-ME₂ intervention

Whole-cell extracts were prepared separately from TRAMP prostate tumors (three individual animals receiving normal drinking water) or prostate tissue (from three individual animals receiving 2-ME₂) was used in immunoblot analysis with the indicated antibodies. Equal loading of protein was confirmed with β actin antibody. Bound antibody was detected by enhanced chemiluminescence using Western lightning western chemiluminescence reagent plus (enhanced luminol) following the manufacturer's directions (PerkinElmer Life and Analytical Sciences, Shelton, CT). The blots were imaged using Syngene G Box Fredrick MD and quantified using Gene tools software. Graphical representation of the quantified data is shown.

Figure 6. Over expression of FLIP enhances anchorage independent growth of PC-3 cells

PC-3 cells were plated in triplicate in 35 mm dishes on 0.5% agarose containing media as described in methods. Following 14-d incubation, cells were stained with 0.5 ml of 0.02% p-iodonitrotetrazolium and colonies containing at least 50 cells were counted in 10 different fields from each plate. The results are expressed as mean values $\pm$ sd and is a representative of two independent experiments.