Mechanistic Elucidation of the Antitumor Properties of Withaferin A in Breast Cancer

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Abstract

Withaferin A (WFA) is a steroidal lactone with antitumor effects manifested at multiple levels that are mechanistically obscure. Using a phospho-kinase screening array, we discovered that WFA activated phosphorylation of the S6 kinase RSK (ribosomal S6 kinase) in breast cancer cells. Pursuing this observation, we defined activation of extracellular signal-regulated kinase (ERK)–RSK and ETS-like transcription factor 1 (Elk1)–CHOP (C-EBP homologous protein) kinase pathways in upregulating transcription of the death receptor 5 (DR5). Through this route, WFA acted as an effective DR5 activator capable of potentiating the biologic effects of celecoxib, etoposide, and TRAIL. Accordingly, WFA treatment inhibited breast tumor formation in xenograft and mouse mammary tumor virus (MMTV)-neu mouse models in a manner associated with activation of the ERK/RSK axis, DR5 upregulation, and elevated nuclear accumulation of Elk1 and CHOP. Together, our results offer mechanistic insight into how WFA inhibits breast tumor growth. Cancer Res; 74(9); 1–13. ©2014 AACR.

Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide. Despite improvements in early diagnosis, and development of various targeted therapeutic approaches, breast cancer–related mortality still remains at a high level. Major limitations associated with available therapeutic approaches are high toxicity, lower efficacy, therapeutic resistance, and therapy-related morbidity. A more promising approach seems to be the development of more effective, nonendocrine, nontoxic therapeutic strategies using active constitutive agents in natural products owing to their cancer preventive as well as therapeutic potential. Historically, natural products have played an important role in the discovery and development of novel anticancer agents (1, 2). Withania somnifera has been successfully used in traditional ayurvedic medicine to treat various diseases owing to its broad spectrum pharmacologic efficacy (3–5). The root extract of Withania somnifera, is composed of 14 bioactive compounds known as withanolides (6, 7). Withaferin A (WFA) is the most abundant molecule, an issue, we addressed by systematically elucidating the underlying molecular mechanisms how WFA-mediated signaling networks in breast cancer growth inhibition and uncover its efficacy and help in clinical development of this bioactive molecule, an issue, we addressed by systematically elucidating the underlying mechanisms. Because many cellular signaling events involve induced phosphorylation of key targets, in the present study, we used phosphokinase arrays to gain insight into the intricacies of WFA-mediated signaling networks and discovered that WFA induces ribosomal S6 kinase (RSK) phosphorylation in breast cancer cells. We designed this study to examine the role of RSK and the underlying molecular mechanisms how WFA-mediated activation of RSK leads to growth inhibition of breast cancer cells. Here, we provide strong evidence for extracellular signal–regulated kinase (ERK)/RSK as an important mediator in WFA-induced breast cancer growth inhibition and uncover
a novel mechanism of WFA action through activation of the ETS-like transcription factor 1 (Elk1)/C-EBP homologous protein (CHOP) axis leading to death receptor 5 (DR5) activation.

Materials and Methods

Cell culture and reagents
The human breast cancer cell lines, MCF7, MDA-MB-231, T47D, and MDA-MB-468 were obtained from the American Type Culture Collection, resuscitated from early passage liquid nitrogen vapor stocks as needed and cultured according to the supplier's instructions. Cells were cultured for less than 3 months before reintroducing cultures and were routinely inspected microscopically for stable phenotype. WFA was procured from Calbiochem. Fluoromethyl ketone–methoxymethylamine (fmk–MEA), a specific p90-RSK inhibitor was kindly provided by Dr. Jack Taunton (University of California at San Francisco, Cellular and Molecular Pharmacology, San Francisco, CA; ref. 21). U0126 and trichostatin A (TSA) were procured from Sigma. CHOP overexpression construct was purchased from OriGene Technologies, Inc. Wild-type and S383A-mutant Elk1 plasmids were kindly provided by Dr. Andrew D. Sharrocks (University of Manchester, Manchester, UK; ref. 22). ERK plasmids were kindly provided by Dr. Paul Shapiro, University of Maryland (Baltimore, MD). Celecoxib was obtained from LKT laboratories. TRAIL was obtained from Millipore. Etoposide was purchased from Sigma.

Clonogenicity, anchorage-independent growth, and cell viability assays
To perform clonogenicity assay (23), breast cancer cells were treated with WFA as indicated for 10 days; colonies were counted. Anchorage-independent growth of breast cancer cells in the presence of WFA was assayed by colony formation in soft agar (24). Cell viability assay was performed using a commercially available XTT Assay Kit (Roche Applied Science).

Breast tumorigenesis assay
MDA-MB-231, MDA-MB-231-pLKO.1, MDA-MB-231-DR5shRNA1, and MDA-MB-231-DR5shRNA2 xenografts were generated as previously described (24), grouped in two experimental groups (8 mice/group) and treated with intra-peritoneal injections of either vehicle (10% dimethyl sulfoxide, 40% cremophor-EL, and 50% PBS) or vehicle containing 4-mg WFA (ChromaDex Inc.)/kg body weight 5 d/wk for 5 weeks. The dose and route of WFA administration were selected from previous study documenting in vivo efficacy of WFA (8). Tumors were collected after 4 weeks of treatment; measured, weighed, and subjected to further analysis by immunohistochemistry, real-time PCR (RT-PCR), and Western blotting. At least four random, nonoverlapping representative images from each tumor section from eight tumors of each group were captured using ImagePro software for quantitation of pERK (phosphorylated-ERK), pRSK (phosphorylated-RSK), CHOP, pElk1 (phosphorylated-Elk1), and DR5 expression. MMTV-neu mice model–mammary tumor tissues from our previously published prevention study in MMTV-neu mice (11) were also used to determine the expression of these proteins by Western blotting. In this study, WFA administration resulted in a statistically significant decrease in macroscopic mammary tumor size, microscopic mammary tumor area (11). All animal studies were in accordance with the guidelines of Johns Hopkins University Institutional Animal Care and Use Committee (IACUC) and University of Pittsburgh IACUC.

Phospho-antibody array analysis
Breast cancer cells were treated with WFA and the phospho-antibody array analysis was performed using the Proteome Profiler Human Phospho-Kinase Array Kit ARY003 from R&D Systems according to the manufacturer's instructions. Array images were analyzed using the GeneTools image analysis software (Syngene).

Subcellular fractions, immunoblotting, transfection, RNA interference, immunofluorescence, and confocal imaging
Cellular cytosolic and nuclear fractions were prepared following previously published protocol (25). Immunoblotting was carried out as described (26). The blots are representative of multiple independent experiments and bar diagrams are included showing quantitation of Western blot signals. Breast cancer cells were transfected with ERK, CHOP, Elk1-WT, and Elk1-S383A–mutant vectors using Lipofectamine-2000 (Invitrogen) and treated with WFA as indicated. For RNA interference, cells were transfected at 50% confluency with 100 nmol/L of control siRNA or ERK1/2 siRNA (SignalSilence) using Oligofectamine (Cell Signaling Technology). Breast cancer cells were subjected to immunofluorescence analysis as described previously (24).

Chromatin immunoprecipitation and RNA isolation, RT-PCR
Chromatin immunoprecipitation (ChIP) analyses were performed using our published procedure (27). Total cellular RNA was extracted using the TRIzol Reagent Kit (Life Technologies, Inc.). RT-PCR was performed using specific sense and antisense PCR primers.

Stable knockdown using lentiviral short hairpin RNA
Five to six premade lentiviral DR5, CHOP and RSK short hairpin RNA (shRNA) constructs, and a negative control construct created in the same vector system (pLKO.1) were purchased from Open Biosystems. Constructs were used for transient transfections using Fugene or Lipofectamine. Paired stable knockdown cells were generated following our previously established protocol (25).

Statistical analysis
All experiments were performed thrice in triplicates. Statistical analysis was performed using Microsoft Excel software. Significant differences were analyzed using the Student t test and two-tailed distribution. Results were considered to be statistically significant if P < 0.05. Results were expressed as mean ± SE between triplicate experiments performed thrice.
Results

**WFA treatment inhibits clonogenicity, anchorage-independent growth of breast cancer cells and inhibits breast tumor progression in athymic nude mice**

We first examined the effect of WFA on clonogenic potential and anchorage-independent growth of breast cancer cells. Treatment with 5 μmol/L WFA resulted in approximately 50% to 60% inhibition in clonogenicity and soft agar colony formation, whereas higher concentrations were more inhibitory (Fig. 1A and B). Exposure of breast cancer cells to WFA led to decreased cell viability (Supplementary Fig. S1A–S1D). WFA-mediated inhibition of cancer cell growth is associated with induction of apoptosis (8, 12, 17). Members of inhibitor-of-apoptosis protein family, survivin and X-linked inhibitor-of-apoptosis protein (XIAP), mainly function to suppress apoptosis by inhibiting caspases (28) and are associated with increased aggressiveness, higher recurrence rate, and unfavorable breast cancer outcome (28–30). Decreased expression of survivin and XIAP was observed in breast cancer cells treated with WFA (Supplementary Fig. S2A). An induction of cleaved PARP was observed in the presence of 5 and 10 μmol/L WFA (Supplementary Fig. S2A). Treatment of breast cancer cells with 5 μmol/L WFA for various time intervals showed a decrease in XIAP expression along with an induction of PARP cleavage (Supplementary Fig. S2B).

We further investigated the _in vivo_ physiologic relevance of our _in vitro_ findings by evaluating whether WFA had inhibitory effects on the development of breast carcinoma in nude mouse models. Tumor growth was significantly inhibited in the WFA-treated experimental group in comparison with the control group (Fig. 1C). Ki-67, a nuclear nonhistone protein, is one of the major markers of tumor proliferation (31) used as a decision-making tool for adjuvant therapy (32). The immunohistochemical assessment of tumor proliferation showed higher Ki-67 in the control group as compared with the WFA-treated group (Fig. 1D and E). In our _in vitro_ analysis, we found that WFA modulated the expression of survivin and XIAP in breast cancer cells. Corroborating the _in vitro_ findings,
the tumors from the WFA-treated mice exhibited lower expression of survivin and XIAP (Fig. 1D and E). The number of TUNEL-positive apoptotic cells was increased in tumors from the WFA-treated mice compared with the vehicle control group (Fig. 1F). Collectively, these results show that WFA treatment results in suppression of tumor growth, inhibition of cellular proliferation, and increased apoptosis in the breast tumors.

**WFA-dependent changes in phosphorylation of signaling mediators in breast cancer cells**

Phosphorylation of kinases is fundamentally important to multiple aspects of signaling networks and cellular functions. To identify cellular signal transduction pathways involved in WFA-induced inhibition of breast carcinogenesis, we interrogated 46 specific Ser/Thr/Tyr phosphorylation sites of 38 selected proteins using phosphoprotein arrays. Breast cancer cells were treated with 5 μmol/L WFA for 3 hours and subjected to human phospho-antibody array analyses. Relative levels of protein phosphorylation (normalized intensity for each antibody) were calculated for each untreated and treated sample. *, *P < 0.001, compared with untreated controls. C, immunoblot analysis of pRSK-Ser380 and total RSK in breast cancer cells treated with WFA as indicated. D, breast cancer cells were treated with 5 μmol/L WFA for indicated time intervals. Total lysates were immunoblotted for pRSK and total RSK expression. E, breast cancer cells were treated as in C; lysates were examined for pERK44/42 and total ERK. F, breast cancer cells were treated as in D; total lysates were immunoblotted for pERK and total ERK expression. G, breast cancer cells were transiently transfected with siERK-siRNAs for 48 hours followed by immunoblot analysis of ERK levels. H, breast cancer cells were transfected with siERK as in G, followed by WFA treatment (5 μmol/L, 3 hours) and immunoblot analysis of pRSK and total RSK levels. C, vehicle-treated cells.

Figure 2. Human phospho-antibody array analyses reveal WFA-induced increased phosphorylation of RSK and ERK and WFA activates RSK in an ERK-dependent manner. A and B, MCF7 and MDA-MB-231 breast cancer cells were treated with 5 μmol/L WFA for 3 hours and subjected to human phospho-antibody array analyses. Relative levels of protein phosphorylation (normalized intensity for each antibody) were calculated for each untreated and treated sample. *, *P < 0.001, compared with untreated controls. C, immunoblot analysis of pRSK-Ser380 and total RSK in breast cancer cells treated with WFA as indicated. D, breast cancer cells were treated with 5 μmol/L WFA for indicated time intervals. Total lysates were immunoblotted for pRSK and total RSK expression. E, breast cancer cells were treated as in C; lysates were examined for pERK44/42 and total ERK. F, breast cancer cells were treated as in D; total lysates were immunoblotted for pERK and total ERK expression. G, breast cancer cells were transiently transfected with siERK-siRNAs for 48 hours followed by immunoblot analysis of ERK levels. H, breast cancer cells were transfected with siERK as in G, followed by WFA treatment (5 μmol/L, 3 hours) and immunoblot analysis of pRSK and total RSK levels. C, vehicle-treated cells.
onscogenic role of RSK, multiple studies have shown RSK as a potential tumor suppressor, a participant in p53-dependent cell growth arrest, showing an important role in decreasing cancer cell proliferation, invasion, and metastasis (33–35). We further explored the molecular mechanisms how WFA activates RSK and the biologic significance of RSK activation in WFA-mediated inhibition of breast carcinogenesis.

**WFA treatment activates p90-RSK via ERK-dependent manner in breast cancer cells**

To confirm whether WFA can activate RSK as indicated by phosphoprotein array results, and to clarify its dose- and time-dependent response, we treated breast cancer cells with WFA. Immunoblot analysis showed that phosphorylation of RSK increased in a concentration-dependent manner in both MCF7 and MDA-MB-231 cells (Fig. 2C). Treatment of breast cancer cells with 5 μmol/L WFA for various time intervals exhibited striking increase in RSK phosphorylation within 30 minutes (Fig. 2D and Supplementary Fig. S4A), whereas total RSK remained unchanged. RSK activation is controlled by the canonical Ras/mitogen-activated protein kinase (MAPK) pathway via direct phosphorylation of RSK by ERK (34). ERK itself represents a major survival signaling pathway that promotes cancer cell survival via inhibiting apoptosis; however, a proapoptotic role of ERK signaling has also been shown (36). Our phosphoprotein array analysis showed elevated phosphorylation of ERK in breast cancer cells treated with WFA. Because of these interesting attributes, we explored the effect of WFA on ERK activation. Increased phosphorylation of ERK was observed in response to WFA treatment as compared with untreated cells (Fig. 2E). Breast cancer cells, treated with 5 μmol/L WFA for various intervals of time, showed that WFA increased ERK phosphorylation within 30 minutes of WFA treatment (Fig. 2F and Supplementary Fig. S4B). We questioned whether ERK is a key player in WFA-mediated RSK activation and toward this end, we used ERK siRNA to silence ERK (Fig. 2G) and then investigated its impact on WFA-induced RSK activation. Immunoblot analysis for phosphorylated RSK levels after ERK silencing and WFA treatment showed that indeed ERK silencing inhibited WFA-induced RSK phosphorylation (Fig. 2H and Supplementary Fig. S4C). Inhibition of ERK phosphorylation using U0126 also resulted in abrogation of WFA-mediated RSK activation (Supplementary Fig. S5A). Next, we examined the importance of ERK and RSK activation in WFA-mediated apoptosis in breast cancer cells using MEK/ERK inhibitor, U0126, and RSK inhibitor, fmk–MEA. WFA treatment resulted in elevated PARP cleavage, indicating increased apoptotic response in breast cancer cells, which was inhibited in breast cancer cells cotreated with U0126 or fmk–MEA (Supplementary Fig. S5B and S5C). ERK silencing abrogated WFA-mediated inhibition of clonogenicity of breast cancer cells and reintroduction of ERK in ERK-silenced cells resensitized them to WFA (Supplementary Fig. S6A). RSK inhibition using RSK–shRNA(s) (Supplementary Fig. S6B) also rendered breast cancer cells nonresponsive to WFA-mediated inhibition of clonogenicity of breast cancer cells (Supplementary Fig. S6C). Collectively, these pieces of evidence support the notion that RSK and ERK activation plays an important role in mediating biologic effects of WFA in breast cancer cells.

**WFA induces concomitant upregulation and nuclear translocation of Elk1 and CHOP via the ERK/RSK signaling axis in breast cancer cells**

The ETS domain transactivation factor Elk1 is a direct target of the MAPK pathways and phosphorylation of the Elk1 transcriptional activation domain by MAPKs triggers its activation, which further induces apoptosis and growth inhibition (37). Phosphorylation at serine 383 plays a critical role in Elk1 activation. Increased Elk1 phosphorylation was observed within 30 minutes of WFA treatment, which was diminished after 6 hours posttreatment (Fig. 3A and B). We examined whether WFA-induced Elk1 phosphorylation involves ERK. Indeed, pretreatment of breast cancer cells with ERK siRNA reduced WFA-induced phosphorylation of Elk1, indicating a direct regulatory role of ERK (Supplementary Fig. S7A and S7B). Our studies discovered the involvement of RSK in WFA function, therefore, we investigated whether RSK is integral for WFA-mediated Elk1 phosphorylation. RSK-specific inhibitor fmk–MEA inhibited phosphorylation of RSK (Fig. 3C). Pretreatment of breast cancer cells with fmk–MEA followed by WFA treatment showed that inhibition of RSK phosphorylation inhibited WFA-induced Elk1 phosphorylation (Fig. 3D and Supplementary Fig. S7C). Phosphorylation can also affect the subcellular localization of Ets proteins (37, 38). As evident in Fig. 3E, WFA treatment did not affect the total expression level of Elk1 in either cytoplasmic or nuclear fraction; however, increased level of phosphorylated Elk1 was observed in nuclear fraction within 30 minutes after treatment, which remained elevated for the duration of the treatment (Fig. 3E and Supplementary Fig. S7D). Immunofluorescence analysis of breast cancer cells showed nuclear phosphorylated Elk1 in WFA-treated cells (Fig. 3F).

Induction of apoptotic response by many pharmacologically active compounds and anticancer drugs is achieved by activation of endoplasmic reticulum (ER) stress. ER stress signaling system induces transcription of CHOP, a key proapoptotic transcription factor that binds with other transcription factors and induces proapoptotic genes (39). We found that WFA increased expression of CHOP in a temporal manner with a significant increase observed within 30 minutes after treatment (Fig. 4A and B). To evaluate the contribution of ERK in WFA-induced CHOP overexpression, we examined CHOP expression after ERK silencing using ERK siRNA. Results indicated that indeed ERK silencing abrogated WFA-induced CHOP expression (Fig. 4C and D), suggesting the involvement of ERK in the CHOP-stimulatory effect of WFA. Once upregulated, CHOP translocates to the nucleus and participates in transcriptional modulation of responsive genes critical for tumor cell apoptosis (39). Analysis of nuclear and cytoplasmic fractions from WFA-treated cells showed that WFA treatment increased nuclear accumulation of CHOP (Fig. 4E and F). Immunostaining of MCF7 and MDA-MB-231 cells showed that WFA treatment increases nuclear accumulation of CHOP, whereas untreated cells showed predominantly cytoplasmic localization of CHOP (Fig. 4G). CHOP inhibition using
CHOP–shRNA(s) (Supplementary Fig. S8A) rendered breast cancer cells nonresponsive to WFA-mediated inhibition of clonogenicity of breast cancer cells (Supplementary Fig. S8B), exhibiting importance of CHOP in WFA function. These findings suggest that the ERK/RSK signaling axis plays an important role in regulating upregulation and activation of CHOP and Elk1 in breast cancer cells.

**Elk1 and CHOP contribute to WFA-induced upregulation of DR5 in breast cancer cells**

DR5 is a death domain–containing transmembrane receptor that triggers apoptotic response upon overexpression or activation by certain stimuli, including small-molecule anticancer drugs and bioactive molecules (40, 41). We found that WFA induced the expression of DR5 in breast cancer cells (Fig. 5A and Supplementary Fig. S9A–S9D). The fact that DR5 promoter contains cis-acting CHOP-like binding sequence prompted us to explore the connection between WFA-mediated CHOP induction and DR5 upregulation. We investigated whether enforced overexpression of CHOP (Fig. 5B) using CHOP overexpression construct alters WFA-mediated DR5 expression. Overexpression of CHOP in MCF7 and MDA-MB-231 cells increased DR5 expression potentiating the effect of WFA (Fig. 5C and Supplementary Fig. S9E). In a reciprocal approach, CHOP was silenced using CHOP–shRNA approach (Supplementary Fig. S8A) followed by WFA treatment. Silencing of CHOP in...
breast cancer cells inhibited WFA-mediated induction of DR5 expression (Fig. 5D). A recent study reported that DR5 promoter contains a putative Elk1-binding site and mutations in this binding site affected DR5 transactivation, suggesting a link between DR5 and Elk1 (42). Because there was no previous study linking Elk1 to DR5 expression in breast cancer cells, we asked whether Elk1 was indeed involved in regulating WFA-mediated DR5 expression. Transactivation potential of Elk1 is enhanced by S383 phosphorylation (38), which is increased by WFA (Fig. 3A and B); therefore, we focused on the key phosphoacceptor residue Ser383. Breast cancer cells were transfected with wild-type or phospho-deficient (S383A) Elk1 constructs. Expression of wild-type Elk1 increased DR5 expression, whereas phospho-deficient Elk1 did not affect DR5 expression (Fig. 5E). To examine whether Elk1 contributes to WFA-mediated DR5 upregulation, we transfected breast cancer cells with wild-type or phospho-deficient Elk1 constructs followed by WFA treatment. Wild-type Elk1 potentiated, whereas phospho-deficient Elk1 inhibited WFA-mediated DR5 expression (Fig. 5F and Supplementary Fig. S9F) demonstrating the

Figure 4. WFA increases CHOP expression via ERK and promotes its nuclear translocation. A, immunoblot analysis of CHOP in breast cancer cells treated with 5 μmol/L WFA for indicated time intervals. B, bar diagram, quantitation of Western blot signals from multiple independent experiments. *P < 0.001, compared with untreated controls. C, breast cancer cells were transfected with siERK for 48 hours followed by WFA (5 μmol/L, 3 hours) treatment and immunoblot analysis using anti-CHOP antibodies. C, control-transfected cells. D, bar diagram, quantitation of Western blot signals from multiple independent experiments. *P < 0.001, compared with untreated controls; **P < 0.05, compared with WFA-treated cells. E, immunoblot analysis of CHOP in cytoplasmic and nuclear fractions of breast cancer cells treated with 5 μmol/L WFA for indicated time intervals. F, bar diagram, quantitation of Western blot signals from multiple independent experiments. *P < 0.05, compared with untreated controls. G, breast cancer cells were treated with 5 μmol/L WFA for 6 and 24 hours and subjected to immunofluorescence analysis of CHOP. Nuclei were visualized with DAPI staining. C, vehicle-treated cells.
important role of p-Ser383-Elk1 in WFA action. Our studies also showed an important role of RSK phosphorylation in WFA-mediated Elk1 phosphorylation (Fig. 3D and Supplementary Fig. S7C); therefore, we examined whether RSK inhibitor fmk–MEA could modulate WFA-mediated DR5 upregulation. Indeed, inhibition of RSK phosphorylation resulted in inhibition of WFA-induced DR5 expression (Fig. 5G). RSK was silenced using RSK–shRNA approach (Supplementary Fig. S6B) followed by

Figure 5. CHOP and Elk1 contribute to WFA-induced upregulation of DR5 in breast cancer cells. A, immunoblot analysis of DR5 in breast cancer cells treated with 5 μmol/L WFA for indicated time intervals. B, breast cancer cells were transfected with CHOP overexpression plasmid (CHOPOE) and analyzed for CHOP expression using immunoblot analysis. C, immunoblot analysis of CHOP and DR5 in breast cancer cells transfected with CHOPOE followed by WFA (5 μmol/L, 3 hours) treatment (CHOPOE + WFA). D, breast cancer cells were transfected with CHOP-shRNA1 followed by WFA (5 μmol/L, 3 hours) treatment. Total lysates were analyzed for DR5 expression. E, breast cancer cells were transfected with Elk1 wild-type (Elk1 WT) and S383A phospho-mutant Elk1 (Elk1M) plasmids. Total lysates were analyzed for pElk1, total Elk1, and DR5 expression. F, MCF7 cells were transfected with Elk1 WT and Elk1M as in E, followed by WFA (5 μmol/L, 3 hours) treatment. C, vehicle-treated controls. Total lysates were analyzed for pElk1, total Elk1, and DR5 expression. G, immunoblot analysis of pRSK, total RSK, and DR5 in MDA-MB-231 cells pretreated with fmk–MEA (6 μmol/L, 3 hours) followed by WFA (5 μmol/L, 3 hours) treatment. H, breast cancer cells were transfected with RSK–shRNA1 followed by WFA (5 μmol/L, 3 hours) treatment. Total lysates were analyzed for DR5 expression. I, MCF7 cells were treated with 5 μmol/L WFA for 3 and 6 hours and subjected to ChIP assay using CHOP, pElk1, HDAC1, and acetylated-H4 antibodies. The purified DNA was analyzed by PCR using specific primers spanning the CHOP and Elk1-binding sites on DR5 promoter. C, vehicle-treated cells.
WFA treatment. Silencing of RSK in breast cancer cells inhibited WFA-mediated induction of DR5 expression (Fig. 5H). In an effort to better understand the molecular events involved in WFA-induced DR5 expression, we used ChIP analyses to examine the recruitment of pElk1 and CHOP to the DR5 promoter. pElk1 and CHOP were associated with the DR5 promoter in the presence of WFA. DR5 in repressed state has been shown to be associated with HDAC1 (43). Intriguingly, we observed release of HDAC1 from DR5 promoter in response to WFA treatment and a significant increase in histone H4 acetylation, indicating active chromatin conformation (Fig. 5L and Supplementary Fig. S10A). Treatment of breast cancer cells with HDAC inhibitor, TSA, resulted in increased expression of DR5 (Supplementary Fig. S10B) supporting a functional role of HDAC1 in regulation of DR5 expression. Taken together, these data show that WFA induces DR5 expression in breast cancer cells through a mechanism involving CHOP and Elk1.

**WFA is a potent inducer of DR5 and silencing of DR5 abrogates WFA-mediated growth inhibition of breast cancer cells**

DR5, upon induction, mediates enhancement of TRAIL-induced apoptosis and contributes to apoptosis by other small-molecule drugs (44). Using established small-molecule DR5 inducers (TRAIL, celecoxib, and etoposide), we compared the efficacy of WFA to induce DR5 expression in breast cancer cells. It was interesting to note that WFA treatment resulted in a greater induction of DR5 expression in comparison with TRAIL, celecoxib, and etoposide (Fig. 6A and Supplementary Fig. S11A). To determine whether WFA enhances the effect of small-molecule DR5 inducers, we treated breast cancer cells with WFA in combination with TRAIL, etoposide, and celecoxib and assessed modulation of DR5 expression, clonogenicity, and soft agar colony formation. Combination treatment with WFA enhanced etoposide-, celecoxib-, and TRAIL-induced DR5 expression (Fig. 6B and Supplementary Fig. S11B) and resulted in significantly higher induction of clonogenicity and soft agar colony formation (Fig. 6C and D). These results show that WFA is a potent inducer of DR5 that can act as an effective bioactive alternative to TRAIL, celecoxib, and etoposide as well as enhance their effect when used in combination.

To directly examine the role of DR5 in WFA-mediated growth inhibition of breast cancer cells, we used DR5shRNA lentiviruses and puromycin to select for stable pools of MCF7 and MDA-MB-231 cells with DR5 depletion. We analyzed plKO.1 and DR5shRNA stable MCF7 and MDA-MB-231 cell pools for DR5 protein expression, and found that DR5 protein expression was significantly knocked down in DR5shRNA1–3 cells as compared with plKO.1 control cells (Fig. 6E). WFA increased PARP cleavage in plKO.1 cells, whereas no change in cleaved PARP was observed in DR5shRNA MCF7 and DR5shRNA MDA-MB-231 cells (Fig. 6F). WFA treatment efficiently inhibited clonogenicity and soft agar colony formation of plKO.1 breast cancer cells but not of DR5shRNA cells (Fig. 6G–I). We further investigated the in vivo physiologic relevance of our in vitro findings by evaluating whether DR5 is integral for the inhibitory effects of WFA on the development of breast carcinoma in nude mouse models. MDA-MB-231-plKO.1 and MDA-MB-231-DR5shRNA1 and shRNA2 were used in the xenograft athymic nude mice model. Tumor growth was significantly inhibited in WFA-treated MDA-MB-231-plKO.1 (the vector control group), whereas WFA was unable to inhibit tumor growth in MDA-MB-231-DR5shRNA1 and shRNA2 groups (Fig. 6J–L). These results showed that WFA-induced DR5 overexpression is indeed a crucial component of the signaling machinery used by WFA in inhibiting growth of breast cancer cells.

**WFA administration modulates activation of ERK/RSK and CHOP/Elk1 axes in vivo**

Our studies show that WFA treatment inhibits breast tumor progression in vivo (Fig. 1C). We used tumor tissue from the same experiment to determine the effect of WFA treatment on the expression and activation of important signaling molecules. Tumors from WFA-treated mice exhibited increased phosphorylation of ERK, RSK, Elk1 as well as higher expression of CHOP and DR5 in comparison with the vehicle-treated group (Fig. 7A). Also, WFA-treated tumors showed transcriptional upregulation of DR5 (Fig. 7B). Immunohistochemical analysis showed that tumors from WFA-treated mice exhibited higher number of tumor cells showing increased expression of pERK, pRSK, CHOP, pElk1, and DR5 as compared with tumors from the vehicle-treated group (Fig. 7C and D) providing physiologic relevance to our in vitro findings. Earlier studies from our group have shown that WFA treatment prevents mammary cancer development in the MMTV-neu mouse model, tumor weight in the WFA treatment group was lower by 50% in comparison with the control group (11). Mammary tumor tissues from this study were used to examine the ERK/RSK signaling axis and DR5. Tumors from the WFA-treated group exhibited increased expression of phosphorylated ERK and RSK as well as higher expression of DR5 in comparison with the control group (Fig. 7E). Collectively, the findings presented here suggest that WFA inhibits breast tumor progression and provide in vitro as well as in vivo evidence for the involvement of RSK as an important mediator, and uncover a novel mechanism of WFA action through activation of Elk1 leading to DR5 activation.

**Discussion**

The realization of the ability of bioactive small-molecule agent WFA to effectively inhibit carcinogenesis in a nontoxic, nonendocrine manner has made this agent of potential interest in the treatment of breast cancer and sparked a new interest in understanding the underlying molecular mechanisms. Deciphering the key nodes of WFA action can help in establishing surrogate biomarkers for its efficacy and help in clinical development of this bioactive molecule. We found that WFA effectively inhibits the growth of breast cancer cells in vitro and in vivo but our phosphokinase array studies led us to novel discoveries that extend beyond the popular proliferative and oncogenic role of certain kinases and inhibition of these kinases by anticancer agents to achieve tumor growth inhibition. Both p90-RSK and ERK are popularly known for their oncogenic role but this study illustrates that WFA despite efficiently inhibiting breast tumor growth, increases phosphorylation of RSK in
breast cancer cells via activation of ERK. These studies provide an interesting mechanism by which WFA-induced ERK/RSK, in a concerted action, results in concomitant upregulation/activation of CHOP and Elk1. We also identify WFA as a potent DR5 activator in breast cancer whose effects mimic molecular and cell biological outcomes of ligand-dependent DR5 activation. Mechanistically, this process is associated with recruitment of CHOP and Elk1 to DR5 promoter, increased histone acetylation in conjunction with clearance of histone deacetylase HDAC1. In vivo analyses of tumor xenografts and tumors from MMTV-neu mice provide further evidence of involvement of ERK/RSK and CHOP/Elk1 axes and an integral role of DR5. On the basis of these data, we provide a schematic diagram depicting a series of events, including a feed-forward interaction of ERK and RSK, direct involvement of CHOP/Elk1/HDAC1 in DR5 upregulation, which is operative in WFA-induced DR5-dependent growth inhibition of breast cancer cells (Fig. 7F).

Our studies offer the first evidence of the ability of WFA to activate RSK. RSK directly phosphorylates transcription factors, hence, regulating the transactivation of multiple gene targets. RSK positively regulates diverse cellular processes, including transformation, cell cycle, cell proliferation, cell survival, and migration in response to various growth factors, chemokines and other stimuli (45). RSK has been implicated in...
apoptosis inhibition achieved in part by phosphorylation of Bad, CEBPβ and DAPK (34), as well as apoptosis induction via phosphorylating Nur77 (46). We show that the ability of RSK to mediate activation of Elk1 and CHOP leading to upregulation of DR5 is critical to WFA outcomes in breast cancer cells. This provides a new mechanism by which WFA can achieve effective breast tumor growth inhibition. Also, these discoveries have potentially important implications in regard to increasing interest in targeting breast cancer through manipulation of DR5.

Activation of death receptors to induce apoptosis in tumor cells has been recognized as an effective way to therapeutically target epithelial cell–derived cancers but this notion is marred by the toxic effects of death receptor ligands, FASL and TNF. Over the few years, several small-molecule activators of the death receptor family have been reported with some more potent than others (44). WFA has been shown to sensitize TRAIL-induced apoptosis and activates DR5 expression in human renal cancer cells (Caki cells, but not in human normal mesangial cells; ref. 41). Here, we show that WFA is more effective than three other known DR5 activators, TRAIL, etoposide, and celecoxib, in activation of DR5 in breast cancer cells, hence, seems to be a good candidate for further development as an effective DR5 inducer. It is interesting to note that in recent years, many phase I and II single agent and combination clinical trials have been conducted to examine the efficacy and safety of recombinant human TRAIL (dulanermin; Amgen/Genentech), and the agonistic monoclonal antibodies to DR5 [lexatumumab (Human Genome Sciences), conatumumab (Amgen), drozitumab (Genentech), tigatuzumab (Daichi-Sankyo), and LBY135 (Novartis); refs. 44, 47]. Studies with these proapoptotic receptor agonists (PARA) have been encouraging but no full phase III studies have been performed, suggesting the need for novel PARAs with improved properties.

Moreover, we report a novel finding that ERK activation is important for WFA action in breast cancer cells. Generally considered a survival signaling pathway, ERK-mediated
induction of apoptotic pathways has also been reported (36, 48). Kinetics and duration of ERK activation are important for these unusual effects of ERK. A transient activation of ERK inhibits cell death (49), whereas prolonged ERK activation is associated with the proapoptotic effect of ERK (36). WFA induces a sustained ERK activation in breast cancer cells, which was observed for the duration of experiment (until 24 hours). Very recently, it was reported that WFA increases ERK phosphorylation in MCF7 and SUM159 cells (50). It is known that the ERK protein directly phosphorylates Elk1 (38). Hence, the finding of the involvement of ERK in regulation of WFA-induced cell death resulted in our subsequent novel finding that WFA activates Elk1, which contributes to DR5 induction. In our study, inhibition of ERK, RSK, or Elk1 with small-molecule inhibitors and siRNA- or shRNA-mediated gene silencing abolishes WFA-mediated DR5 induction.

In conclusion, we uncovered a novel mechanism by which WFA inhibits growth of breast cancer cells in vitro and in vivo, which involves activation of RSK and ERK. We also demonstrate a feed-forward loop of ERK and RSK that results in concurrent activation of CHOP and pElk1, their recruitment to DR5 promoter leading to DR5 activation. Our results, thus, demonstrate the integral role of a previously unrecognized functional cross-talk between WFA and ERK/RSK and CHOP/Elk1 axes in breast tumor growth inhibition. Also, our findings may potentially open new avenues of research on the role of WFA as a novel PARA.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Nagalingam, D. Sharma, N.K. Saxena
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Nagalingam, S.V. Singh, N.K. Saxena
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Nagalingam, P. Kuppusamy, D. Sharma, N.K. Saxena

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