Published in final edited form as:

Mol Cell Endocrinol. 2015 May 5; 406: 102–114. doi:10.1016/j.mce.2015.02.020.

Epigenetic reactivation of $p21^{CIP1/WAF1}$ and *KLOTHO* by a combination of bioactive dietary supplements is partially ER α -dependent in ER α -negative human breast cancer cells

Sonam Sinha^a, Samriddhi Shukla^a, Sajid Khan^a, Trygve O. Tollefsbol^b, and Syed M. Meeran^{a,c,*}

^aLaboratory of Cancer Epigenetics, Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow 226 031, India

^bDepartment of Biology, University of Alabama at Birmingham, AL 35294, USA

^cAcademy of Scientific and Innovative Research (AcSIR), New Delhi, India

Abstract

Available treatment strategies against estrogen receptor (ER)-negative breast cancer patients are limited due to their poor response to hormonal therapy. We have shown previously that the combinations of green tea polyphenols (GTPs), a dietary DNA methyltransferase inhibitor, and sulforaphane (SFN), a dietary histone deacetylase inhibitor, reactivate ER α expression in ER α -negative MDA-MB-231 cells. Here, we investigated the functional significance of ER α reactivation in the reactivation of silenced tumor suppressor genes (TSGs) in ER α -negative human breast cancer cells. We found that the treatment of MDA-MB-231 cells with the combinations of GTPs and SFN leads to the reactivation of silenced TSGs such as $p21^{CIP1/WAF1}$ and *KLOTHO* through active chromatin modifications. Further, GTPs- and SFN-mediated reactivation of TSGs was, at least in part, dependent on ER α reactivation in ER α -negative MDA-MB-231 cells. Collectively, our findings suggest that a novel combination of bioactive dietary supplements could further be explored as an effective therapeutic option against hormonal refractory breast cancer.

Keywords

Green tea; Sulforaphane; Estrogen receptor; Tumor suppressor genes; Epigenetics; Breast cancer

1. Introduction

Breast cancer is the most common cancer and a major cause of cancer-associated mortality among females worldwide (Jemal et al., 2011). One of the important classifications of breast cancer is based on the presence or absence of hormonal receptors. The presence of estrogen receptor- α (ER α) is an important determinant for hormonal therapy using available selective estrogen receptor modulators (SERMs) (Chang et al., 2000). Recently, many

*Corresponding author. Laboratory of Cancer Epigenetics, Division of Endocrinology, CSIR-Central Drug Research Institute (CSIR-CDRI), Jankipuram Extn., Sector-10, Sitapur Road, Lucknow-226 031, India. Tel.: +91 522 2772450; fax: +91 522 2771941. s.musthapa@cdri.res.in (S.M. Meeran).

investigators including us have demonstrated the possibilities of the epigenetic reactivation of ER α in ER α -negative human breast cancer cells for the effective treatment with available SERMs (Li et al., 2013; Meeran et al., 2012; Sharma et al., 2005; Yang et al., 2001). Previous studies have shown that *ER α* promoters as well as promoters of various tumor suppressor genes (TSGs) such as *p16^{INK4A}* and *p21^{CIP1/WAF1}* are in general hypermethylated for their transcriptional silencing in cancer (Herman and Baylin, 2003; Jones, 2002; Majid et al., 2008). In addition to DNA hypermethylation, chromatin modifications, which include enrichment of inactive chromatin markers and down-regulation of active chromatin markers, are associated with transcriptional repression of TSGs (Fang et al., 2003; Herman and Baylin, 2003; Majid et al., 2008; Rubinek et al., 2012).

Tumor suppressor proteins such as BRCA1, *p16^{INK4A}* and *p21^{CIP1/WAF1}* regulate cellular proliferation and apoptosis through various mechanisms including cell cycle regulation (Kawai et al., 2002; Mandal and Davie, 2010). Tumor suppressor *p21^{CIP1/WAF1}* is a cyclin-dependent kinase inhibitor and its upregulation causes cell cycle arrest at G₁ or G₂/M phase, followed by the inhibition of cellular proliferation and induction of cellular apoptosis (Chen et al., 2013a; Kawai et al., 2002; Mandal and Davie, 2010). Expression of *p21^{CIP1/WAF1}* has been shown to be epigenetically silenced in many cancers including breast cancer (Chen et al., 2013a; Chew et al., 2012). In addition to *p21^{CIP1/WAF1}*, KLOTHO, a novel 1012-amino acid transmembrane protein, has also been shown to play major role as a tumor suppressor, mainly by inhibiting IGF-1/insulin pathways in many cancer subtypes (Doi et al., 2011; Lee et al., 2010; Wolf et al., 2008; Xie et al., 2013). In addition, upregulation of KLOTHO has been positively correlated with the suppression of cancer metastasis through inhibition of transforming growth factor- β 1 signaling (Doi et al., 2011). Therefore, upregulation of TSGs have central importance in both cancer prevention and therapy.

Epigenetic modalities of gene regulations have drawn considerable attention in the past few years and have been advanced for cancer prevention and therapy. Studies have shown that DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors, either alone or in combinations, alter the expression status of TSGs in many human cancers (Brueckner et al., 2005; Fang et al., 2003; Majid et al., 2008). However, the use of synthetic inhibitors is generally associated with too many adverse side effects and many are not delivered well to solid tumors which limits the practical applications to chemoprevention and therapy. Therefore, we have chosen a combination of bioactive dietary components, green tea polyphenols (GTPs), a dietary DNMT inhibitor, and sulforaphane (SFN), a dietary HDAC inhibitor, to study their chemotherapeutic effect in hormonal refractory breast cancer. The major active component of GTPs, epigallocatechin-3-gallate (EGCG), a known DNMT inhibitor, has been shown to possess anti-cancer properties through induction of cellular apoptosis and cell cycle arrest in many cancer cells (Ahmad et al., 1997; Fang et al., 2003). SFN, an active component present in many cruciferous vegetables, has been shown to have HDAC inhibitory activities (Dashwood and Ho, 2007; Myzak et al., 2006a). The HDAC inhibitory activity of SFN has been demonstrated to alter the histone acetylation status of a number of genes, thereby inhibiting cellular proliferation and inducing cellular apoptosis in many human cancers (Meeran et al., 2010; Myzak et al., 2006a). Previously, we have found that the combinations of GTPs and SFN synergistically reactivate ER α expression in ER α -negative human breast cancer cells through active chromatin modifications (Meeran et al.,

2012). In this study, we investigated the role of ER α reactivation in the upregulation of silenced TSGs and their underlying molecular mechanisms in ER α -negative MDA-MB-231 cells.

Our present study demonstrates that the treatment with combinations of GTPs and SFN synergistically reactivates *p21^{CIP1/WAF1}* and *KLOTHO* expressions through active DNA demethylation and histone modifications at the *p21^{CIP1/WAF1}* and *KLOTHO* promoters in human breast cancer cells. Further, our *ER α* -knockdown study revealed that GTPs- and SFN-mediated reactivation of TSGs is, at least in part, dependent on the epigenetic reactivation of *ER α* in ER α -negative MDA-MB-231 cells. Furthermore, our findings strengthen the idea of novel dietary combinations of DNMT and HDAC inhibitors for the effective treatment of hormonal refractory breast cancer.

2. Material and methods

2.1. Cell culture and treatment

Human breast cancer MDA-MB-231 and MDA-MB-453 cells were purchased from American Type Cell Culture Collection (ATCC, Manassas, VA). MDA-MB-231 and MDA-MB-453 cells were cultured and maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% dextran-charcoal-stripped FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich) in a humidified incubator at 37 °C with 5% CO₂. GTPs and R, S-sulforaphane were procured from LKT Laboratories (Minneapolis, MN) and working stocks were prepared as described previously (Meeran et al., 2012).

2.2. Analysis of cell cycle progression

Approximately 2×10^5 MDA-MB-231 and MDA-MB-453 cells were plated in each well of 6-well plates. After overnight serum starvation, cells were treated with either GTPs or SFN or the combinations of both at the indicated concentrations. Cells were also treated with a combination of 10 μ M 5-aza-2'-deoxycytidine (5-AZA), a standard DNMT inhibitor, and 500 nM suberoylanilide hydroxamic acid (SAHA), a standard HDAC inhibitor. Cells were harvested after 48 h, washed with cold PBS and were fixed in 100% methanol at -20 °C overnight. After washing, cells were suspended in 0.1% RNase A and stained with 50 μ g/mL propidium iodide. Cell cycle distribution was analyzed using a FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software. The results were represented as percentage of cells in each phase of cell cycle.

2.3. Quantitative real-time PCR

Human breast cancer MDA-MB-231 and MDA-MB-453 cells were treated with either GTPs or SFN or the combinations of both at the indicated concentrations for 12, 24, 48 and 72 h. Total RNA was extracted at approximately 70–80% confluence using the Trizol–RNA isolation protocol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Two micrograms of RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR was carried out in a lightcycler-480 thermocycler (Roche, Germany) using SsoFast EvaGreen Supermix detection system (Bio-Rad). The primers specific for *p21^{CIP1/WAF1}*, *KLOTHO* and

glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were obtained from Sigma-Aldrich. The sequences of primers with their respective annealing temperatures (T_A) are given in Supplementary Table S1. The reaction conditions were 30 s at 94 °C followed by 45 cycles (5 s at 94 °C; 20 s at T_A °C; 30 s at 72 °C) and extension at 72 °C for 2 min. The relative levels of mRNA expression were calculated using the cycle threshold (C_t) method: $2^{-C_t} = 2^{\{C_t(\text{untreated control}) - C_t(\text{Gene of interest})\}}$, where $C_t = C_t(\text{Gene of interest}) - C_t(\text{GAPDH})$.

2.4. Western blot analysis

Cells were treated with either GTPs or SFN and the combinations of both for 12, 24, 48 and 72 h. After incubation, protein was extracted from cultured cells at approximately 70–80% confluence using RIPA-protein extraction buffer (Millipore, Billerica, MA). For western blot analysis, 40 µg of protein was subjected to SDS–PAGE using 12% Tris–glycine gels and transferred electrophoretically onto 0.45 µm PVDF membranes (Millipore). Non-specific sites were blocked by incubating with blocking buffer for 1 h and the membranes were incubated overnight with primary antibodies specific for p21^{CIP1/WAF1} (1:500), CDK4 (1:500), CDK6 (1:500), ERα (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA), KLOTHO (1:1000) (Sigma-Aldrich), β-actin (1:5000), phospho-p44/42 MAPK (Erk1/2) (Thr202/ Tyr204) (1:1000), p44/42 MAPK (Erk1/2) (1:1000), phospho-Akt (ser473) (1:1000), total-pan-Akt (1:1000), phospho-IGF-1R (1:1000) and IGF-1R (1:1000) (Cell Signaling Technologies, Danvers, MA), Cyclin B1 (1:1000), CDC25C (1:1000) (Millipore) and CDK1 (1:2000) (BD Biosciences). After washing with TBS containing 0.1% (v/v) Tween-20, the membrane was incubated with appropriate secondary antibody conjugated with horseradish peroxidase for 1 h. Protein expressions were visualized using the chemiluminescence detection substrate (Millipore) using Imagequant LAS 4010 chemidocumentation system (GE Healthcare, Amersham, UK).

2.5. Bisulfite modification and methylation analysis by methylation-specific PCR (MSP)

The DNA methylation status of *p21^{CIP1/WAF1}* and *KLOTHO* promoters was determined by MSP as described elsewhere (Rubinek et al., 2012). MDA-MB-231 cells were treated with either GTPs or SFN or the combinations of both as described earlier. Genomic DNA was extracted at approximately 70–80% confluence using the Qiagen DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 1 µg of genomic DNA was subjected to bisulfite conversion using the EpiTect Bisulfite modification kit (Qiagen) according to the manufacturer's protocol. For MSP analysis, two sets of specific primers were designed (methylated or unmethylated) for the *p21^{CIP1/WAF1}* and *KLOTHO* promoters. The primer sequences and MSP conditions are given in Supplementary Table S2. PCR amplification was performed for 2 min at 94 °C followed by 35 cycles (30 s at 94 °C; 30 s at T_A °C; 30 s at 72 °C) and extension at 72 °C for 2 min using platinum DNA Taq polymerase (Invitrogen). After amplification, PCR products were resolved in 2% agarose gels and visualized by staining with ethidium bromide using ImageQuant LAS4010 chemidocumentation system.

2.6. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) analysis was performed using the EZ-ChIP kit according to the manufacturer's instructions (Millipore) as described previously (Li et al.,

2013; Meeran et al., 2010, 2012). The ChIP-validated anti-acetyl-histone H3 (AcH3), anti-acetyl-histone H4 (AcH4), anti-acetyl-histone H3K9 (AcH3K9), anti-trimethyl-histone H3K9 (H3K9me3), anti-trimethyl-histone H3K27 (H3K27me3) (Millipore) and anti-MeCP2 (Abcam, Cambridge, MA) antibodies were used at 5 µg/mL dilutions. Input DNA was used as an internal control and no specific antibody IgG control was used to check ChIP efficiency. ChIP-purified DNA was quantified by using real-time PCR using primers spanning the CpG-rich region of the distal promoter regions of *p21^{CIP1/WAF1}* and *KLOTHO*. PCR amplification was performed in a Light Cycler (Roche) using SsoFast EvaGreen Supermix detection system (Bio-Rad). The sequences of primers with their respective annealing temperature (T_A) are given in Supplementary Table S3. Real-time PCR amplification was performed for 30 s at 94 °C followed by 45 cycles (5 s at 94 °C; 20 s at T_A °C; 30 s at 72 °C).

2.7. Small interfering RNA (siRNA) knockdown of ERα

siRNA transfection experiments were performed as described previously (Meeran et al., 2012). Approximately 2.2×10^5 cells per well were seeded in a 6-well plate and allowed to incubate overnight. *ERα*-siRNA (Santa Cruz Biotechnology) was made into 10 µM stock using RNase-free water and 40 nM siRNA was delivered to the cells using the siRNA transfection kit (Santa Cruz Biotechnology) according to the manufacturer's protocol. siCONTROL Non-Targeting siRNA (Santa Cruz Biotechnology) was used as a negative control. Cells were treated with the combinations of GTPs and SFN alone or with tamoxifen (TAM) for an additional 48 h. Cells were harvested and checked for *ERα* knockdown using western blot analysis as described earlier.

2.8. Cell proliferation assay

Approximately 5×10^3 cells were treated with the indicated concentrations of GTPs and SFN with or without TAM for 48 h. At the end of the incubation time, MTT assay was performed for assessing cellular proliferation as described previously (Meeran et al., 2012).

2.9. Statistical analysis

The DNA and protein band intensity was analyzed by using ImageJ software version 1.47 h (<http://imagej.nih.gov/ij>) and normalized with respective loading control. The statistical significance of differences between the values of treated samples and controls were determined by using one-way ANOVA with Dunnet's post-hoc test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). The statistical significance among all pairs was determined by one-way ANOVA with Bonferroni's multiple comparison tests. In each case, $P < 0.05$ was considered statistically significant.

3. Results

3.1. GTPs and SFN induce G₂/M phase cell cycle arrest in human breast cells

Previously we have shown that treatment with the 20 µg/mL GTPs and 10 µM SFN alone or in combinations significantly induced cell growth inhibition in ERα-negative human breast cancer cells (Meeran et al., 2012). However, these concentrations of GTPs and SFN did not induce cellular toxicity in control MCF10A cells (Meeran et al., 2012). Therefore, for

subsequent studies, we chose to use the same concentrations of GTPs and SFN to explore their mechanism of action of growth inhibition and ER α -reactivation in ER α -negative MDA-MB-231 and MDA-MB-453 breast cancer cells. Further, to elaborate the mechanism of GTPs- and SFN-mediated inhibition of cellular proliferation, we analyzed cell cycle distribution in these human breast cancer cells. As shown in Fig. 1A, treatment with either 20 μ g/ mL GTPs or 10 μ M SFN alone had no significant effect on cell cycle progression as compared to vehicle-treated control. However, the combinations of 20 μ g/mL GTPs and 10 μ M SFN resulted in a significant (58.46%; $P < 0.001$) arrest in the G₂/M phase of cell cycle progression as compared to control (24.87%). In addition, increased octaploidy was observed with the combinational treatment of 20 μ g/mL GTPs and 10 μ M SFN in MDA-MB-231 cells which indicated the occurrence of mitotic catastrophe. Furthermore, analysis of cell cycle distribution in another ER α -negative MDA-MB-453 cells also revealed that the combinations of GTPs and SFN resulted in a significant arrest in the G₂/M phase of cell cycle progression as compared to control (Supplementary Fig. S1B). Interestingly, the dietary bioactive combinations of 20 μ g/mL GTPs and 10 μ M SFN induced a significantly greater effect on cell cycle arrest in G₂/M phase compared with a combination of standard DNMT and HDAC inhibitors against respective controls in MDA-MB-231 cells (39.25%, $P < 0.01$) as well as in MDA-MB-453 cells (18.44%, $P < 0.01$) (Supplementary Fig. S1B).

Cyclins and cyclin-dependent kinases (CDKs) play important regulatory roles in the cell cycle progression (Meeran and Katiyar, 2008). To further validate GTPs- and SFN-induced G₂/M phase cell cycle arrest, we analyzed various cell cycle regulatory proteins such as Cyclin B1, CDK1 and CDC25C which regulate the G₂/M phase of the cell cycle and are also involved in mitotic catastrophe-mediated cell death (Castedo et al., 2002, 2004). We found marginal reductions in Cyclin B1, CDK1 and CDC25C expressions in cells with GTPs- and SFN-treatments alone and the effects were significantly pronounced when treated with the combinations of GTPs and SFN in MDA-MB-231 cells (Fig. 1B). However, levels of G₁ phase regulators, CDK4 and CDK6, were not altered significantly. Thus, these results suggest that treatment of ER α -negative MDA-MB-231 breast cancer cells with the combinations of GTPs and SFN induced a G₂/M phase cell cycle arrest by down-regulation of cell cycle regulatory proteins which, at least in part, is responsible for the inhibition of cellular proliferation.

3.2. GTPs and SFN reactivate p21^{CIP1/WAF1} and KLOTHO expressions in breast cancer cells

To further investigate the role of GTPs- and SFN-mediated cell cycle arrest, we analyzed the expression levels of key cell cycle regulatory proteins in MDA-MB-231 and MDA-MB-453 cells. *KLOTHO*, a novel cell-cycle regulatory TSG, has been reported to be transcriptionally silenced in MDA-MB-231 cells (Wolf et al., 2008; Xie et al., 2013). Treatment with the combinations of 20 μ g/mL GTPs and 10 μ M SFN significantly ($P < 0.001$) reactivated mRNA expression of *KLOTHO* by 11.2-, 11.4- and 20.2-fold in MDA-MB-231 cells at 24, 48 and 72 h, respectively, as compared to their respective controls (Fig. 2A). Further, western blot analysis showed that the combinations of GTPs and SFN significantly ($P < 0.001$) reactivated KLOTHO expression in MDA-MB-231 cells at 24, 48 and 72 h (Fig. 2B). Similarly, the combinations of GTPs and SFN also significantly up-regulated the expressions of KLOTHO in another ER α -negative MDA-MB-453 cell line (Supplementary Fig. S1C).

Similar to MDA-MB-231 cells, the optimum reactivation of KLOTHO was achieved by the combinatorial treatment of 20 µg/mL GTPs and 10 µM SFN in MDA-MB-453 cells.

Upregulation of p21^{CIP1/WAF1} has been known to play an important role in inducing G1 phase cell cycle arrest. However, there are multiple reports where p21^{CIP1/WAF1} upregulation was also found to be involved in the induction of G₂/M phase cell cycle arrest (Chen et al., 2013a; Chew et al., 2012; Meeran and Katiyar, 2008). Therefore, we further looked for the GTPs- and SFN-mediated alteration of p21^{CIP1/WAF1} expression in both human breast cancer MDA-MB-231 and MDA-MB-453 cells. We found that treatment with either 20 µg/mL GTPs alone or in combination with 5 µM SFN significantly reactivated mRNA expressions of *p21^{CIP1/WAF1}* by 3.96-fold ($P < 0.05$) and 8.4-fold ($P < 0.001$), respectively, in MDA-MB-231 cells as compared to control at 48 h (Fig. 2C). Similar concentrations of GTPs and SFN significantly ($P < 0.001$) reactivated p21^{CIP1/WAF1} protein expression in MDA-MB-231 cells at 24, 48 and 72 h (Fig. 2D) and in MDA-MB-453 cells at 48 h (Supplementary Fig. S1C). In contrast with MDA-MB-231, MDA-MB-453 cells treated with 10 µM SFN alone could also significantly induce p21^{CIP1/WAF1} expression at 48 h. The p21^{CIP1/WAF1} expression induced by the combinations of 20 µg/mL GTPs and 5 µM SFN was well correlated with the concentrations at which the ERα reactivation was observed in ERα-negative MDA-MB-231 (Meeran et al., 2012) as well as in MDA-MB-453 cells (Supplementary Fig. S1C). These results drove us to validate the correlation between the epigenetic reactivation of ERα and TSGs in ERα-negative breast cancer cells by the combinations of GTPs- and SFN-treatment.

3.3. GTPs and SFN altered DNA methylation of the KLOTHO and p21^{CIP1/WAF1} promoters

Silencing of TSGs mediated by their promoter hypermethylation and hypoacetylation is a common occurrence in most of the human cancers (Fang et al., 2003; Fuks, 2005; Herman and Baylin, 2003; Jones, 2002; Majid et al., 2008). Therefore, we sought to determine the GTP- and SFN-mediated demethylation effect for the reactivation of TSGs in MDA-MB-231 cells. We used MSP to analyze the DNA methylation status of the *p21^{CIP1/WAF1}* and *KLOTHO* promoters in MDA-MB-231 cells treated with GTPs and SFN. As shown in Fig. 3, control cells maintain high levels of methylation at the promoters of *p21^{CIP1/WAF1}* and *KLOTHO*. Treatment with the combinations of GTPs and SFN induced a significant demethylation on the *p21^{CIP1/WAF1}* and *KLOTHO* promoters at 24 h and 48 h (Fig. 3A and B). These results are consistent with mRNA as well as protein expressions of p21^{CIP1/WAF1} and KLOTHO observed in Fig. 2A and B. Further, consistent with mRNA expression, the combinations of 20 µg/mL GTPs and 5 µM SFN induced a higher level of DNA demethylation at the *p21^{CIP1/WAF1}* promoter than the combinations of 20 µg/mL GTPs- and 10 µM SFN-treated MDA-MB-231 cells at 48 h (Fig. 3B). Overall, the combinations of GTPs and SFN have more pronounced demethylating effect at the *p21^{CIP1/WAF1}* and *KLOTHO* promoters than when treated separately in MDA-MB-231 cells. The combination-mediated demethylating effects could be important contributing factors in altering the expressions of silenced TSGs in human breast cancer cells.

3.4. GTPs and SFN altered chromatin modification of the *KLOTHO* and *p21^{CIP1/WAF1}* promoters

In addition to DNA methylation, histone modifications also play an important role in gene activation and repression (Fuks, 2005; Meeran et al., 2010; Nandakumar et al., 2011). Therefore, we further investigated key chromatin modifications such as histone acetylation and methylation at the *KLOTHO* and *p21^{CIP1/WAF1}* promoters. We designed two sets of primers designated as ChIP1 (–988 to –888) and ChIP2 (–534 to –470) for the *KLOTHO* promoter upstream of the transcriptional start site (Fig. 4A). We found that GTPs- and SFN-treatment resulted in a significant enrichment of active chromatin markers such as AcH3, AcH4 and AcH3K9 at promoter regions of *KLOTHO* in MDA-MB-231 cells at 24 h of post-treatment (Fig. 4B–D). In contrast, we found a significant decrease in the enrichment of inactive chromatin markers such as H3K9me3 and H3K27me3 in MDA-MB-231 cells with GTPs- and SFN-treatments (Fig. 4E and F). Methyl CpG binding protein 2 (MeCP2), comprising a methyl CpG binding domain (MBD), is well known to bind specifically to the methylated DNA resulting in gene repression (Majid et al., 2008; Meeran et al., 2012). We found a significant decrease in the binding of MeCP2 at the promoter regions of *KLOTHO* after treatment with 20 µg/mL GTPs and 10 µM SFN combinations at 24 h (Fig. 4G). Overall, the enrichment of active chromatin markers and down-regulation of inactive chromatin markers binding at the *KLOTHO* promoter were significantly pronounced in the cells treated with the combinations of GTPs and SFN, suggesting the importance of dietary combination-mediated reactivation of *KLOTHO* expression in MDA-MB-231 cells through histone modifications.

Similarly, we designed two sets of primers designated as ChIP1 (–1985 to –1557) and ChIP2 (–1575 to –1159) for the *p21^{CIP1/WAF1}* promoter upstream of the transcriptional start site (Fig. 5A). The primer pair amplifying ChIP1 flanks a Sp1-binding site on the *p21^{CIP1/WAF1}* promoter. We found a significant enrichment of active chromatin markers such as AcH3 and AcH4 after combinatorial treatment of 20 µg/mL GTPs and 10 µM SFN in MDA-MB-231 cells (Fig. 5B and D). In the case of AcH3K9, the maximum enrichment was observed after combinatorial treatment of 20 µg/mL GTPs and 5 µM SFN which is consistent with mRNA and protein expressions of *p21^{CIP1/WAF1}* (Fig. 5C). In contrast, inactive chromatin markers showed significantly decreased enrichments with the combinations of GTPs and SFN as compared to their respective control at 24 h of post-treatment (Fig. 5E and F). We observed a significant decrease in the binding of MeCP2, a methylation-sensitive gene repressor, at the ChIP2 promoter regions of *p21^{CIP1/WAF1}* with the individual as well as combinatorial doses of GTPs and SFN in MDA-MB-231 cells. Unlike significantly enriched active chromatin modifications observed at ChIP1 regions of the *p21^{CIP1/WAF1}*, the inhibition of MeCP2 binding was more pronounced at ChIP2 regions of the *p21^{CIP1/WAF1}* promoter, which might be due to the GTPs- and SFN-mediated demethylation in this CpG-rich region in MDA-MB-231 cells. From these results, we may conclude that the reactivations of *KLOTHO* and *p21^{CIP1/WAF1}* with the combinatorial treatment of GTPs and SFN might be due to the active chromatin modifications occurring at their respective promoter regions.

3.5. GTPs- and SFN-induced expression of KLOTHO inhibits the activation of IGF-1 pathways in MDA-MB-231 cells

The IGF-1 pathway has been reported to be involved in the regulation of cellular proliferation in breast cancer (Yavari et al., 2010; Yee, 2006). Since KLOTHO is a potent inhibitor of the insulin receptor (IR) and the IGF-1 receptor (IGF-1R), we tested the downstream changes of KLOTHO reactivations mediated by GTPs and SFN in MDA-MB-231 cells. It was found that GTPs- and SFN-treatment decreased the expression of phospho-IGF-1R and the effect was considerably pronounced by the combinatorial dose of 20 µg/mL GTPs and 10 µM SFN while the expressions of total IGF-1R remained unaltered or marginally increased at 12, 24, 48 and 72 h (Fig. 6A–D). Further, to analyze the effect of GTPs and SFN treatment on downstream effectors of this pathway, phosphorylated and total protein expressions of ERK1/2 and Akt were analyzed in MDA-MB-231 cells. In accordance with down-regulation of phospho-IGF-1R, the downstream phosphorylation of ERK1/2 and Akt were also down-regulated with the combinations of GTPs and SFN-treatment in MDA-MB-231 cells at 12, 24, 48 and 72 h. (Fig. 6). We also found that the expressions of total ERK1/2 and Akt remained unaltered at this dose of GTPs and SFN after 12, 24, 48 and 72 h post-treatment (Fig. 6). These results suggest that the reactivation of *KLOTHO* by the combinations of GTPs and SFN leads to the inhibition of IGF-1 and its downstream cascade in human breast cancer cells.

3.6. GTPs- and SFN-mediated reactivations of KLOTHO and p21^{CIP1/WAF1} are partially dependent on the ERα-reactivation in MDA-MB-231 cells

As the combinational treatment of GTPs and SFN reactivated ERα expression, we wanted to confirm the functional significance of ERα on the expressions of p21^{CIP1/WAF1} and *KLOTHO* in MDA-MB-231 cells. Knockdown of *ERα* using siRNA was performed in MDA-MB-231 cells treated with the combinations of GTPs and SFN for 48 h. Knockdown of *ERα* considerably suppressed the GTPs- and SFN-mediated ERα reactivation in ERα-negative MDA-MB-231 cells (Fig. 7A). Previously, we have reported that the combinations of 20 µg/mL GTPs and 5 µM SFN optimally reactivated ERα expression in ERα-negative MDA-MB-231 cells. In accordance, we have also observed the reactivation of ERα and p21^{CIP1/WAF1} at this dose with control siRNA-treated MDA-MB-231 cells. Further, the optimum reactivation of KLOTHO was observed at a combination of 20 µg/mL GTPs and 10 µM SFN with control siRNA-treated MDA-MB-231 cells. Interestingly, when the GTPs- and SFN-mediated reactivation of ERα was silenced using ERα-siRNA, p21^{CIP1/WAF1} expression was significantly down-regulated along with partial downregulation of KLOTHO. This gives experimental evidence that the reactivation of p21^{CIP1/WAF1} and KLOTHO is, at least in part, dependent on GTPs- and SFN-mediated reactivation of ERα in ERα-negative MDA-MB-231 cells (Fig. 7A).

We have previously shown that the combinations of GTPs- and SFN-treatment sensitize ERα-negative breast cancer cells to tamoxifen therapy through epigenetic reactivation of ERα (Meeran et al., 2012). Therefore, we investigated the effect of ERα-knockdown in cellular proliferation of MDA-MB-231 cells treated with the combinations of GTPs and SFN alone or with 1 µM TAM (Fig. 7B). We found that MDA-MB-231 cells treated with a combination of GTPs and SFN inhibited a considerable level of cellular proliferation in both

control as well as ER α -knockdown cells. However, cells treated with 20 μ g/mL GTPs and 5 μ M SFN combined with TAM had significantly reduced cellular proliferation, which is likely due to the optimal epigenetic reactivation of ER α and p21^{CIP1/WAF1} in MDA-MB-231 cells. Conversely, ER α knockdown MDA-MB-231 cells treated with GTPs and SFN combined with TAM induced a significantly lesser inhibition of cellular proliferation than the control siRNA-treated cells ($P < 0.05$). The combinations of 20 μ g/mL GTPs and 10 μ M SFN with or without TAM have shown a similar level of inhibition of cellular proliferation, which might be due to the insignificant reactivation of ER α in ER α -negative MDA-MB-231 cells for the TAM therapy. Collectively, these results indicated that the dietary combinations of GTPs and SFN can induce the expressions of silenced TSGs in addition to the reactivations of ER α in ER α -negative human breast cancer, for the effective treatment of hormonal refractory breast cancer.

4. Discussion

Many studies have demonstrated the chemopreventive and therapeutic potentials of GTPs and SFN through genetic and epigenetic mechanisms (Ahmad et al., 1997; Chen et al., 2013a; Chew et al., 2012; Dashwood and Ho, 2007; Fang et al., 2003; Meeran et al., 2010; Myzak et al., 2006a). EGCG, an active ingredient of GTPs, has been shown to inhibit DNMT activity and alter the expressions of oncogenes and TSGs (Fang et al., 2003; Meeran et al., 2011; Nandakumar et al., 2011). Similarly, SFN, a dietary HDACs inhibitor, has been found to modulate the expression of histone acetylation status of the tumor-promoter and oncogenes in many cancers including breast cancer (Chen et al., 2013a; Meeran et al., 2010, 2012; Myzak et al., 2006a). Since both DNA methylation and histone modifications are of central importance in the cancer prevention and therapy, we studied the effects of dietary combinations of DNMT and HDAC inhibitors for the effective treatment of hormonal refractory breast cancer. The inhibitors of DNMTs and HDACs have been shown to reactivate the silenced TSGs in cancer cells, which have been found to be one of the effective treatment strategies against cancer. Dietary EGCG has been shown to reactivate several known key TSGs in various cancer cell lines (Fang et al., 2003; Nandakumar et al., 2011). Similarly, EGCG in combination with TSA, a HDAC inhibitor, synergistically reactivates p16^{INK4a} and ER α expressions in human lymphoma and breast cancer cells, respectively, through active chromatin modifications (Li et al., 2010; Wu et al., 2013). Therefore, the focus of the present study was to investigate the effect of the combinations of dietary DNMT and HDAC inhibitors toward the epigenetic reactivation of key TSGs and its ER α dependence in ER α -negative human breast cancer cells.

In common, most of the anti-cancer agents including bioactive dietary supplements such as GTPs and SFN have been shown to have multiple targets for their anti-cancer activities (Ahmad et al., 1997; Chen et al., 2013a; Chew et al., 2012; Dashwood and Ho, 2007; Meeran et al., 2010; Myzak et al., 2006a). Increasing evidences suggest that anti-cancer agents also induce polyploidy, which promotes mitotic catastrophe-mediated cell death in various cancer cells (Cornago et al., 2014; Jackson and Singletary, 2004a; Xu et al., 2005). Several HDAC inhibitors including sulforaphane have been shown to induce early mitotic arrest, which was also accompanied by aberrant mitosis and micronucleation, characteristic of cell death by mitotic catastrophe in breast cancer cells (Jackson and Singletary, 2004a,

2004b). In accordance, we also observed an increased octaploid peak with the combinations of 20 µg/mL GTPs and 10 µM SFN in MDA-MB-231 cells. This is possibly due to the DNA damage responses induced when cells are treated with higher concentrations of natural compounds. In accordance, GTPs and sulforaphane have also shown to inhibit cellular proliferation through multiple pathways including inhibition of topoisomerases, suppression of microtubular dynamics, and induction of reactive oxygen species-mediated mitotic arrest (Ferreira de Oliveira et al., 2014; Park et al., 2014; Timmel et al., 2013).

In this study, we have used 20 µg/mL GTPs, which contain about 24 µM of EGCG, shown to be a sub-optimal dose used against many cancers including breast cancer (Chen et al., 2013b; Meeran et al., 2011, 2012). Similarly, 10 µM SFN or even higher doses of SFN have been demonstrated to have target-specific anti-cancer effects in different cancer cell lines including breast cancer (Hu et al., 2004; Meeran et al., 2012; Myzak et al., 2006b). More evidently, the combinations of GTPs and SFN used in this study have specifically induced cell death and inhibited cellular proliferations in human breast cancer cells significantly but not in human transformed non-tumorigenic mammary epithelial cells (Meeran et al., 2012). In general, achieving the physiological concentrations of two different dietary supplements is much more feasible than the uptake of higher concentrations of individual dietary supplements, if the combination displays synergistic therapeutic effect. The use of dietary GTPs is more advantageous over the use of EGCG not only due to the cost-effective approach, but also due to the presence of other dietary catechins and their synergistic anti-cancer effects (Mukhtar and Ahmad, 2000). The doses of GTPs and SFN adopted in this investigation are physiologically achievable in humans through daily consumption of a few cups (~3–4) of green tea and one cup (~68 g) of broccoli sprouts (Chen et al., 2013a, 2013b; Myzak et al., 2006a, 2007). It has been demonstrated that the peak plasma concentration achievable in rats was 20 µM after a single oral dose of 50 µM SFN, which is higher than the dose used in the current study (Hu et al., 2004). Further, consumption of ~6 µM SFN per day for 10 weeks have been shown to significantly inhibit the HDAC activity and increased acetylated histone in the prostate (Myzak et al., 2006a, 2006b).

Upregulation of key TSGs and cell cycle regulatory proteins are linked with cell cycle arrest and inhibition of cellular proliferations in human cancers (Chen et al., 2013a; Kuzumaki et al., 1998; Meeran and Katiyar, 2008). Our findings suggest that the combinational treatment of GTPs and SFN synergistically induces mRNA and protein expressions of p21^{CIP1/WAF1} and KLOTHO in breast cancer cells. Although p21^{CIP1/WAF1} upregulation has been mainly associated with G1 arrest, it has also been shown to induce G₂/M phase cell cycle arrest through the formation of trimeric complexes with CyclinB1/CDK1 in different cancer cells (Chew et al., 2012; Meeran and Katiyar, 2008; Suppipat et al., 2012). In addition, studies also suggest that inhibitors of cell cycle check point kinases such as CDK1 can sensitize cancer cells to the induction of mitotic catastrophe and subsequent apoptosis (Castedo et al., 2002, 2004). SFN has been shown to up regulate p21^{CIP1/WAF1} expression-associated G₂/M arrest in both p53-dependent and -independent manners (Chen et al., 2013a; Chew et al., 2012; Myzak et al., 2007; Suppipat et al., 2012). In accordance with previous results, we have also found that the upregulation of p21^{CIP1/WAF1} induced by the combinations of GTPs and SFN was well correlated with the G₂/M arrest in these human breast cancer cells (Ahmad et al., 1997; Chen et al., 2013a; Suppipat et al., 2012). Similarly, KLOTHO, a novel

tumor suppressor, has been shown to play an important role in the inhibition of cellular proliferation and induction of cell cycle arrest in human cancers through various mechanisms including insulin-like growth factor-1 (IGF-1) signaling (Lee et al., 2010; Pan et al., 2011; Wang et al., 2011; Wolf et al., 2008). Studies have shown that insulin/IGF-1 signaling activates the MAPK and Akt pathways, thereby regulating cellular proliferation and survival of cancer cells (Pollak, 2008; Wolf et al., 2008; Yee, 2006). In accordance, GTPs- and SFN-mediated upregulation of *KLOTHO* has been shown to inhibit IGF-1 signaling and cellular proliferation in MDA-MB-231 cells.

Studies have shown that the CpG hypermethylation at the promoter regions of TSGs are in general associated with their transcriptional repression (Majid et al., 2008; Nandakumar et al., 2011; Rubinek et al., 2012). Therefore, we examined the CpG island methylation status of *p21^{CIP1/WAF1}* and *KLOTHO* promoter regions with GTPs- and SFN-treated MDA-MB-231 cells. Our results demonstrate that the combinations of GTPs- and SFN induced active demethylation at the promoters of TSGs, which decreased the binding of MeCP2, a methylation-dependent transcriptional repressor, at the *p21^{CIP1/WAF1}* and *KLOTHO* promoters leading to their transcriptional upregulation in MDA-MB-231 cells. Further, this is in accordance with previous reports that the promoter demethylations of TSGs are in general associated with their transcriptional upregulation in many cancers including breast cancer (Herman and Baylin, 2003; Jones, 2002; Majid et al., 2008; Nandakumar et al., 2011). In addition, the enrichment of active and inactive histone modifications at the promoters of TSGs plays important role in their transcriptional regulation (Li et al., 2013; Majid et al., 2008; Nandakumar et al., 2011; Rubinek et al., 2012). These histone modifications were significantly more pronounced with the combinations of GTPs and SFN than the treatment of either of the dietary compounds in MDA-MB-231 cells. This is in agreement with our previous observations that the combinations of GTPs and SFN synergistically induced ER α expression in ER α -negative human breast cancer cells through active chromatin modifications (Meeran et al., 2012).

Interestingly, optimum reactivation of *p21^{CIP1/WAF1}* was observed at the same GTPs and SFN concentrations where optimum ER α reactivation was observed in ER α -negative human breast cancer cells (Meeran et al., 2012). Hence, we studied the correlations between GTPs- and SFN-mediated epigenetic reactivations of ER α and *p21^{CIP1/WAF1}* in MDA-MB-231 cells using ER α -knockdown study. Unlike *KLOTHO*, the upstream promoter region of *p21^{CIP1/WAF1}* contains several estrogen response elements (EREs). Binding of the estrogen at the EREs promotes the recruitment of ER α and other transcription factors to the upstream promoter region of *p21^{CIP1/WAF1}* (Majid et al., 2008; Mandal and Davie, 2010). Hence, the GTPs- and SFN-mediated reactivation of *p21^{CIP1/WAF1}* is, at least, partially dependent on ER α reactivation, while upregulation of *KLOTHO* was either in part or independent of ER α reactivation. Further, the optimum reactivation of *p21^{CIP1/WAF1}* was observed with the combinations of 20 μ g/mL GTPs and 5 μ M SFN and slightly decreased with the combination of 20 μ g/mL GTPs and 10 μ M SFN, which might be due to the mRNA instability as well as higher rate of protein turnover observed in case of these cell cycle regulators (Boisvert et al., 2012; Reed, 2003). In addition, the combinations of 20 μ g/mL GTPs and 10 μ M SFN induced a minimal reactivation of ER α and its associated TAM-mediated inhibition of cellular proliferation, in spite of a significant cell cycle arrest, which

might be due to the demonstration of DNA damage responses (Ferreira de Oliveira et al., 2014; Park et al., 2014). However, this study is the first of its kind to report the GTPs and SFN combination-mediated epigenetic reactivations of key TSGs and their ER α -dependence in ER α -negative human breast cancer cells. Further, these findings are of importance not only for the understanding of epigenetic regulations of the TSGs but they also provide evidence for the synergistic effect of the dietary combinations of GTPs and SFN against breast cancer.

5. Conclusion

In this study, we have clearly demonstrated that the dietary combinations of GTPs and SFN not only potentiate the tamoxifen therapy by reactivating ER α expression in ER α -negative human breast cancer cells, but also up-regulated silenced-TSGs by active chromatin modifications in hormonal refractory breast cancer. Therefore, we conclude that the combinations of GTPs and SFN not only pronounced the effect of individual compounds, but were also shown to function synergistically through multi-targeting, thereby enhancing the treatment efficiency in hormonal refractory breast cancer. These findings are of importance for understanding the potential of dietary combinations against hormonal refractory breast cancer. However, further pre-clinical *in vivo* studies are warranted to confirm the efficacy of these dietary combinations against hormonal refractory breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported with funds from the Science and Engineering Research Board (SR/FT/LS-80/2011), Department of Biotechnology-Twinning Programme (BCIL/NER-BPMC/2013/722), New Delhi, India and CSIR-EpiHeD-Network Scheme (BSC0118) to SMM. Part of this work was also supported from grants by the NCI (ROI CA178441) and the American Institute for Cancer Research (316184) to TOT. SS and SK are thankful for Senior Research fellowship grants from the Council of Scientific and Industrial Research (CSIR), India. CSIR-CDRI communication number-8930.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.02.020.

References

- Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J Natl Cancer Inst.* 1997; 89:1881–1886. [PubMed: 9414176]
- Boisvert FM, Ahmad Y, Gierli ski M, Charrière F, Lamont D, Scott M, et al. A quantitative spatial proteomics analysis of proteome turnover in human cells. *Mol Cell Proteomics.* 2012; 11:M111.011429.
- Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res.* 2005; 65:6305–6311. [PubMed: 16024632]
- Castedo M, Perfettini JL, Roumier T, Kroemer G. Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death Differ.* 2002; 9:1287–1293. [PubMed: 12478465]

- Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene*. 2004; 23:2825–2837. [PubMed: 15077146]
- Chang J, Powles TJ, Allred DC, Ashley SE, Makris A, Gregory RK, et al. Prediction of clinical outcome from primary tamoxifen by expression of biologic markers in breast cancer patients. *Clin Cancer Res*. 2000; 6:616–621. [PubMed: 10690547]
- Chen H, Landen CN, Li Y, Alvarez RD, Tollefsbol TO. Enhancement of cisplatin-mediated apoptosis in ovarian cancer cells through potentiating G2/M arrest and p21 upregulation by combinatorial epigallocatechin gallate and sulforaphane. *J Oncol*. 2013a; 2013:872957. [PubMed: 23476648]
- Chen H, Landen CN, Li Y, Alvarez RD, Tollefsbol TO. Epigallocatechin gallate and sulforaphane combination treatment induce apoptosis in paclitaxel-resistant ovarian cancer cells through hTERT and Bcl-2 down-regulation. *Exp Cell Res*. 2013b; 319:697–706. [PubMed: 23333498]
- Chew YC, Adhikary G, Wilson GM, Xu W, Eckert RL. Sulforaphane induction of p21(Cip1) cyclin-dependent kinase inhibitor expression requires p53 and Sp1 transcription factors and is p53-dependent. *J Biol Chem*. 2012; 287:16168–16178. [PubMed: 22427654]
- Cornago M, Garcia-Alberich C, Blasco-Angulo N, Vall-Llaura N, Nager M, Herreros J, et al. Histone deacetylase inhibitors promote glioma cell death by G2 checkpoint abrogation leading to mitotic catastrophe. *Cell Death Dis*. 2014; 5:e1435. [PubMed: 25275596]
- Dashwood RH, Ho E. Dietary histone deacetylase inhibitors: from cells to mice to man. *Semin Cancer Biol*. 2007; 17:363–369. [PubMed: 17555985]
- Doi S, Zou Y, Togao O, Pastor JV, John GB, Wang L, et al. Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem*. 2011; 286:8655–8665. [PubMed: 21209102]
- Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, et al. Tea polyphenol (–)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. *Cancer Res*. 2003; 63:7563–7570. [PubMed: 14633667]
- Ferreira de Oliveira JM, Remédios C, Oliveira H, Pinto P, Pinho F, Pinho S, et al. Sulforaphane induces DNA damage and mitotic abnormalities in human osteosarcoma MG-63 cells: correlation with cell cycle arrest and apoptosis. *Nutr Cancer*. 2014; 66:325–334. [PubMed: 24405297]
- Fuks F. DNA methylation and histone modifications: teaming up to silence genes. *Curr Opin Genet Dev*. 2005; 15:490–495. [PubMed: 16098738]
- Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med*. 2003; 349:2042–2054. [PubMed: 14627790]
- Hu R, Hebbar V, Kim BR, Chen C, Winnik B, Buckley B, et al. In vivo pharmacokinetics and regulation of gene expression profiles by isothiocyanate sulforaphane in the rat. *J Pharmacol Exp Ther*. 2004; 310:263–271. [PubMed: 14988420]
- Jackson SJ, Singletary KW. Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. *Carcinogenesis*. 2004a; 25:219–227. [PubMed: 14578157]
- Jackson SJ, Singletary KW. Sulforaphane inhibits human MCF-7 mammary cancer cell mitotic progression and tubulin polymerization. *J Nutr*. 2004b; 134:2229–2236. [PubMed: 15333709]
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011; 61:69–90. [PubMed: 21296855]
- Jones PA. DNA methylation and cancer. *Oncogene*. 2002; 21:5358–5360. [PubMed: 12154398]
- Kawai H, Li H, Chun P, Avraham S, Avraham HK. Direct interaction between BRCA1 and the estrogen receptor regulates vascular endothelial growth factor (VEGF) transcription and secretion in breast cancer cells. *Oncogene*. 2002; 21:7730–7739. [PubMed: 12400015]
- Kuzumaki T, Kobayashi T, Ishikawa K. Genistein induces p21(Cip1/WAF1) expression and blocks the G1 to S phase transition in mouse fibroblast and melanoma cells. *Biochem Biophys Res Commun*. 1998; 251:291–295. [PubMed: 9790949]
- Lee J, Jeong DJ, Kim J, Lee S, Park JH, Chang B, et al. The anti-aging gene KLOTHO is a novel target for epigenetic silencing in human cervical carcinoma. *Mol Cancer*. 2010; 9:109. [PubMed: 20482749]
- Li Y, Yuan YY, Meeran SM, Tollefsbol TO. Synergistic epigenetic reactivation of estrogen receptor- α (ER α) by combined green tea polyphenol and histone deacetylase inhibitor in ER α -negative breast cancer cells. *Mol Cancer*. 2010; 9:274. [PubMed: 20946668]

- Li Y, Meeran SM, Patel SN, Chen H, Hardy TM, Tollefsbol TO. Epigenetic reactivation of estrogen receptor- α (ER α) by genistein enhances hormonal therapy sensitivity in ER α -negative breast cancer. *Mol Cancer*. 2013; 12:9. [PubMed: 23379261]
- Majid S, Kikuno N, Nelles J, Noonan E, Tanaka Y, Kawamoto K, et al. Genistein induces the p21WAF1/CIP1 and p16INK4a tumor suppressor genes in prostate cancer cells by epigenetic mechanisms involving active chromatin modification. *Cancer Res*. 2008; 68:2736–2744. [PubMed: 18413741]
- Mandal S, Davie JR. Estrogen regulated expression of the p21 Waf1/Cip1 gene in estrogen receptor positive human breast cancer cells. *J Cell Physiol*. 2010; 224:28–32. [PubMed: 20301197]
- Meeran SM, Katiyar SK. Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Front Biosci*. 2008; 13:2191–2202. [PubMed: 17981702]
- Meeran SM, Patel SN, Tollefsbol TO. Sulforaphane causes epigenetic repression of hTERT expression in human breast cancer cell lines. *PLoS ONE*. 2010; 5:e11457. [PubMed: 20625516]
- Meeran SM, Patel SN, Chan TH, Tollefsbol TO. A novel prodrug of epigallocatechin-3-gallate: differential epigenetic hTERT repression in human breast cancer cells. *Cancer Prev Res (Phila)*. 2011; 4:1243–1254. [PubMed: 21411498]
- Meeran SM, Patel SN, Li Y, Shukla S, Tollefsbol TO. Bioactive dietary supplements reactivate ER expression in ER-negative breast cancer cells by active chromatin modifications. *PLoS ONE*. 2012; 7:e37748. [PubMed: 22662208]
- Mukhtar H, Ahmad N. Tea polyphenols: prevention of cancer and optimizing health. *Am J Clin Nutr*. 2000; 71:1698S–1702S. [PubMed: 10837321]
- Myzak MC, Dashwood WM, Orner GA, Ho E, Dashwood RH. Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc-minus mice. *FASEB J*. 2006a; 20:506–508. [PubMed: 16407454]
- Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. *Carcinogenesis*. 2006b; 27:811–819. [PubMed: 16280330]
- Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E. Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. *Exp Biol Med*. 2007; 232:227–234.
- Nandakumar V, Vaid M, Katiyar SK. (–)-Epigallocatechin-3-gallate reactivates silenced tumor suppressor genes, Cip1/p21 and p16INK4a, by reducing DNA methylation and increasing histones acetylation in human skin cancer cells. *Carcinogenesis*. 2011; 32:537–544. [PubMed: 21209038]
- Pan J, Zhong J, Gan LH, Chen SJ, Jin HC, Wang X, et al. Klotho, an anti-senescence related gene, is frequently inactivated through promoter hypermethylation in colorectal cancer. *Tumour Biol*. 2011; 32:729–735. [PubMed: 21523445]
- Park HS, Han MH, Kim GY, Moon SK, Kim WJ, Hwang HJ, et al. Sulforaphane induces reactive oxygen species-mediated mitotic arrest and subsequent apoptosis in human bladder cancer 5637 cells. *Food Chem Toxicol*. 2014; 64:157–165. [PubMed: 24296129]
- Pollak M. Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer*. 2008; 8:915–928. [PubMed: 19029956]
- Reed SI. Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. *Nat Rev Mol Cell Biol*. 2003; 4:855–864. [PubMed: 14625536]
- Rubinek T, Shulman M, Israeli S, Bose S, Avraham A, Zundeleovich A, et al. Epigenetic silencing of the tumor suppressor klotho in human breast cancer. *Breast Cancer Res Treat*. 2012; 133:649–657. [PubMed: 22042362]
- Sharma D, Blum J, Yang X, Beaulieu N, Macleod AR, Davidson NE. Release of methyl CpG binding proteins and histone deacetylase 1 from the estrogen receptor alpha (ER) promoter upon reactivation in ER-negative human breast cancer cells. *Mol Endocrinol*. 2005; 19:1740–1751. [PubMed: 15746193]
- Suppipat K, Park CS, Shen Y, Zhu X, Lacorazza HD. Sulforaphane induces cell cycle arrest and apoptosis in acute lymphoblastic leukemia cells. *PLoS ONE*. 2012; 7:e51251. [PubMed: 23251470]

- Timmel MA, Byl JA, Osheroff N. Epimerization of green tea catechins during brewing does not affect the ability to poison human type II topoisomerases. *Chem Res Toxicol*. 2013; 26:622–628. [PubMed: 23514406]
- Wang L, Wang X, Jie P, Lu H, Zhang S, Lin X, et al. Klotho is silenced through promoter hypermethylation in gastric cancer. *Am J Cancer Res*. 2011; 1:111–119. [PubMed: 21969138]
- Wolf I, Levanon-Cohen S, Bose S, Ligumsky H, Sredni B, Kanety H, et al. Klotho: a tumor suppressor and a modulator of the IGF-1 and FGF pathways in human breast cancer. *Oncogene*. 2008; 27:7094–7105. [PubMed: 18762812]
- Wu DS, Shen JZ, Yu AF, Fu HY, Zhou HR, Shen SF. Epigallocatechin-3-gallate and trichostatin A synergistically inhibit human lymphoma cell proliferation through epigenetic modification of p16INK4a. *Oncol Rep*. 2013; 30:2969–2975. [PubMed: 24064951]
- Xie B, Chen J, Liu B, Zhan J. Klotho acts as a tumor suppressor in cancers. *Pathol Oncol Res*. 2013; 19:611–617. [PubMed: 23828695]
- Xu WS, Perez G, Ngo L, Gui CY, Marks PA. Induction of polyploidy by histone deacetylase inhibitor: a pathway for antitumor effects. *Cancer Res*. 2005; 65:7832–7839. [PubMed: 16140952]
- Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, Davidson NE. Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. *Cancer Res*. 2001; 61:7025–7029. [PubMed: 11585728]
- Yavari K, Taghikhani M, Ghannadi Maragheh M, Mesbah-Namin SA, Babaei MH. Downregulation of IGF-IR expression by RNAi inhibits proliferation and enhances chemosensitization of human colon cancer cells. *Int J Colorectal Dis*. 2010; 25:9–16. [PubMed: 19669768]
- Yee D. Targeting insulin-like growth factor pathways. *Br J Cancer*. 2006; 94:465–468. [PubMed: 16450000]

Fig. 1.

GTPs and SFN induced G2/M phase cell cycle arrest in ER α -negative MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated with the indicated concentrations of GTPs and SFN as well as the combinations of both the compounds for 48 h. Cell cycle analysis was performed by propidium iodide staining followed by FACS analysis. (A) Representative figure of cell cycle analysis and the graphical representation of the percentage of cells present in different phases of cell cycle. (B) Expression analysis of cell cycle regulatory proteins using western blot. Graphical representations are indicative of relative band

intensity of the respective blots, normalized with β -actin. β -actin was used as an equal loading control. These results are representative of two independent experiments mean $\pm$ SEM. Statistical significance, * P < 0.05; ** P < 0.01; *** P < 0.001.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2.

GTPs and SFN synergistically reactivated *KLOTHO* and *p21^{CIP1/WAF1}* expressions in MDA-MB-231 cells. MDA-MB-231 cells were treated with the indicated concentrations of GTPs and SFN as well as the combinations of both the compounds for 12, 24, 48 and 72 h. (A) Real-time mRNA expression of *KLOTHO* in MDA-MB-231 cells. (B) Protein expression of *KLOTHO* in MDA-MB-231 cells. Graphical representations are indicative of relative band intensity of *KLOTHO* expression in MDA-MB-231 cells, normalized with respective β -actin. Values are mean band intensity of two independent experiments $\pm$ SEM. (C) Real-time mRNA expression of *p21^{CIP1/WAF1}* in MDA-MB-231 cell. (D) Protein expression of *p21^{CIP1/WAF1}* in MDA-MB-231 cells. Graphical representations are indicative of relative band intensity of *p21^{CIP1/WAF1}* expression in MDA-MB-231 cells, normalized with respective β -actin. Values are mean band intensity of two independent experiments $\pm$

SEM. Real-time mRNA data were obtained from three independent experiments, normalized to *GAPDH*. The values were plotted against control as mean relative fold induction $\pm$ SEM. Significance against control, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2016 December 01.

independent experiments with mean $\pm$ SEM. Statistical significance, $*P < 0.05$ with respect to control.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

GTPs and SFN altered pattern of histone modification and binding of MeCP2 at the *KLOTHO* promoter of MDA-MB-231 cells. (A) Two different CpG-rich regions of the *KLOTHO* promoter, ChIP1 (covering nucleotides -988 to -888) and ChIP2 (-534 to -470 nucleotides) regions were used for ChIP-qPCR analysis. (B) MDA-MB-231 cells were treated with the indicated concentrations of GTPs and SFN for 24 h. Histone modifications were analyzed by ChIP-qPCR for active chromatin markers including acetyl-H3 (panel B), acetyl-H3K9 (panel C), acetyl-H4 (panel D) and repressive chromatin markers including trimethyl-H3K9 (panel E), trimethyl-H3K27 (panel F) and MeCP2 (panel G) in the promoter regions of *KLOTHO*. The X-axis represents the different treatment groups, and the Y-axis represents the relative enrichment of individual binding factors. The data are represented in fold-change enrichment compared with respective control. The experiment was repeated

three times in triplicate with real-time PCR and each point indicates the mean $\pm$ SEM.
Statistical significance, * $P < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5.

GTPs and SFN altered patterns of histone modification and binding of MeCP2 at the *p21^{CIP1/WAF1}* promoter of MDA-MB-231 cells. (A) Two different CpG-rich regions of the *p21^{CIP1/WAF1}* promoter, ChIP1 (covering nucleotides -1985 to -1557) and ChIP2 (-1575 to -1159 nucleotides) regions were used for ChIP-qPCR analysis. (B) MDA-MB-231 cells were treated with the indicated concentrations of GTPs and SFN for 24 h. Histone modifications were analyzed by ChIP-qPCR for active chromatin markers including acetyl-H3 (panel B), acetyl-H3K9 (panel C), acetyl-H4 (panel D) and repressive chromatin markers including trimethyl-H3K9 (panel E), trimethyl-H3K27 (panel F) and MeCP2 (panel G) in the promoter regions of *p21^{CIP1/WAF1}*. The X-axis represents the different treatment groups, and the Y-axis represents the relative enrichment of individual binding factors. The data are represented in fold-change enrichment compared with respective control. The experiment was repeated three times in triplicate with real-time PCR and each point indicates the mean $\pm$ SEM. Statistical significance, * $P < 0.05$.

Fig. 6.

Treatment of MDA-MB-231 cells with GTPs and SFN down-regulates insulin-like growth factor-1 (IGF-1) signaling. MDA-MB-231 cells were treated with the indicated concentrations of GTPs and SFN as well as the combinations of both the compounds for 12, 24, 48 and 72 h. Cell lysates were prepared for the expression analysis of various proteins involved in the IGF-1 signaling. Panels A–D show the protein expressions at 12, 24, 48 and 72 h, respectively. The blots were representative of three independent experiments.

Fig. 7. GTPs- and SFN-mediated reactivation of *p21^{CIP1/WAF1}* and *KLOTHO* is dependent on ER α reactivation in MDA-MB-231 cells. (A) MDA-MB-231 cells were subjected to treatment with 40 nM of ER α -siRNA or control-siRNA as described in Section 2. Control- and ER α -siRNA transfected cells were treated with the indicated concentrations of the combinations of GTPs and SFN for 48 h. Cell lysates were prepared for the detection of ER α , *p21^{CIP1/WAF1}* and *KLOTHO* expressions by western blot. Relative band intensity of the respective blots was normalized with respect to β -actin, used as a loading control. The results are representative of two independent experiments mean band intensity $\pm$ SEM. Statistical significance, * P < 0.05 against control, # P < 0.05 against respective control siRNA group. (B) Control- and ER α -siRNA transfected cells were treated with indicated concentrations of the combinations of GTPs and SFN with or without 1 μ M TAM for 48 h. Cellular proliferation was determined and plotted against percent control. Results were obtained from three independent experiments, mean $\pm$ SEM, Statistical significance, * P < 0.05 against control, # P < 0.05 against respective control siRNA group, and ^a P < 0.05 against respective group without TAM.