Published in final edited form as:

Neuroscience. 2009 September 29; 163(1): 286–295. doi:10.1016/j.neuroscience.2009.06.037.

COMBINATION OF N-(4-HYDROXYPHENYL) RETINAMIDE AND GENISTEIN INCREASED APOPTOSIS IN NEUROBLASTOMA SK-N-BE2 AND SH-SY5Y XENOGRAFTS

S. KARMAKAR^a, S. ROY CHOUDHURY^a, N. L. BANIK^b, and S. K. RAY^{a,*}

^aDepartment of Pathology, Microbiology, and Immunology, University of South Carolina School of Medicine, Columbia, South Carolina

^bDepartment of Neurosciences, Medical University of South Carolina, Charleston, South Carolina

Abstract

Neuroblastoma is the childhood malignancy that mainly occurs in adrenal glands and found also in neck, chest, abdomen, and pelvis. New therapeutic strategies are urgently needed for successful treatment of this pediatric cancer. In this investigation, we examined efficacy of the retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) and the isoflavonoid genistein (GST) alone and also in combination for controlling the growth of human malignant neuroblastoma SK-N-BE2 and SH-SY5Y xenografts in nude mice. Combination of 4-HPR and GST significantly reduced tumor volume *in vivo* due to overwhelming apoptosis in both neuroblastoma xenografts. Time-dependently, combination of 4-HPR and GST caused reduction in body weight, tumor weight, and tumor volume. Combination of 4-HPR and GST increased Bax:Bcl-2 ratio, mitochondrial release of Smac, down regulation of BIRC-2 and BIRC-3, and activation of caspase-3 and AIF. Further, down regulation of NF- κ B, VEGF, and FGF2 were also detected. *In situ* immunofluorescent labelings of tumor sections showed overexpression of calpain, caspase-12, and caspase-3, and also AIF in course of apoptosis. Combination therapy increased apoptosis in the xenografts but did not induce kidney and liver toxicities in the animals. Results demonstrated that combination of 4-HPR and GST induced multiple molecular mechanisms for apoptosis and thus could be highly effective for inhibiting growth of malignant neuroblastoma in preclinical animal models.

Keywords

apoptosis; caspases; genistein; N-(4-hydroxyphenyl) retinamide; neuroblastoma

INTRODUCTION

Neuroblastoma is the most frequent extracranial childhood solid tumor, which mostly occurs in adrenal glands and frequently metastasizes to chest, neck, lymph nodes, pelvis, liver, and bone (Brodeur, 2003). Neuroblastoma is characterized by biological heterogeneity (Brodeur, 2003; Voigt and Zintl, 2003). Current treatment modalities for this childhood malignancy

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* **Corresponding author:** Swapan K. Ray, Ph.D., Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Building 2, Room C11, 6439 Garners Ferry Road, Columbia, SC 29209, USA. Tel.: 803-733-1593; fax: 803-733-3192; swapan.ray@uscmed.sc.edu.

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include surgery, radiation, and chemotherapy. However, in most cases therapeutic inefficacy of neuroblastoma accounts for approximately 15% of all childhood cancer deaths (Maris and Matthay, 1999; Goldschneider et al., 2006). So, development of new therapeutic strategies is highly warranted for treating neuroblastoma.

Retinoids modulate a wide variety of biological processes including differentiation, proliferation, and apoptosis in different cancers (Sun and Lotan, 2002). Cell differentiating capability and anti-cancer activity of the retinoids such as all-*trans* retinoic acid (ATRA) and 13-*cis* retinoic acid (13-CRA) are well established in several *in vitro* and *in vivo* models (Lotan, 1996; Okuno et al., 2004). N-(4-Hydroxyphenyl) retinamide (4-HPR), also known as fenretinide, is a promising anti-cancer agent among the synthetic and natural retinoids including ATRA and 13-CRA (Sabichi et al., 2003). 4-HPR shows broad spectrum anti-cancer properties against a variety of *in vitro* and *in vivo* animal studies (Hail et al., 2006). Most of the *in vitro* studies using 4-HPR reported its anti-cancer activity due to induction of apoptosis (Hail et al., 2006) with increased Bax:Bcl-2 ratio and caspase-3 activation in glioblastoma cells (Das et al., 2008) and it also induced apoptosis in neuroblastoma cells (Hewson et al., 2005). The effects of 4-HPR are dose-dependent and relatively low concentrations of 4-HPR induce neuronal differentiation in human retinal pigment epithelial (ARPE-19) cells (Chen et al., 2003) as well as in neuroblastoma SH-SY5Y cells due to differential expression of a variety of key proteins (Das et al., 2009). The differentiating and growth inhibitory properties of 4-HPR indicate that it can be therapeutically useful in combination with another cytotoxic agent against neuroblastoma.

Genistein (GST), an isoflavonoid primarily from soy products, inhibits the growth of various cancer cells through modulation of genes that control cell cycle and apoptosis. GST induced apoptosis with alterations of Bax and Bcl-2 levels and increase in caspase-3 activity in human breast cancer MDA-MB-231 cells (Li et al., 2008). GST induced endoplasmic reticulum (ER) stress and mitochondrial damage in human hepatoma Hep3B cells (Yeh et al., 2007) and Ca²⁺-mediated calpain/caspase-12-dependent apoptosis in breast cancer MCF-7 cells (Sergeev, 2004). Previously, we reported that GST induced activation of calpain and caspases for apoptosis in human neuroblastoma SH-SY5Y cells (Das et al., 2006). Both 4-HPR (Ribatti et al., 2001; Shishodia et al., 2005) and GST (Kim, 2003; Wang et al., 2006) showed anti-proliferative and anti-angiogenic properties due to inhibition of NF- κ B, VEGF, and FGF2 in a variety of cancer cell lines.

In this study, we explored the efficacy of 4-HPR for prevention of cell proliferation and GST for induction of apoptosis in human neuroblastoma SK-N-BE2 and SH-SY5Y xenografts in nude mice. There has not yet been any report on this combination therapy in neuroblastoma. Our results showed that combination of 4-HPR and GST produced better efficacy than either treatment alone for activation of multiple molecular mechanisms for apoptosis in human malignant neuroblastoma SK-N-BE2 and SH-SY5Y xenografts in nude mice.

EXPERIMENTAL PROCEDURES

Tumor cell lines and culture conditions

Both SK-N-BE2 (Baumann Kubetzko et al., 2004) and SH-SY5Y (Bian et al., 2001; Baumann Kubetzko et al., 2004) cell lines stand for human malignant (N-type) neuroblastoma. SK-N-BE2 cell line harbors amplified N-Myc (Vandesompele et al., 2003; Baj and Tongiorgi, 2009) and mutant p53 (Goldschneider et al., 2006), simulating a clinical situation found in neuroblastoma patients with relapse after cytotoxic chemotherapy. On the other hand, SH-SY5Y cell line contains single copy N-Myc (Vandesompele et al., 2003, Baj and Tongiorgi, 2009) and wild-type p53 (Goldschneider et al., 2006), representing a

clinically relevant condition of neuroblastoma patients at the time of initial diagnosis. Both SK-N-BE2 and SH-SY5Y cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in 75-cm² flasks containing 10 ml of 1x RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a fully-humidified incubator containing 5% CO₂ at 37°C. 4-HPR and GST were purchased from Sigma Chemical (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was used as vehicle to make stock solutions of 4-HPR and GST and all aliquots of stock solutions were stored at -20°C until ready to use. For animal treatments, drugs were appropriately diluted in 0.9% NaCl (saline) before treatments. Primary IgG antibody against β -actin (monoclonal clone AC-15) was obtained from Sigma Chemical (St. Louis, MO, USA). All other primary IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). We used horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary IgG antibody and HRP-conjugated goat anti-rabbit secondary IgG antibody (ICN Biomedicals, Aurora, OH, USA) for detecting monoclonal primary IgG antibody and polyclonal primary IgG antibody, respectively.

Neuroblastoma xenografts in nude mice

Six weeks-old female athymic nu/nu mice were obtained from Charles River Laboratories (Wilmington, MA, USA). All animal studies were conducted according to the NIH guidelines and also approved by our Institutional Animal Care and Use Committee (IACUC). Each of neuroblastoma SK-N-BE2 and SH-SY5Y cells (6×10^6) in 100 μ l of 1:1 mixture with Matrigel (BD Biosciences, San Jose, CA, USA) was implanted by subcutaneous (sc) injection on the flank of each mouse under isoflurane anesthesia condition. Palpable xenografts were developed within 6 to 8 days, tumors were measured using an external caliper, and tumor volume was calculated using the formula: $4\pi/3 \times (\text{length}/2) \times (\text{width}/2)^2$. Animals with 3 weeks-old neuroblastoma xenografts were randomly divided into 4 groups: control (CTL), 4-HPR, GST, and 4-HPR + GST. Animals in CTL group did not receive any therapy but similar amount DMSO-saline mixture. Each animal in other groups received intraperitoneally (ip) a daily dose of 4-HPR (20 μ g/kg/day), GST (2 mg/kg/day), or 4-HPR (20 μ g/kg/day) + 4 h later GST (2 mg/kg/day) for 8 or 15 days. After treatments for 8 or 15 days, we determined tumor volume and weight. For time-course studies, once in every week we monitored animal body weight and tumor volume for 21 days before the treatments and during the treatments for next 15 days.

Histopathological examination of xenograft sections

After completion of treatment schedule, xenografts were excised. One half of each tumor was immediately frozen in liquid nitrogen and stored at -80°C. The other half of tumor was immediately frozen (-80°C) in Optima Cutting Temperature media (Fisher Scientific, Suwanee, GA, USA) and 5 μ m sections were cut with a cryostat. These sections were subjected to hematoxylin and eosin (H&E) staining for examination of changes in histopathology following treatments.

In situ immunofluorescent labeling for detecting expression of pro-apoptotic protein

Tumor sections were blocked with 2% (v/v) horse and goat sera (Sigma Chemical, St. Louis, MO, USA) in PBS for 1 h and then probed with a primary IgG antibody (1:100) for 1 h and rinsed in PBS. Slides were then incubated with either fluorescein isothiocyanate (FITC)-conjugated or Texas red-conjugated secondary IgG antibody (1:75) (Vector Laboratories, Burlingame, CA, USA) for 1 h and rinsed in PBS and then water. Slides were mounted with the anti-fade medium Vectashield (Vector Laboratories) and viewed promptly under a fluorescence microscope at 200x magnification (Olympus, Japan). The digital pictures were captured using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA), as we reported previously (Karmakar et al., 2007).

Combined TUNEL and double immunofluorescent staining for detection of apoptosis and upregulation of pro-apoptotic protein

We pre-fixed 5 μ m sections in 95% ethanol (10 min) and 4% methanol-free formaldehyde (10 min) (Polysciences, Warrington, PA, USA) and washed in PBS. After equilibration in terminal deoxynucleotidyl transferase (TdT) buffer (Promega, Madison, WI, USA), sections were incubated with digoxigenin (DIG)-labeled nucleotides (Roche, Indianapolis, IN, USA) and recombinant TdT (Promega) at 37°C for 1 h. TUNEL reaction was stopped and unbound nucleotides were removed by washing in PBS. Slides were blocked with 2% normal goat and horse sera, incubated with a primary IgG antibody (1:100) for 1 h, washed in PBS, and incubated with fluoresceinated antibodies such as Texas red-conjugated anti-DIG antibody (1:50) (Roche, Indianapolis, IN, USA) and FITC-conjugated secondary antibody (1:75) (Vector Laboratories) for 30 min. Slides were then washed in PBS and water, mounted with VectaShield (Vector Laboratories), and examined under a fluorescence microscope (Olympus) at 200x magnification to capture images using Image-Pro Plus Software (Media Cybernetics, Bethesda, MD, USA), as we reported recently (Karmakar et al., 2008).

Western blotting

Briefly, protein samples were extracted following the lysis of control and drug-treated tumor tissues, quantitated spectrophotometrically, denatured in boiling water for 5 min, and loaded onto the SDS-polyacrylamide gradient (4-20% or 5%) gels (Bio-Rad, Hercules, CA, USA). All proteins were resolved by electrophoresis and then electroblotted to the membranes. The blots were incubated with a primary IgG antibody followed by incubation with an alkaline horseradish peroxidase (HRP)-conjugated secondary IgG antibody. Subsequently, specific protein bands were detected by HRP/H₂O₂-catalyzed oxidation of luminol in alkaline conditions using the enhanced chemiluminescence system and autoradiography (Karmakar et al., 2006).

Biochemical determination of organ toxicity in mice with SK-N-BE2 xenografts

Kidneys and livers from all groups were surgically removed and stored at -80°C until used. Tissue homogenates were prepared in chilled 0.1 M phosphate buffer (pH 7.4) and then centrifuged at 10,000 $\times g$ for 10 min at 4°C to obtain supernatant. Serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase, acid phosphatase, creatinine, and creatinine kinase were estimated spectrophotometrically using the commercially available kits (Biotron Diagnostics, Hemet, CA, USA).

Statistical analysis

Data were analyzed using Minitab® 15 Statistical Software (Minitab., State College, PA, USA), expressed as mean $\pm$ standard error of mean (SEM) of separate experiments ($n \geq 3$), and compared by one-way analysis of variance (ANOVA) with Fisher post-hoc test. Significant difference between control (CTL) and a treatment was indicated by * $p < 0.05$ or ** $p < 0.001$.

RESULTS

Tumor volume, weight, and histopathological evaluations

We used two strategies in neuroblastoma xenografts for exploring the efficacy of treatments (Fig. 1). In the first strategy (left panels), we treated SK-N-BE2 xenografts in nude mice for 8 days; and in the second strategy (right panels), we treated SH-SY5Y xenografts in nude mice for 15 days (Fig. 1A). Compared with CTL or a monotherapy, combination of 4-HPR and GST showed significant reductions in tumor volume (Fig. 1B and 1C). Following

treatments for 8 or 15 days, H&E staining of tumor sections showed that CTL tumors maintained characteristic growth, 4-HPR alone inhibited tumor cell proliferation, GST alone induced death to some extent, and 4-HPR + GST increased cell death; and extent of cell death was more due to treatment with 4-HPR + GST for 15 days than for 8 days (Fig. 1D). Also, time-dependently 4-HPR + GST caused reduction in animal body weight (Fig. 2A), tumor volume (Fig. 2B), and tumor weight (Fig. 2C) in neuroblastoma SH-SY5Y xenografts, compared with corresponding CTL groups. The time-dependent drug effects were also evident from the increase in percent regression in tumor volume (Fig. 2D) because combination therapy for 15 days showed more regression in tumor volume than that for 8 days (Fig. 2D).

Combination therapy increased Bax:Bcl-2 ratio

Western blotting showed alterations in the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins in neuroblastoma SK-N-BE2 and SH-SY5Y xenografts (Fig. 3). Expression of 42 kD β -actin was used as an internal control in Western blotting (Fig. 3A). Treatment with 4-HPR + GST increased the Bax:Bcl-2 ratio in both xenografts (Fig. 3B). Increase in Bax:Bcl-2 ratio could cause mitochondrial release of pro-apoptotic molecules to trigger activation of down stream processes of apoptosis.

Mitochondrial release of pro-apoptotic molecules, activation of caspase-3, and inhibition of survival and angiogenetic factors

Western blotting (Fig. 4) showed the most increases in mitochondrial release of 25 kD Smac into the cytosol and down regulation of 72 kD BIRC-2 (cIAP1) and 68 kD BIRC-3 (cIAP2) to favor activation of caspase-3 (Fig. 4A) for apoptosis following combination therapy in SK-N-BE2 xenografts. An increase in cytosolic level of 67 kD AIF following treatment with 4-HPR + GST (Fig. 4A) indicated activation of mitochondria-mediated caspase-independent pathway of apoptosis as well.

Also, 4-HPR + GST caused the highest inhibition of the cell survival factor 65 kD NF- κ B and the angiogenetic factors such as 21 kD VEGF and 17 kD FGF2 and also activation of caspase-3 for apoptosis in neuroblastoma SH-SY5Y xenografts (Fig. 4B). Further, colorimetric assay showed that 4-HPR + GST most significantly induced caspase-3 activity in neuroblastoma SH-SY5Y xenografts (Fig. 4C).

Involvement of calpain and caspase-12 in apoptosis in SK-N-BE2 xenografts

We employed single immunofluorescent (SIF) staining to detect expression of a specific pro-apoptotic protein (Fig. 5) whereas double immunofluorescent (DIF) staining to detect expression of a specific pro-apoptotic protein and DNA fragmentation in apoptotic cells in the xenograft sections (Fig. 5). SIF staining showed a significant increase in expression of calpain (Fig. 5A) and DIF staining detected significant overexpression of calpain and increase in DNA fragmentation in neuroblastoma SK-N-BE2 xenografts following treatment with 4-HPR + GST (Fig. 5B), indicating calpain upregulation for apoptosis in neuroblastoma SK-N-BE2 xenografts following combination therapy.

Also, SIF staining detected significant increase in expression of caspase-12 in neuroblastoma SK-N-BE2 xenografts following combination therapy (Fig. 5C). DIF staining showed a prominent role for caspase-12 in cell death (Fig. 5D). We identified significant overexpression of caspase-12 and increase in DNA fragmentation in neuroblastoma SK-N-BE2 xenografts following treatment with 4-HPR + GST (Fig. 5D).

Activation of caspase-dependent and caspase-independent pathways for apoptosis

We used SIF staining to examine expression of caspase-3 and found significant overexpression of caspase-3 in the neuroblastoma SK-N-BE2 xenografts after treatment with 4-HPR + GST (Fig. 6A). DIF staining showed significant increases in expression of caspase-3 and DNA fragmentation after the combination therapy, indicating an essential role for caspase-3 in apoptotic DNA fragmentation in the xenografts (Fig. 6B).

To identify caspase-independent mechanism of apoptosis, we examined expression of AIF using SIF staining and found significant increase in expression of AIF in the neuroblastoma SK-N-BE2 xenografts after treatment with 4-HPR + GST (Fig. 6C). Overexpression of AIF and increase in DNA fragmentation occurred most significantly in the xenografts after combination therapy (Fig. 6D). These observations showed the highest activation of caspase-dependent pathway as well as caspase-independent pathway for apoptosis in neuroblastoma SK-N-BE2 xenografts after treatment with 4-HPR + GST.

Evaluation of kidney and liver toxicities after the treatments

We spectrophotometrically determined biochemical markers such as SGOT, SGPT, alkaline phosphatase, acid phosphatase, creatinine, creatinine kinase in the kidneys and livers from the animals with neuroblastoma SK-N-BE2 xenografts after the treatments (Fig. 7). None of the treatments significantly altered these biochemical markers, when compared with CTL animals (Fig. 7). Thus, treatments did not induce toxicity or interfere with the normal metabolism in such highly metabolic organs as kidney and liver.

DISCUSSION

In this investigation, we explored the efficacy of 4-HPR + GST for controlling the growth of human malignant neuroblastoma SK-N-BE2 and SH-SY5Y xenografts in nude mice. We showed that 4-HPR + GST produced significant anti-tumor efficacy with activation of multiple molecular mechanisms for induction of apoptosis without inducing kidney and liver toxicities in the *in vivo* preclinical models of neuroblastoma.

We found that 4-HPR + GST significantly reduced the tumor volume in both neuroblastoma SK-N-BE2 and SH-SY5Y xenografts due to prevention cell proliferation and induction of cell death. Previous studies reported that 4-HPR or GST induced apoptosis in a variety of cell lines (Ribatti et al., 2001; Hail et al., 2006) including neuroblastoma (Ribatti et al., 2001; Hewson et al., 2005; Das et al., 2009) but this is the first report showing that treatment with 4-HPR + GST showed better efficacy than monotherapy in two neuroblastoma xenografts. In both neuroblastoma xenografts, there were significant increases in Bax:Bcl-2 ratio, a key determining feature for induction of apoptosis. Previous studies reported that GST induced alterations in Bax and Bcl-2 levels in breast (Li et al., 2008) and neuroblastoma (Das et al., 2006) cells. Recent reports showed that GST switched Bcl-2 from an anti-apoptotic protein into a proapoptotic protein in breast cancer cells for apoptosis (Tophkhane et al., 2007). An increase in Bax and its translocation to mitochondria could induce mitochondrial release of pro-apoptotic molecules (Makin et al., 2001). Treatment with 4-HPR + GST increased mitochondrial release of Smac into the cytosol. We also found down regulation of the anti-apoptotic BIRC-2 (cIAP1) and BIRC-3 (cIAP2) proteins in neuroblastoma SK-N-BE2 xenografts. BIRC proteins are upregulated to cause tumor cell survival and resistance to radiation and chemotherapies (LaCasse et al., 1998). Overexpression of BIRC-2 and BIRC-3 also causes inhibition of caspase activation and lack of apoptosis (Wang et al., 1998), indicating their ability to act as potential oncogenes. On the contrary, Smac acted as an indirect activator of caspases by inhibition of the BIRC proteins (Du et al., 2000). We found that 4-HPR + GST caused mitochondrial release of Smac into

the cytosol to down regulate BIRC-2 and BIRC-3 and facilitate apoptotic process with activation of caspase-3 in neuroblastoma SK-N-BE2 xenografts.

Mitochondria also induce caspase-independent pathway of apoptosis by releasing AIF (Cregan et al., 2004). We explored involvement of caspase-independent pathway of apoptosis in neuroblastoma SK-N-BE2 xenografts by determining the cytosolic levels of AIF. Treatment of the xenografts with 4-HPR + GST increased cytosolic levels of AIF, suggesting that AIF translocation to nucleus could cause caspase-independent nuclear DNA fragmentation for apoptosis. The anti-proliferative and anti-angiogenic potential of the treatment with 4-HPR + GST in SH-SY5Y xenografts was due to inhibition of cell survival factor NF- κ B and angiogenic factors VEGF and FGF2. These data agreed with previous reports where 4-HPR (Ribatti et al., 2001; Shishodia et al., 2005) and GST (Kim, 2003; Wang et al., 2006) produced similar effects in a variety of cancers. Thus, efficacy of 4-HPR + GST also caused inhibition of the survival and anti-angiogenic factors to facilitate apoptosis with increasing caspase-3 activation and activity in neuroblastoma SH-SY5Y xenografts.

The anti-inflammatory property of isoflavones including GST is due to inhibition of NF- κ B activation (Gong et al., 2003; Kang et al., 2005). GST also inhibited both constitutive and inducible NF- κ B activation in human cystic fibrosis bronchial gland cells (Tabary et al., 1999). Both NF- κ B activation and cytokine release were inhibited by GST in human monocytes (Geng et al., 1993) and alveolar macrophages (Carter et al., 1998). GST treatment significantly inhibits NF- κ B and also NF- κ B-associated genes in dendritic cells, which control the immune response (Dijsselbloem et al., 2007). Thus, GST is not only useful as an anti-cancer agent but also as an important dietary tool to control unnecessary cellular immune response and cytokine production following transplantation. Role of GST in controlling immune disorders is an open field for further investigation.

Our results indicated significant overexpression of both calpain and caspase-12 in course of apoptotic cell death in neuroblastoma SK-N-BE2 xenografts. Calpain is known to be involved in mitochondrial release of AIF (Polster et al., 2005) and endoplasmic reticulum (ER) stress-mediated activation of caspase-12 for apoptosis (Sergeev, 2004). Active caspase-12 directly promotes sequential activation of caspase-9 and caspase-3 for induction of apoptosis (Rao et al., 2002).

We found that significant upregulation of caspase-3 was associated with apoptotic DNA fragmentation in neuroblastoma SK-N-BE2 xenografts following treatment with 4-HPR + GST. Also, significant overexpression of AIF in TUNEL-positive cells strongly implicated involvement of AIF in apoptosis. 4-HPR causes elevation of Bax to induce mitochondrial release of cytochrome c for activation of caspase-3 and also to release AIF into the cytosol and its nuclear translocation for apoptosis (Tiwari et al., 2006). In another study, GST increased intracellular free $[Ca^{2+}]$ due to its release from ER Ca^{2+} storage, causing activation of the Ca^{2+} -dependent calpain and thereby caspase-12 for apoptosis in breast cancer cells (Sergeev, 2004). We previously reported that GST increased intracellular free $[Ca^{2+}]$ to cause activation of calpain, caspase-12, and caspase-3 in neuroblastoma SH-SY5Y cells for apoptosis (Das et al., 2006). Importantly, treatment with 4-HPR + GST did not impair metabolism in the kidney and liver, indicating absence of toxicities in these highly metabolic organs in the preclinical model of neuroblastoma.

There are scanty reports about the effect of 4-HPR or GST on the central nervous system (CNS) cells. However, 4-HPR induced apoptosis in human glioblastoma cells but not in normal astrocytes, suggesting its cancer cell-specific action (Tiwari et al., 2006). The toxicity profile of 4-HPR has also been studied, suggesting that 4-HPR has low overall

toxicity (Camerini et al., 2001). Chemoprevention clinical trials of 4-HPR showed no hematological toxicity but depicted decrease in night vision due to decrease in plasma retinol levels (Reynolds and Lemons, 2001; Reynolds et al., 2003). Some studies suggest that GST protects normal CNS cells due to its free radicals scavenging property (Wei et al., 1993). GST protects human cortical neuronal HCN1-A and HCN2 cells from oxidative stress (Sonee et al., 2004) and also primary cortical neurons from iron-induced free radical reaction and lipid peroxidation (Ho et al., 2003). More recent study reports that GST protects dopaminergic neurons from lipopolysaccharide-induced injury by inhibiting microglia activation (Wang et al., 2005). Overall, 4-HPR and GST are highly regarded for their anti-cancer effects and they hardly affect human normal CNS cells. Our current study suggests that 4-HPR + GST can be useful for controlling *in vivo* growth of human malignant neuroblastomas without inducing kidney and liver toxicities.

CONCLUSION

Taken together, our studies demonstrated that 4-HPR + GST effectively induced multiple molecular mechanisms for increasing amounts of apoptosis in neuroblastoma SK-N-BE2 and SH-SY5Y xenografts in nude mice.

Acknowledgments

This investigation was supported in part by the R01 grants (NS-57811 and CA-91460) from the National Institutes of Health (Bethesda, MD, USA).

Grant support: NIH grants R01 NS-57811 and R01 CA-91460 (S. K. Ray).

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Fig. 1.

Regression of neuroblastoma tumors and changes in histopathological features. Treatments: control (CTL), 4-HPR (20 μ g/kg/day), GST (2 mg/kg/day), and 4-HPR (20 μ g/kg/day) + 4 h later GST (2 mg/kg/day). (A) Mice with neuroblastoma SK-N-BE2 and SH-SY5Y xenografts after treatments. (B) Representative samples showing regression of tumor volume after treatments. (C) Determination of tumor volume. (D) Evaluation of histopathological changes in the xenografts after the treatments. Mice with xenografts were treated for 8 or 15 days. We used 6 animals per group. Significant difference between CTL and treatment was indicated * $p < 0.05$ or ** $p < 0.001$.

Fig. 2.

Time-dependent reduction in animal body weight, tumor volume, and tumor weight in SH-SY5Y xenografts following treatments. Treatments (for 8 or 15 days): control (CTL), 4-HPR (20 µg/kg/day), GST (2 mg/kg/day), and 4-HPR (20 µg/kg/day) + 4 h later GST (2 mg/kg/day). (A) Changes in body weight following therapies. (B) Changes in tumor volume following therapies. (C), Changes in tumor weight following therapies. (D) Time-dependent drug effects in the percent regression of tumor volume. Combination therapy showed best efficacy in reducing animal body weight and tumor volume. We used 6 animals per group. Significant difference between CTL and treatment was indicated * $p < 0.05$ or ** $p < 0.001$. Significant difference between monotherapy and combination therapy was indicated by ## $p < 0.001$.

Fig. 3.

Western blotting for determination of Bax:Bcl-2 ratio in neuroblastoma SK-N-BE2 and SH-SY5Y xenografts. Treatments (for 8 or 15 days): control (CTL), 4-HPR (20 μ g/kg/day), GST (2 mg/kg/day), and 4-HPR (20 μ g/kg/day) + 4 h later GST (2 mg/kg/day). (A) Representative Western blots (n≥3) showed expression of 42 kD β -actin, 21 and 24 kD Bax, and 26 kD Bcl-2. (B) Changes in Bax:Bcl-2 ratio in SK-N-BE2 and SH-SY5Y xenografts. Significant difference between CTL and a treatment was indicated by *p<0.05 or **p<0.001.

Fig. 4.

Pro-apoptotic and anti-angiogenic effects of the treatments in neuroblastoma xenografts. Treatments (for 8 or 15 days): control (CTL), 4-HPR (20 μ g/kg/day), GST (2 mg/kg/day), and 4-HPR (20 μ g/kg/day) + 4 h later GST (2 mg/kg/day). (A) Representative Western blots ($n \geq 3$) showed expression of 42 kD β -actin, 25 kD Smac/Diablo, 72 kD BIRC-2, 68 kD BIRC-3, 20 and 12 kD active caspase-3, and 67 kD AIF in SK-N-BE2 xenografts. (B) Representative Western blots ($n \geq 3$) showed levels of 42 kD β -actin, 65 kD NF- κ B, 21 kD VEGF, 17 kD FGF2, and 32 and 20 kD caspase-3 in SH-SY5Y xenografts. (C) Colorimetric assay for determination of caspase-3 activity in SH-SY5Y xenografts following treatments. Significant difference between CTL and a treatment was indicated by * $p < 0.05$ or ** $p < 0.001$.

Fig. 5.

In situ single and double immunofluorescent labelings to detect increases in expression of calpain and caspase-12 for apoptosis (TUNEL-positive cells) in the xenografts after the treatments. Treatments (for 8 days): control (CTL), 4-HPR (20 μ g/kg/day), GST (2 mg/kg/day), and 4-HPR (20 μ g/kg/day) + 4 h later GST (2 mg/kg/day). (A) Combination therapy most significantly increased calpain expression in neuroblastoma SK-N-BE2 xenografts. (B) Calpain expression in TUNEL-positive cells. (C) Combination therapy most significantly increased caspase-12 expression in SK-N-BE2 xenografts. (D) Caspase-12 expression in TUNEL-positive cells. In bar graphs: 1 = CTL, 2 = 4-HPR, 3 = GST, and 4 = 4-HPR + GST. Significant difference between CTL and a treatment was indicated by * $p < 0.05$ or ** $p < 0.001$.

Fig. 6.

In situ single and double immunofluorescent labelings to detect increases in expression of caspase-3 and AIF for apoptosis (TUNEL-positive cells) in the xenografts after the treatments. Treatments (for 8 days): control (CTL), 4-HPR (20 $\mu\text{g/kg/day}$), GST (2 mg/kg/day), and 4-HPR (20 $\mu\text{g/kg/day}$) + 4 h later GST (2 mg/kg/day). (A) Combination therapy most significantly increased caspase-3 expression in neuroblastoma SK-N-BE2 xenografts. (B) Caspase-3 expression in TUNEL-positive cells. (C) Combination therapy most significantly increased AIF expression in SK-N-BE2 xenografts. (D) Overexpression of AIF in TUNEL-positive cells. In bar graphs: 1 = CTL, 2 = 4-HPR, 3 = GST, and 4 = 4-HPR + GST. Significant difference between CTL and a treatment was indicated by * $p < 0.05$ or ** $p < 0.001$.

Fig. 7.

Determination of kidney and liver toxicity profiles in mice with SK-N-BE2 xenografts after the treatments. Treatments (for 8 days): control (CTL), 4-HPR (20 μ g/kg/day), or GST (2 mg/kg/day), and 4-HPR (20 μ g/kg/day) + 4 h later GST (2 mg/kg/day). Following treatments, kidneys and livers from all groups were collected to make homogenates. Supernatants from all groups were prepared separately for biochemical analyses of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase, acid phosphatase, creatinine, and creatinine kinase. No significant changes were found in combination treatment group, compared with CTL, suggesting that minimal or no kidney and liver toxicity occurred after the treatments.