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Original Article

Sorafenib downregulates ERK/Akt and STAT3 survival pathways and induces apoptosis in a human neuroblastoma cell line

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Abstract: Neuroblastoma is a common solid tumor in children and its tumorigenicity is enhanced by the expression of survival pathways such as Akt and signal transducer and activator of transcription 3 (STAT3). Sorafenib is a multi-kinase inhibitor that also inhibits STAT3 signaling and induces apoptosis. In this study, we will examine the efficacy of sorafenib on a human neuroblastoma cell line (SK-N-AS) and also investigate its possible mechanisms. After cells reached 50-60% confluence, they were treated with various concentrations of sorafenib (0, 0.1, 1, 5, 10 and 20 μM) for different periods of time. The cell viability and apoptosis were determined by MTS colorimetric assay and TUNEL, respectively. Phosphorylation of Akt1/2/3 (p-Akt1/2/3), extracellular signal-regulated kinase 1/2 (p-ERK1/2), STAT3 (p-STAT3), and AMP-activated protein kinase alpha subunit (p-AMPKα) were determined with Western blot. The results indicate that as early as 2 hours post-treatment, cell viability was significantly decreased at 10 μM concentration. In 24 hours or longer treatment groups, sorafenib at 5 μM and above significantly decreased cell viability. TUNEL assay showed a significant increased of apoptosis in 5 and 20 μM treatment groups 24 hours after treatment. Western blots showed a decrease of p-ERK1/2, p-Akt1/2/3, p-STAT3, and p-AMPKα expression levels in various sorafenib treatment groups. Our results indicate that sorafenib significantly decreased cell viability and increased apoptosis in human neuroblastoma cell line in association with down-regulation of p-ERK1/2, p-Akt, p-STAT3 survival pathways. These data suggested potential clinical application of sorafenib in the treatment of neuroblastoma.

Keywords: Neuroblastoma; Sorafenib; Apoptosis; Akt1/2/3 Protein Kinase; Signal Transducer and Activator of Transcription 3

Introduction

Neuroblastoma ranks second among extracranial solid cancers in childhood and affects children at very young age. It is reported with about 650 new cases each year in the Unites States [1], accounting for about 15% of cancer mortality in children [2]. The five-year relative survival rate has increased from 54% for patient diagnosed in 1975-1984 to 68.5% for those diagnosed in 1996-2006 [3, 4]. However, the clinical course is variable and depends on substantial variation in age of onset/diagnosis, extend of disease, histology, and most importantly, specific genetic abnormalities [1, 4]. In particular, various levels of the phosphorylation of AMP-activated protein kinase (AMPK) and Akt (protein kinases B) have been reported in neuroblastoma cell lines and may related to the cancer development as well as the prognosis [5-7].

Sorafenib is the first oral multikinase inhibitor that targets cancer-specific pathways and affects tumor signaling and the tumor vasculature [8]. It has been approved by the U.S. Food and Drug Administration for the treatment of primary kidney cancer (advanced renal cell carcinoma, 2005) and advanced primary liver cancer (hepatocellular carcinoma, 2007). Recent studies have showed its effects on those non-receptor tyrosine kinases that relay signals through the mitogen-activated protein kinase (MAPK) intracellular signal transduction pathway, which is comprised of Raf, extracellular signal-regulated kinase (ERK) as well as others

[8-11]. Due to its highly evolutionary conserved nature, MAPK signaling pathway has been linked with the regulation of cell survival, differentiation, proliferation and other critical functions, through relaying extracellular growth-factor signals to multiple downstream nuclear effectors.

In this study, we investigated the anti-proliferative potential of sorafenib on the SK-N-AS neuroblastoma cell line. The effects of sorafenib on phosphorylation of various growth and apoptosis-related proteins were also studied. We discuss our findings as a rationale for the potential application of sorafenib in the treatment of neuroblastoma.

Materials and methods

Chemicals and reagents

Sorafenib was purchased from LC laboratories (Woburn, MA). CellTiter Aqueous One Solution Cell Proliferation Assay kit and DeadEnd Fluorometric TUNEL System were purchased from Promega (Madison, WI). Antibodies against phospho-STAT3 (Ser 727, p-STAT3), phospho-AMP-activated kinase alpha (p-AMPKα) and RadioImmuno Precipitation Assay (RIPA) buffer were purchased from Cell Signaling (Danvers, MA). Antibodies against phospho-Akt1/2/3 (Thr308, p-Akt) and secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA). Antibodies against phospho-ERK1/2 (p-ERK), beta-actin (β-actin) and other chemicals and culture supplies were purchased from Sigma (St. Louis, MO) unless specified.

Cell culture

Human neuroblastoma cell line SK-N-AS was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. They were maintained as a monolayer and sub-cultured when reached 80-90% confluence. For viability assay, cells were grown in 96-well plates (5,000 cells per well). For TUNEL assays, cells were grown in 4-well chamber slides. For Western blot assay, cells were grown in 10 cm culture plates. Experiments were done in duplicate, with 3 to 5 plates used for each condition. Sorafenib was dissolved in 100% dimethyl sulfoxide (DMSO) and further diluted in DMEM to the desired concentration with final DMSO concentrations ranging between 0.001- 0.2% (v/v). Our preliminary study showed that DMSO at concentrations less than 0.5% has no effect on cell viability and survival after 72 hours (data not shown). Throughout this project, DMEM with 0.2% (v/v) DMSO was used as solvent controls (control).

Cell viability

Cells were plated at 5,000 per well in 96-well plates and incubate in DMEM with 10% FBS. When they reached 50-60% confluence, DMEM with various concentrations of sorafenib (0, 0.1, 1, 5, 10 and 20 μM) was added. Plates were incubated for various periods of time (2, 6, 24, 48 and 72 hours). Cell viability was assessed by CellTiter AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's instructions. In brief, 20 μl of MTS reagent was added into each well of the 96-well assay plate containing the samples in 100μl of culture medium. Plates were incubated at 37 °C in a humidified, 5% CO_2 atmosphere for another 1-2 hours. Absorbance at 490 nm was read by means of an ELISA plate reader. Each experiment was repeated at least three times. Final data were normalized and presented as percentage of controls.

TUNEL assay

Cells were plated on 4-well chamber slides (Sigma, St. Louis, MO). After reaching 50-60% confluence, cells were exposed to sorafenib (0, 1, 10, 20 μM) for 24 hours. Apoptosis was quantitated by a fluorometric TUNEL system (Promega) according to the manufacturer's instructions. Slides were fixed by 4% formaldehyde in ice-cold phosphate buffered saline (PBS) and permeabilized with 0.2% Triton X-100 solution. After equilibration, slides were immersed in incubation buffer at 37°C for 60 minutes inside the humidified chamber to allow the tailing reaction to occur. Reaction was stopped by 2X saline sodium citrate (SSC) and slides were mounted in VECTASHIELD with DAPI (Vector Lab) for nuclei counterstaining. Slides were analyzed with a Nikon microscope and images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using the program Meta View (Universal Images). Ten (10) high-power fields from each slide were randomly

selected and numbers of TUNEL-positive cells were counted. Each experiment was repeated at least two times.

Western blot analysis

After treatment, total protein was isolated from cells using RIPA buffer following manufacturer's instruction. Protein concentration was determined using the Bradford protein assay with bovine serum albumin as standard. The same amount of proteins (24 μg) was resolved electrophoretically by one-dimensional SDS-PAGE (10% polyacrylamide) for approximately 1 hour at 120 V. Subsequently, the gel was equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.03% SDS, and 20% methanol) and the proteins were electrophoretically transferred at 30 V overnight to the Polyvinylidene Fluoride (PVDF) filter. Filter was blocked using 3% bovine serum albumin in Tris buffer saline (TBS) with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature. Phosphorylation of ERK1/2, Akt1/2/3, AMPKα, and STAT3 was detected using antibodies listed above. β-actin was used as loading control. Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo, Rockford, IL) and analyzed with NIH ImageJ software.

Statistical analysis

Data from the cell viability assay, TUNEL assay, Western blot assay between the treated and control groups were compared using the paired Student's t test (two-tails) using statistic software MiniTab (State College, PA). Significance was considered if $p<0.05$.

Results

Sorafenib induces decreases of neuroblastoma cell viability through increasing apoptosis

Neuroblastoma cell line SK-N-AS was cultured with various concentrations of sorafenib for different periods of time. Numbers of viable cells were assessed by CellTiter AQueous One Solution Cell Proliferation Assay kit. We observed a significant decrease in viable cell treated with 5, 10 or 20 μM of sorafenib for 24 or 48 hours (**Figure 1A**, $p<0.01$), as compared with control groups. The time course study showed that a single dose of sorafenib caused decrease in number of viable cells started as early as 2 hrs (10 μM concentration) or 6 hrs (5 μM concentration) after treatment and lasted for at least 72 hours (**Figure 1B**, $p<0.01$), as compared with control groups.

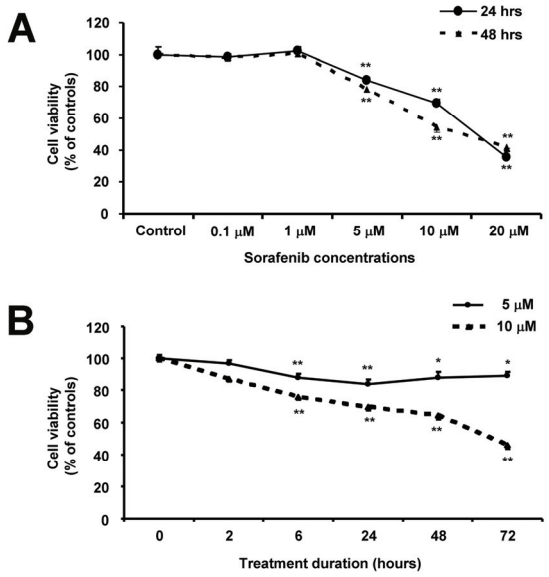

Figure 1. Effects of sorafenib on cell viability in SK-N-AS. SK-N-AS cells were cultured with different concentrations of sorafenib for different periods of time. Cell viability was assessed by CellTiter AQueous One Solution Cell Proliferation Assay kit according to the manufacturer's instructions. A, Sorafenib (5, 10 or 20 μM) significantly decreased cell viability after 24 or 48 hours treatment, as compared with controls (**P<0.01). B, When cultured with 5 or 10 μM of sorafenib for 6, 24, 48 or 72 hours, cells viability decreased significantly as compared with controls (*P<0.05, **P<0.01).

There are two types of programmed cell death, apoptosis and autophagy [12]. In this study, we focused on the role of apoptosis in sorafenib-induced SK-N-AS cell death. TUNEL assay showed a significant increase of apoptosis (159% and 282%, respectively) in cell treated with 10 or 20 μM of sorafenib for 24 hrs (**Figure 2A**, 2B, $p<0.01$), as compared with control groups.

Sorafenib induces decreases of Akt1/2/3 phosphorylation

The Akt signaling pathway is one of the most critical pathways in regulating cell survival [13].

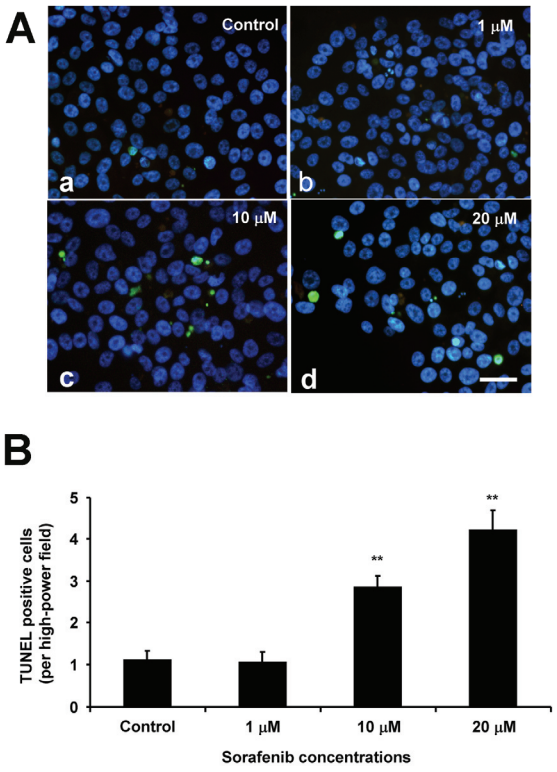

Figure 2. Effects of sorafenib on apoptosis in SK-N-AS. SK-N-AS cells were cultured with various concentrations of sorafenib for 24 hours. Apoptosis was studied using TUNEL assay. A, Representative images of TUNEL staining (100X). Scare bar = 50 μM. B, Average of numbers of TUNEL positive cells per each high power field (100X). Significantly increased numbers of TUNEL positive cells were seen in 10 or 20 μM treatment groups (**P<0.01).

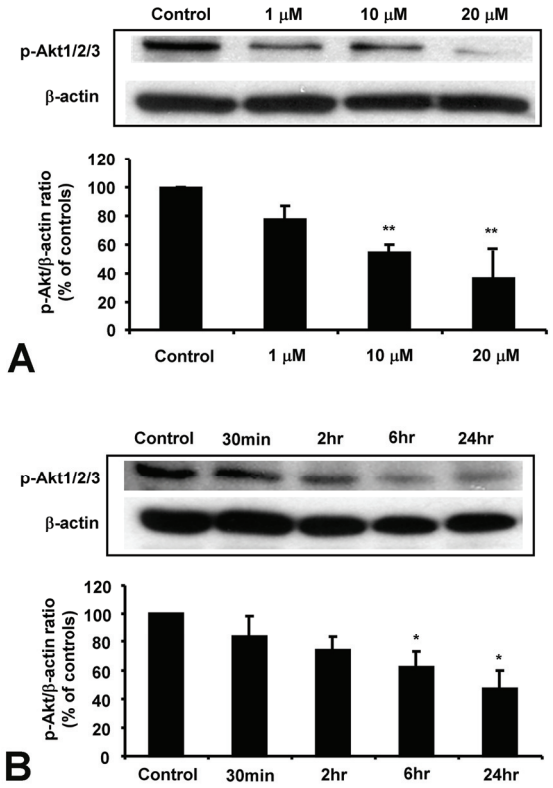

Figure 3. Effects of sorafenib on Akt1/2/3 phosphorylation in SK-N-AS. A, Representative bands of p-Akt1/2/3 and β-actin Western blot staining. Quantitative band density ratios showed that 24 hours treatment of sorafenib at 10 or 20 μM significantly reduced the phosphorylated Akt1/2/3 protein levels, as compared with controls (**P<0.01). B, Representative bands of p-Akt1/2/3 and β-actin Western blot staining. Quantitative band density ratios showed that 6 or 24 hours treatment of sorafenib at 10 μM significantly reduced the phosphorylated Akt1/2/3 protein levels, as compared with controls (*P<0.05).

Phosphorylation of Akt provides cells with a survival signal that allows them to withstand apoptotic stimuli [14]. Our data showed that sorafenib significantly decreased phosphorylation of Akt1/2/3 by 45% and 63% at 10 or 20 μM concentrations, respectively, after 24 hour treatment (**Figure 3A**, p<0.01), as compared with controls. Time course study showed that this inhibitory effect started as 6 hours post treatment (10 μM, **Figure 3B**, p<0.05).

Sorafenib induces decreases of ERK1/2 phosphorylation

The ERK1/2 signaling pathway regulates cell proliferation and cell differentiation [10]. Inhibition of ERK1/2 pathway could lead to the induction of apoptosis [15]. Again, our data showed that sorafenib significantly decreased phosphorylation of ERK1/2 by 45% and 72% at 10 or 20 μM concentrations, respectively, after 24 hours treatment (**Figure 4A**, p<0.05), as compared with controls. Time course study showed similar inhibitory effect 24 hours post treatment (10 μM, **Figure 4B**, p<0.05).

Sorafenib induces decreases of STAT3 phosphorylation

STAT3 has been shown to have anti-apoptotic as well as proliferative effects [16]. Yang, et al.

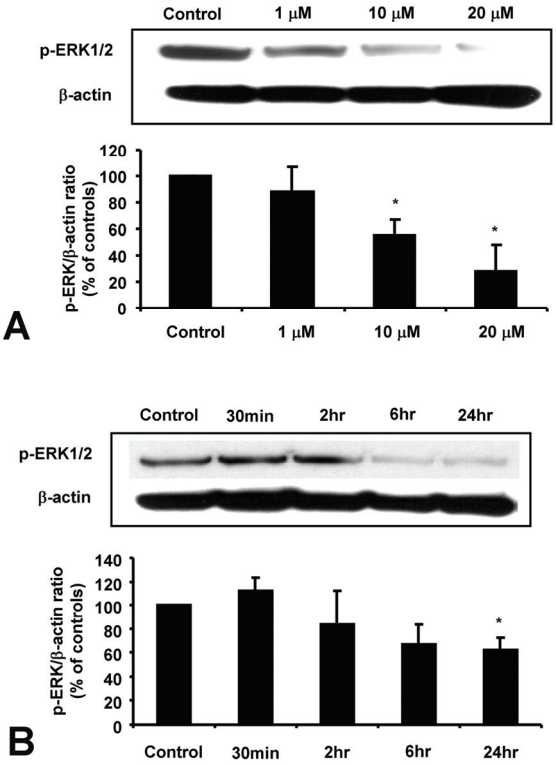

Figure 4. Effects of sorafenib on ERK1/2 phosphorylation in SK-N-AS. A, Representative bands of p-ERK1/2 and β-actin Western blot staining. Quantitative band density ratios showed that 24 hours treatment of sorafenib at 10 or 20 μM significantly reduced the phosphorylated ERK1/2 protein levels, as compared with controls (*P<0.05). B, Representative bands of p-ERK1/2 and β-actin Western blot staining. Quantitative band density ratios showed that 24 hours treatment of sorafenib at 10 μM significantly reduced the phosphorylated Akt1/2/3 protein levels, as compared with controls (*P<0.05).

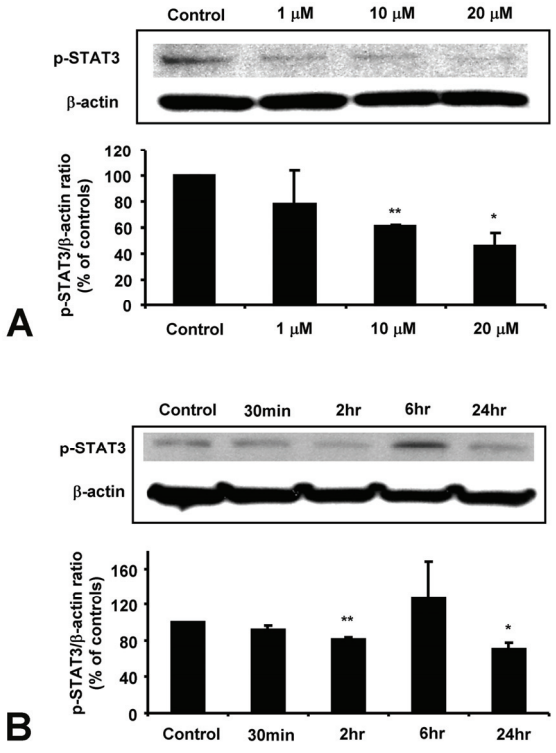

Figure 5. Effects of sorafenib on STAT3 phosphorylation in SK-N-AS. A, Representative bands of p-STAT3 and β-actin Western blot staining. Quantitative band density ratios showed that 24 hours treatment of sorafenib at 10 or 20 μM significantly reduced the phosphorylated STAT3 protein levels, as compared with controls (**P<0.01, *P<0.05). B, Representative bands of p-STAT3 and β-actin Western blot staining. Quantitative band density ratios showed that 2 or 24 hours treatment of sorafenib at 10 μM significantly reduced the phosphorylated STAT3 protein levels, as compared with controls (**P<0.01, *P<0.05). It is interesting that there is no significant change at 6 hours time point. The exact mechanism of this phenomenon is still under investigation.

reported that sorafenib inhibits STAT3 with growth arrest and apoptosis of medulloblastoma [17]. Our data showed that in neuroblastoma cell line, sorafenib also significantly decreased the p-STAT3 level by 39% and 54% in 10 or 20 μM treatment groups, respectively, as compared with controls (**Figure 5A**, p<0.05). Time course study showed significant inhibitory effect 2 or 24 hours post treatment (10 μM, **Figure 5B**, p<0.05), while not at 6 hours (p=0.43, as compared with controls).

Sorafenib induces decreases of AMP-activated protein kinase phosphorylation

AMPK is a sensor of cellular energy status, thus is the primary regulator of the cellular response to lowered ATP levels in eukaryotic cells [18]. We investigated the effects of sorafenib on phosphorylation of AMPK alpha in neuroblastoma cells. Our data indicated that sorafenib can significantly decrease the p-AMPKα level by 41% and 45% in 10 or 20 μM treatment groups, respectively, as compared with controls (**Figure 6A**, p<0.05). Time course study showed similar inhibitory effect 24 hours post treatment (10 μM, **Figure 6B**, p<0.01).

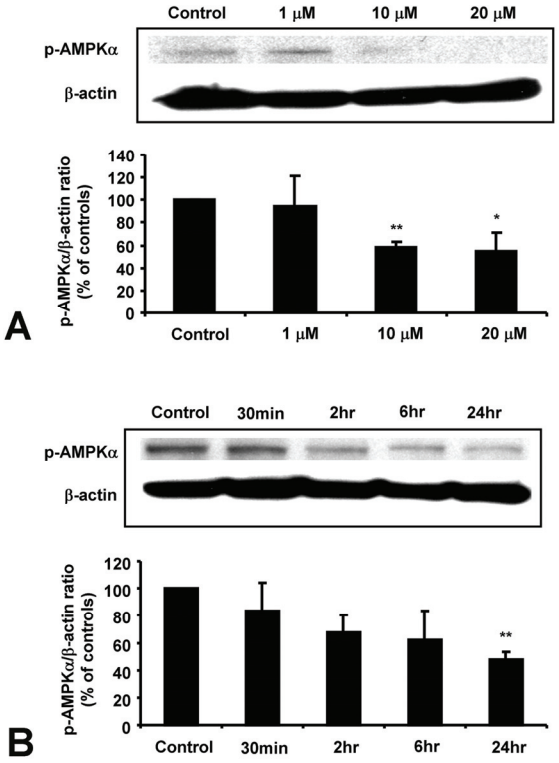

Figure 6. Effects of sorafenib on AMPKα phosphorylation in SK-N-AS. A, Representative bands of p-AMPKα and β-actin Western blot staining. Quantitative band density ratios showed that 24 hours treatment of sorafenib at 10 or 20 μM significantly reduced the phosphorylated AMPKα protein levels, as compared with controls (**P<0.01, *P<0.05). B, Representative bands of p- AMPKα and β-actin Western blot staining. Quantitative band density ratios showed that 24 hours treatment of sorafenib at 10 μM significantly reduced the phosphorylated AMPKα protein levels, as compared with controls (**P<0.01).

Discussions

In this study, we present evidences to prove that sorafenib is able to dose-dependently induce growth suppression in a human neuroblastoma cell line. This effect is associated with induction of apoptosis and suppression of the phosphorylation of several key signaling molecules including Akt, ERK1/2, STAT3 and AMPK. Our data suggested a potential clinical application of sorafenib in the treatment of neuroblastoma.

Sorafenib is a multikinase inhibitor recently approved by FDA for the treatment of advanced renal cancer and hepatocellular carcinoma. Data showed that it inhibits cell proliferation by targeting the Raf/MEK/ERK signaling pathways and exerts an anti-angiogenic effect by inhibition of tumor angiogenesis through VEGFR and PDGFR [15]. Due to its wide array of molecular targets, sorafenib has shown efficacy against a broad-spectrum activity against several preclinical models of human cancer. Publications suggested that sorafenib can inhibit cell proliferation or induce apoptosis in hepatocellular carcinoma [15], melanoma and breast cancer [19], malignant glioma [20], leukemia [21] and medulloblastoma [17]. Our cell viability assay confirmed similar effect of sorafenib on SK-N-AS, a widely used neuroblastoma cell line, in a dose-dependent manner. Preliminary data showed that this effect is not induced by solvent (data not shown). To the best of our knowledge, there has been only one report of sorafenib on two slightly different neuroblastoma cell lines [5]. The authors reported a significant decrease in cell viability when the cells were treated with sorafenib for 24 hours at 2.5 or 5 μM concentrations. They also reported an increase of apoptosis using morphological features, flow cytometry and electron microscopy analysis.

The Ras/Raf/MEK/ERK pathways are conserved signaling pathways which enable cells to respond to external stresses and stimuli [22]. Changes of the ERK pathway can promote effects ranging from apoptosis to malignancy in different cell types. In this study, we observed a decreased p-ERK1/2 in response to sorafenib treatment. This inhibition may inhibit cell growth through effects on cell cycle regulation, which has been described in various cancers including hepatocellular carcinoma [23], pancreatic cancer [24], as well as neuroblastoma [25]. In addition, our data demonstrated that sorafenib effectively down-regulated the phosphorylation of Akt, a known survival factor. Akt has been shown to play important roles in the proliferation and survival of tumor cells [25, 26]. The down-regulation of both p-ERK and p-Akt may lead to the changes in other survival factors including NF-kB [27], which eventually lead to cell apoptosis seen in neuroblastoma cells treated with sorafenib. Further investigations including MAPK blocking assay and other mechanistic experiments are warranted.

The activity of STAT3 is constitutively elevated in many human solid tumors as well as hema-

tologic malignancies. And STAT3 has been shown to actively participate in cell growth and survival [28]. Studies showed that the STAT3 signaling contributes to malignancy at least in part by preventing apoptosis in multiple myeloma cells [29]. Our data suggested that sorafenib treatment may inhibit the phosphorylation of STAT3 at Ser727 site, which is an important site related to cell apoptosis. An interesting finding is that we saw a bi-phasic change in time course study that has not been reported in neuroblastoma cells. The underlying mechanism is still unclear and further investigations are warranted.

Another interesting target is AMPK, which is a serine/threonine protein kinase that serves as an energy sensor in all eukaryotic cell types. Studies indicate that AMPK activation strongly suppresses cell proliferation in non-malignant cells as well as in tumor cells [30]. The AMPK signaling network contains a number of tumor suppressor genes and overcomes growth factor signaling from a variety of stimuli (via growth factors and by abnormal regulation of cellular proto-oncogenes including Akt and ERK). In this study, we reported, for the first time, that sorafenib treatment inhibited the activation of APMK alpha. However, the molecular basis of the relationship between AMPK and apoptosis, if any, is presently unclear.

Taken together, we are showing that sorafenib effectively induced cell death through apoptosis in neuroblastoma cell line. Our results suggested that various signaling pathways involving ERK, Akt, STAT3 and AMPK might be involved. These data suggest the potential clinical application of sorafenib in the treatment of human neuroblastoma.

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