Inhibition of S-adenosylmethionine decarboxylase by inhibitor SAM486A connects polyamine metabolism with p53-Mdm2-Akt/protein kinase B regulation and apoptosis in neuroblastoma

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Abstract

S-adenosylmethionine decarboxylase (AdoMetDC) is an essential enzyme of polyamine (PA) biosynthesis, and both AdoMetDC and PA levels are often up-regulated in cancer cells. The second-generation inhibitor SAM486A inhibits AdoMetDC enzyme activity and has been evaluated in phase II clinical cancer trials. However, little is known about the mechanism of action and potential use of this therapeutic drug in the treatment of the pediatric cancer neuroblastoma (NB). Here, we show that p53 wild-type NB cells are highly sensitive to SAM486A treatment. Most notably, SAM486A treatment resulted in the rapid accumulation of proapoptotic proteins p53 and Mdm2. Concomitant with the increase of proteins at endogenous levels, the in vivo phosphorylation of p53 at residues Ser46/Ser392 and Mdm2 at residue Ser166 was observed. Moreover, the antiapoptotic protein Akt/protein kinase B was down-regulated and also dephosphorylated at residue Ser473 in a dose- and time-dependent manner and NB cells entered apoptotic cell death. The results presented in this study highlight the importance of PA homeostasis and provide a direct link between PA metabolism and apoptotic cell signaling pathways in p53 wild-type NB cells. PA inhibitors such as SAM486A may be effective alternative agents for the treatment of NBs with or without MYCN amplification. [Mol Cancer Ther 2009;8(7):2067–75]

Introduction

Elevated polyamine (PA) levels are sustained in rapidly proliferating cells, and suppression of PA biosynthesis provides an attractive therapeutic target for many cancers (1–6). S-adenosylmethionine decarboxylase (AdoMetDC), a key enzyme in PA biosynthesis, provides the aminopropyl donor, decarboxylated S-adenosylmethionine (dcAdoMet), which is required for the sequential conversions of PAs putrescine to spermidine and then spermine. Ornithine decarboxylase (ODC) is a second rate-limiting enzyme in PA biosynthesis and catalyzes the conversion of ornithine to putrescine.

Both biosynthetic enzymes can be inhibited with specific inhibitors. SAM486A (also known as CGP48664), a derivative of the first-generation AdoMetDC inhibitor mitoguanzone, exerts potent and specific inhibition of AdoMetDC (7–10). The efficacy of SAM486A has been assessed in various cancer cells and animal systems (7, 8, 10–13), and the inhibitor has been evaluated in phase II human clinical trials (14, 15). α-Difluoromethylornithine (DFMO; ref. 5) is a specific suicide inhibitor of ODC that has been intensively studied in human clinical trials as an anticancer or chemopreventive agent (2, 5, 16).

Despite a plethora of information about the effect of these inhibitors in various cancer systems, very little is known about their role and therapeutic use in neuroblastoma (NB), an aggressive cancer of childhood (17). Although ~50% of human tumors harbor p53 mutations, the majority of primary NB tumors are tumor suppressor protein p53 wild-type with intact downstream p53 signaling pathways (18, 19). Our previous work showed that DFMO and DFMO combined with SAM486A (but not SAM486A alone) caused the down-regulation of MYCN protein and p27/Rb-regulated G1 cell cycle arrest without the induction of apoptosis in MYCN-amplified and p53-mutant NB cells (20). In this study, we report that p53 wild-type NB cells, independent of their MYCN amplification status, are highly sensitive to SAM486A. This targeted PA inhibitor induces the rapid accumulation and phosphorylation of p53 and Mdm2, down-regulates...
Akt/protein kinase B (PKB), and induces apoptotic cell death.

Materials and Methods

Chemicals

The AdoMetDC inhibitor SAM486A was provided by Novartis and dissolved in water. Aliquots at 10 mmol/L concentration were sterile filtered and stored frozen at −20°C until used in the experiment. Spermidine, amino-guanidine, and doxycycline were purchased from Sigma Chemical Co.

Cell Lines and Treatment of Cultured Cells

The human NB cell lines SK-N-SH, IMR-32, and SH-SY5Y were obtained from the American Type Culture Collection. MYCN-2, a doxycycline-inducible, MYCN-expressing cell line was kindly provided by Jason Shohet (Texas Children’s Hospital, Houston, TX; ref. 21). Cells were maintained in RPMI 1640 (Biosource) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 IU/mL), and streptomycin (100 μg/mL). If cells were treated with spermidine (10 μmol/L), aminoguanidine (100 μmol/L) was included as an inhibitor of serum PA oxidation. Cells in early log phase were seeded 2 to 3 h before treatment with 0.1 to 10 μmol/L of SAM486A and analyzed after designated time periods. NB cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. The number of viable cells was determined using a hemacytometer in the presence of trypan blue.

Flow Cytometry

For the detection of apoptosis, NB cells were seeded and treated with equal volumes of either sterile water (control) or 10 μmol/L SAM486A. Where indicated, cells were treated with doxycycline (1 μg/mL) or spermidine (10 μmol/L) and aminoguanidine (1 mmol/L). Cells were trypsinized, washed twice in PBS, and counted, and 1 to 2 × 105 cells were suspended in 0.1 mL of 1× assay buffer according to the manufacturer’s instructions (BD Biosciences). Cells were stained with Annexin V-FITC (5 μL) and propidium iodide (5 μL) for 15 min in the dark at room temperature. Assay buffer (0.4 mL) was added, and 5,000 cells were analyzed using a FACScan flow cytometry instrument (Becton Dickinson). The CellQuest program or the FlowJo software (Tree Star, Inc.) was used for data analysis. For the detection of cell cycle phase distribution, SAM486A-treated cells were stained with propidium iodide as previously described (20), and cell cycle distribution was determined using the ModFit software.

Western Blot Analysis

Cell lysates were prepared in radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl (pH 7.5), 0.1% (w/v) sodium lauryl sulfate, 0.5% (w/v) sodium deoxycholate, 135 mmol/L NaCl, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 2 mmol/L EDTA] supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals) and phosphatase inhibitors sodium fluoride (20 mmol/L) and sodium vanadate (0.27 mmol/L). Western blot analysis was done as previously described (20). The total protein concentration was determined using the protein assay dye reagent from Bio-Rad Laboratories. Cell lysates in SDS-sample buffer were boiled for 5 min and equal amounts of total protein were analyzed by 10% SDS-PAGE and Western blotting. The antibodies used in this study are mouse monoclonal p53 (1:250) from Santa Cruz Biotechnology; rabbit polyclonal phospho-p53 (Ser166; 1:1,000), rabbit polyclonal phospho-p53 (Ser392; 1:1,000), rabbit polyclonal Akt/PKB (1:1,000), and rabbit polyclonal phospho-Akt/PKB (Ser473; 1:1,000) from Cell Signaling Technology, Inc.; mouse monoclonal Mdm2 (Ab-4; 1:1,000) and rabbit polyclonal phospho-Mdm2 (Ser106; 1:1,000) from EMD Biosciences; mouse monoclonal β-actin (A5316; 1:5,000) from Sigma Chemical; and mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (6C5; 1:5,000) from Ambion, Inc. Secondary anti-mouse (1:5,000) and anti-rabbit (1:5,000) antibodies coupled to horseradish peroxidase were from Amersham Biosciences. Proteins were detected using the enhanced chemiluminescence Plus reagents (Amersham Biosciences) and Kodak BioMax XAR film (Fisher Scientific). Membranes were stripped at 50°C for 30 min with enhanced chemiluminescence stripping buffer [62.5 mmol/L Tris-HCl (pH 6.7), 2% SDS, 100 mmol/L 2-mercaptoethanol] and sequentially probed. Quantification was done as described previously using a Bio-Rad Fluor-S Multi Imager and Quantity One Quantitation Software, version 4 (Bio-Rad Laboratories).

Microscopy

NB cells were grown in cell culture dishes and treated with inhibitors as described above, and light micrographs were taken after 72 h using an inverted microscope [Nikon Diaphot (Nikon Corp.) or Leica DM IL (Leica Microsystems)]. To visualize p53 using immunofluorescence microscopy, SAM486A-treated or untreated cells were grown on coverslips (1 × 104/mL) in 12-well plates for 72 h. Washed cells were blocked in bovine serum albumin, washed again, and incubated with p53 antibody followed by incubation with secondary Alexa Fluor 488 antibody. To visualize the actin cytoskeleton, NB cells were stained with Texas red-X phalloidin according to the manufacturer’s instructions (Molecular Probes). Cells were washed, fixed in 3% paraformaldehyde for 10 min, and exposed to 0.1% Triton X-100 for 5 min. Fixed cells were washed again, blocked in 1% bovine serum albumin/PBS for 30 min, and incubated with phalloidin for 20 min in the dark at room temperature. Washed coverslips were mounted using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc.), and samples were analyzed with an epifluorescence microscope (Zeiss Axioplan, Carl Zeiss Micro Imaging GmbH).

PA Pool Analysis

Intracellular PA pools were measured in human SK-N-SH cells treated with SAM486A as previously described (13). The samples were normalized in 0.2 N sodium hydroxide and the amount of total protein per sample was measured using the Bio-Rad assay. Data from the PA measurements are represented as nmol per mg of total cellular protein.
The CellTiter 96® AQueous One Solution Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity (Promega). The CellTiter 96® AQueous One Solution Reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] and was used to determine the proliferation rate of NB cell lines. SK-N-SH, IMR-32, SH-SY5Y, and MYCN-2 (±doxycycline) cells were treated with increasing concentrations of SAM486A (0–10 μmol/L) for 72 h or treated with SAM486A (10 μmol/L) ± spermidine (10 μmol/L) for 24 to 72 h, and the proliferation rate was compared with untreated control cells. Briefly, cells were seeded at a density of 750 to 1,000 per well on a transparent, flat-bottom, 96-well plate in a total volume of 10 μL. After cell treatments, 20 μL of CellTiter 96 AQueous One Solution Reagent were added to wells and incubated for 1 to 4 h at 37°C. The absorbance was measured at 490 nm using a 96-well microplate reader.
Results

**SAM486A Induces Apoptosis and Inhibits Cellular Proliferation**

To examine whether the AdoMetDC inhibitor SAM486A induces apoptosis, we tested the effect of SAM486A in NB cells with wild-type p53: SK-N-SH and SH-SY5Y (MYCN nonamplified), IMR-32 (MYCN amplified), and MYCN-2 (MYCN-nonamplified, doxycycline-inducible MYCN). First, we examined the effects of SAM486A using an inverted light microscope and noticed that SAM486A affects the morphology of NB cells as evidenced by profound cell shrinkage and rounding of cells, membrane blebbing, and cell detachment, all of which are typical signs of apoptosis (Fig. 1A). The onset of apoptosis was confirmed by poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 1B) and Annexin V staining (Fig. 1C) as early as 24 hours after treatment with SAM486A using Western blot and flow cytometry analyses, respectively. Spermidine supplementation reversed the SAM486A-induced apoptosis in SK-N-SH cells (Fig. 1C). Comparable effects were observed with MYCN-2 cells and were independent of MYCN expression (Supplementary Fig. S1).5

To examine whether SAM486A inhibits cell proliferation, cells were analyzed by MTS assay. The treatment of cell lines SK-N-SH, SH-SY5Y, IMR-32, and MYCN-2 (±doxycycline) with SAM486A at various concentrations significantly reduced the number of viable cells in a dose-dependent manner (Fig. 2A), an effect that was reversible with spermidine supplementation (Fig. 2B). At doses ≥2.5 μmol/L, SAM486A exhibited cytotoxic effects in cell lines SH-SY5Y and IMR-32. The induction of MYCN expression with doxycycline in MYCN-2 cells did not have an effect on SAM486A-induced growth inhibition (Fig. 2A). Interestingly, the treatment with SAM486A did not significantly disturb cell cycle progression of SK-N-SH cells, indicating that the observed induction of apoptosis is not a consequence of cell cycle arrest (Supplementary Table S1).5 Together, these results confirm that SAM486A induces apoptosis and inhibits cell proliferation in several p53 wild-type NB cell lines independent of their MYCN amplification status.

**SAM486A Modulates Protein Levels and In vivo Phosphorylation of p53, Mdm2, and Akt/PKB**

The tumor suppressor protein p53 plays an important role in regulatory processes including apoptosis and cell cycle progression. To examine the potential effect of SAM486A...
on p53 at endogenous levels, we determined the protein content in SK-N-SH cells. As shown in Fig. 3A, strong accumulation of p53 was evident 24 hours after cell treatment with 10 μmol/L S486A. Extended treatments (48 and 72 hours) under identical conditions did not lead to further accumulation of p53 (Fig. 3A). To examine the phosphorylation status of p53, antibodies specifically recognizing phosphorylated p53 at residues Ser392 and Ser46 were used. S486A increased p53 phosphorylation at Ser392 and Ser46 after 24 hours (Fig. 3A). Extended treatments (48 and 72 hours) under identical conditions did not increase p53 phosphorylation at either phosphorylation site.

Because p53 regulation and its proteasomal degradation are dependent on its association with Mdm2, we did an identical experiment as described in Fig. 3A and measured the total amount of Mdm2 protein. As shown in Fig. 3B, control SK-N-SH cells expressed basal levels of Mdm2, whereas S486A-treated cells accumulated large amounts of Mdm2. Concomitant with the increased protein levels, a large increase in Mdm2 phosphorylation at Ser166 was observed (Fig. 3B). In addition to the Mdm2 protein at 90 kDa, we noticed a previously reported p60 fragment of Mdm2 at 48 hours, which increased at 72 hours.

The antiapoptotic protein Akt/PKB is a cell survival factor and a key regulator in apoptosis. We therefore tested whether S486A treatment of SK-N-SH cells modulates Akt/PKB levels and alters its phosphorylation status. We found that Akt/PKB protein levels as well as its phosphorylation at Ser473 were strongly reduced after 48 hours of treatment, and less so at the beginning of the experiment at 24 hours (Fig. 3C). This observation was confirmed by a decrease of Akt/PKB levels and its phosphorylation in response to increasing concentrations of S486A (Fig. 3D).

**Figure 3.** Analysis of apoptosis-regulating proteins in S486A-treated NB cells. Whole-cell lysates were obtained from SK-N-SH cells exposed to 10 μmol/L S486A for 24, 48, and 72 h or exposed to increasing concentrations of S486A (0, 0.1, 1, 2, 5, and 10 μmol/L) for 72 h and analyzed by Western blot. Resolved proteins were transferred to polyvinylidene difluoride membranes and sequentially probed for (A) total p53, phospho-p53 (Ser392), and phospho-p53 (Ser46); (B) total Mdm2 and phospho-Mdm2 (Ser166); and (C and D) total Akt/PKB and phospho-Akt/PKB (Ser473). S486A treatment increased total p53, phospho-p53 (Ser392), phospho-p53 (Ser46), total Mdm2, and phospho-Mdm2 (Ser166) and decreased total Akt/PKB and phospho-Akt/PKB (Ser473). Total Akt/PKB and phospho-Akt/PKB (Ser473) also decreased in a dose-dependent manner. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin served as loading markers. Data are representative of three independent experiments (n = 3).
PA Levels in SAM486A-Treated Cells

Many of the effects observed with SAM486A in the present study occurred within the first 24 hours after treatment. Therefore, we examined the changes in intracellular PA content in SAM486A-treated and untreated cells over a 24-hour time course. SAM486A exposure led to a large and progressive increase in putrescine content, whereas putrescine levels in control cells changed very little (Fig. 5). The increase in putrescine was observed as early as 8 hours. Spermidine and spermine levels were not significantly altered in response to SAM486A treatments at these early time points. Spermine levels were very low and not detectable in some samples. Of note, this early increase in putrescine content in SAM486A-treated cells directly correlates with the rapid increase of p53 levels at 8 hours.

Discussion

Our previous work showed that DFMO and DFMO/SAM486A combinations effectively inhibit cell cycle progression (without apoptosis) in MYCN-amplified, p53-mutant NB cells (20). In those NB cell lines, SAM486A alone decreased cell viability but otherwise had little or even opposing effects. In a more recent study, we further showed that DFMO activates two opposing pathways in MYCN-amplified and p53-mutant NB cells: one inducing cell survival via the phosphatidylinositol 3-kinase-Akt/PKB signaling pathway and another inducing cell cycle arrest through a mechanism that involves p27Kip1 phosphorylation and accumulation (20, 22). In contrast, the present study shows that SAM486A rapidly induces apoptosis in p53 wild-type NB cells with or without MYCN amplification. SAM486A treatment in NB cells resulted in cell blebbing, cell shrinking, and eventually detachment of cells. These morphologic changes are typical signs of apoptosis, which were confirmed by Annexin V staining (an early apoptosis marker) and PARP cleavage (a late apoptosis marker) via flow cytometry and Western blot analyses, respectively. This suggests that SAM486A is an apoptosis-inducing agent.

We also observed a decrease of the antiapoptotic protein Akt/PKB 48 hours after treatment. However, due to the fact that Annexin V staining and PARP cleavage occurred already within 24 hours of SAM486A treatment, SAM486A may exert its apoptotic effects independent of Akt/PKB in these NB cells. Interestingly, we also found that total PARP protein levels decreased after 48 and 72 hours of SAM486A treatment, suggesting that SAM486A disrupts the synthesis of PARP or increases its degradation (Fig. 1B).

Next, we examined the mechanism by which SAM486A induces apoptosis in these NB cells. SAM486A induced rapid accumulation of native p53 protein and phosphorylation of p53 at Ser392 and Ser46 in a time- and dose-dependent manner. The phosphorylation of p53 at Ser392 occurred within 8 hours of SAM486A treatment and was
represented by a single band in the Western blot analysis. In contrast, the phosphorylation of p53 at Ser\(^{46}\) was represented by two bands, one immediately above the other. These results suggest that the mechanism by which SAM486A induces p53-mediated cell death may involve posttranslational phosphorylation of p53 at Ser\(^{46}\), which stabilizes p53, and subsequent phosphorylation at Ser\(^{46}\), which is a signal for the induction of apoptosis in response to DNA damage, a process that might be mediated by DNA damage–induced ATM. Previous studies on stress-induced, p53-mediated cell death in MCF-7 breast cancer cells resulted in similar findings (23). Furthermore, a recent study reported that p53 may have different functions under conditions that result in DNA damage (camptothecin treatment) compared with conditions that induce PA depletion (DFMO treatment; ref. 24). Both treatments led to the phosphorylation and stabilization of p53. However, whereas camptothecin-induced DNA damage led to p53-dependent apoptosis, DFMO-induced PA depletion protected the cells from undergoing apoptosis by modulating cell cycle checkpoint proteins and decreasing transcription of genes that are involved in apoptosis.

To further elucidate the mechanism of SAM486A-induced p53 accumulation, we examined the effects of SAM486A on Mdm2, an E3 ubiquitin ligase that mediates p53 ubiquitination and subsequent proteasomal degradation. The increase in native Mdm2 protein levels and phosphorylation of Mdm2 at Ser\(^{166}\) may occur as a consequence of p53 accumulation, as Mdm2 is a transcriptional target of p53 (25). However, this increase does not seem to play an antagonistic role in the SAM486A-induced effects observed in the present study. Furthermore, the increase in Mdm2 p60 fragments has been previously shown to occur during p53-mediated apoptosis, as Mdm2 cleavage is mediated by caspases or caspase-like proteases (26).

We previously showed that the inhibition of AdoMetDC by SAM486A in MYCN-amplified, p53-mutant NB cells led to strong accumulation of putrescine levels and depleted spermidine and spermine levels after 48 to 72 hours of treatment (20). However, based on the findings of the present study, SAM486A induced p53 levels very rapidly and as early as 8 hours after treatment (Fig. 4A). Therefore, we were curious to determine whether the intracellular PA pools alter accordingly, thus suggesting that the change in p53 levels is linked to PA levels and modulated by SAM486A. Remarkably, we found that SAM486A-treated SK-N-SH cells indeed accumulated putrescine as early as 8 hours after treatment (Fig. 5). To our knowledge, this is the first time that a significant increase in putrescine pools has been observed within such a short time period and suggests that the early increase in p53 could be due to the SAM486A-induced changes of intracellular putrescine levels. The fact that spermidine and spermine levels were not significantly altered at these early time points was expected, as the depletion of higher PAs in response to PA inhibitors is usually observed after 48 hours in NB cells (20). In contrast, accumulation of putrescine may occur more rapidly as SAM486A-mediated inhibition of AdoMetDC reduces dcAdoMet, the immediate precursor necessary for the conversion of putrescine to spermidine and spermine. In addition, the rapid accumulation of putrescine may be accounted for by the fact that cells in the early log phase of growth exhibit highest ODC enzyme activities and, consequently, produce particularly high levels of putrescine. Finally, SAM486A has been shown to inhibit diamine oxidase and increase ODC activity, possibly further contributing to an increase in putrescine levels (8).

We also attempted to simulate these conditions by first depleting PAs with DFMO and then supplementing with exogenous putrescine. However, the intracellular putrescine content did not increase to the same extent as with SAM486A treatment, and therefore, it was not surprising that we did not observe the same effect on p53 accumulation and phosphorylation (data not shown). An explanation for the observed difference may be that due to modulations in the PA uptake and/or export system, exogenous putrescine is not...
able to effectively enter DFMO-treated NB cells and accumulate to high intracellular levels such as detected in SAM486A-treated cells. Furthermore, the inhibition of AdoMetDC and diamine oxidase as well as the activation of ODC enzyme activity by SAM486A (8) may elevate cellular putrescine levels to a degree much higher than detected in DFMO-treated cells supplemented with exogenous putrescine.

As depicted in the schematic diagram of Fig. 6, our study proposes that putrescine levels increase within 8 hours of SAM486A treatment in NB cells with wild-type p53. In addition, we observed an increase in activation of p53 within 8 hours of SAM486A treatment followed by the onset of apoptosis (Annexin V staining and PARP cleavage) within 24 hours of treatment. In summary, our results suggest that the rapid putrescine accumulation induced by SAM486A may play a role in p53-mediated apoptosis. However, it should be noted that SAM486A may be able to induce the effects observed in the current study through non–PA-mediated mechanisms (11).

Although PA inhibitors such as SAM486A and DFMO have been used in several human cancer trials (2, 3, 15), their efficacy as a treatment for NBs (individually or combined) has never been explored. Based on available clinical data from human trials, both inhibitors are relatively well tolerated, even at higher doses, with the occasional occurrence of temporary ototoxicity (with DFMO), nausea, and mild neutropenia. DFMO is water soluble and may be administered in drinking water, which is a particular advantage for pediatric patients. Based on our findings in this study as well as previous studies (20, 22), PA inhibitors such as SAM486A are effective in killing NB cells, namely, those with intact p53 signaling, thus providing further evidence that these targeted PA inhibitors are possible alternative agents for the treatment of NBs. Both SAM486A and DFMO
should be tested in combination with other chemotherapeutics (possibly at lower doses) to achieve an improved therapeutic outcome with hopefully reduced side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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