THE NEW TELLURIUM COMPOUND SAS INDUCES PC12 DIFFERENTIATION AND DECREASES NEURONS DEATH: MECHANISM OF ACTION

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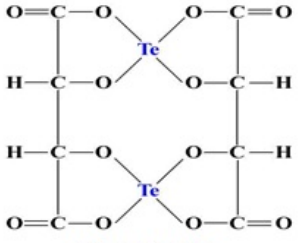
Abstract - Studies show that anti-apoptotic and neurotrophic agents are suitable candidates to prevent delayed neural cell death and/or restore neural function. Here we present the novel nontoxic immunomodulating tellurium compound–octa-O-bis- (R,R)-Tartarate Ditellurane (SAS), with high stability and solubility, which has the ability to induce neurite outgrowth and neural differentiation in PC12 cells in a dose dependent manner. The concentration spectrum of SAS that induces PC12 differentiation is very broad, which contributes superior potential for SAS in clinical research. The pathways which are activated by SAS are p21ras dependent and play an important role in neural differentiation and survival. Interruption of the p21ras cascade by either inhibition p21ras farnesyltion, or depleting c-raf-1 or inhibiting Mek, abolishes SAS-induced PC12 differentiation, whereas inhibition of p38 has not such effect. PI_3K plays also an essential role in SAS induced differentiation since introducing the specific inhibitor LY294002 abrogates this activity. Moreover, we demonstrate that nitric oxide synthase plays an essential role in SAS-induced PC12 differentiation. All investigated proteins are essential in SAS ability to increase amount of p21waf. Depletion of p21waf by antisense abrogates SAS ability to induce PC12 differentiation. Finally, SAS reduces the amount of ongoing cells in growth factor depravation. Our findings may be important in understanding the regulation of survival/apoptosis of neurons deprived of neurotropic support, and in developing new potent small non-toxic agents for restoring neuronal function.

Keywords - Neurotrophic Factor, PC-12, p21ras, Tellurium.

I. INTRODUCTION

During mammalian development and in the mature organism, various extracellular stimulli act to promote survival and suppress cellular suicide programs. Under pathological conditions, as well as deficiency of target-derived survival factors, a lot of neurons in flawed tissues are dying during the development of the disease. The signaling pathways that lead to apoptosis are beginning to be defined, and a number of proteins have been identified that either induce or prevent apoptosis. For example, the activation of the p21ras by different neurotrophic factors, like NGF, is essential for neural differentiation and proliferation of PC12 cells, as well as for neural survival. P21ras phosphorylation leads to activation of c-raf-1 and PI3K [1]. On the one hand, the p21ras-PI3K/Akt pathway leads to deactivation of different pro-apoptotic proteins, like Bad and caspase 9, and to the activation of atypical PKCs, NF-kB, etc. This pathway thus plays a crucial role in cell proliferation and survival [2]. On the other hand, the p21ras-raf-ERK1/2 pathway participates in cell differentiation and Nitric Oxide (NO) production [3]. NO is a unique molecule that plays an important role in both, neural development and degeneration. Studies show that the outcome of Nitric Oxide's effect depends on its concentration, the cell type and its accumulation in a specific stage in the cell cycle. Small amounts of NO can prevent apoptosis of NGF-differentiated PC12 cells after NGF withdrawal. Survival is mediated by NO through p21ras-PI3K signal transduction pathway, or through the direct inhibition of caspase activity by S-nitrosylation [4].
Here we present a new organo-tellurium derivative, octa-O-bis-(R,R)-Tartarate Ditellurane (SAS). (Scheme 1) SAS is a Te^{IV} compound, synthesized in our laboratory [5] This compound contains two tellurium atoms, each liganded by four oxygen atoms, two carboxylate group and two alkoxide groups derived from two tartaric acids [6]. Unlike the extensively studied immunomodulating tellurium compound AS101[7], SAS is highly stable in aqueous solution and exhibits improved properties [8].

The present study investigates the influence of SAS on the differentiation of PC12 cells to neuronal like cells and their survival/apoptosis under conditions of deprivation of trophic support. In addition signal-transduction pathways are determined. Those include ras-ERK1/2 ras-PI3K/Akt1/2 pathways, and iNOS and p21waf protein expression, which resulting in SAS-induced decrease of ongoing NGF-differentiated cells under condition of neurotropic starvation.



Scheme 1: SAS

II. RESULTS

The ability of SAS to induce PC12 differentiation and neurite outgrowth – PC12 differentiation by NGF is accompanied by morphological changes, neurite outgrowth and expression of different neural markers. Figure 1 illustrates that addition of SAS to PC12 cells results in their differentiation.

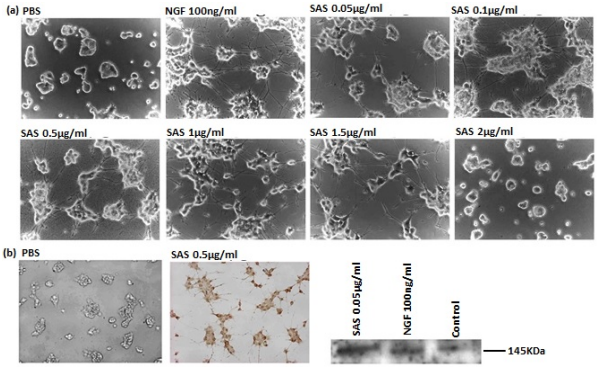


Figure 1: (a) SAS stimulates PC12 cells to induce neurite outgrowth in a dose dependent manner. At the end incubation time cells were observed in a phase contrast microscope (b) SAS-induced differentiated PC12 cells express Neurofilament M. After SAS stimulation, differentiated PC12 cells were washed, fixed and stained with ABC staining system which includes first antibody against neurofilament M.

Differentiation of PC12 cells was microscopically observed following addition of SAS at 0.05-1.5µg/ml concentrations (Fig. 1 (a)). SAS ability to induce PC12 differentiation was confirmed by immunohistochemistry studies showing positive staining to the neuronal marker Neurofilament M, following SAS treatment (Fig. 1 (b)) and by western blot analysis showing elevated amounts of Neurofilament M protein (Fig. 1 (b)).

SAS induces activation of two different p21 ras - dependent cascades – Two important pathways, could be activated by p21ras by growth factors, are the c-raf-1/ERK1/2 and PI3K/Akt1/2 pathways, which are known to play a pivotal role in neuronal differentiation and survival. Since SAS alone leads to the differentiation of PC12 cells, we attempted to elucidate the mechanism of its activity. SAS alone induces ERK and Akt1/2 phosphorylation in a time dependent manner. Phosphorylation of ERK and Akt1/2 induces by SAS was at early (15-30min) and late (6-12h) stimulation time (Fig. 2 (a)). Whereas, phosphorylation of p38 protein, other MAPK protein, was reduced by SAS along 1/2 to 6h of SAS stimulation time, following first ERK1/2 activation induced by SAS (Figure 2 (a)). In order to confirm that c-raf-1/ERK and PI3K/Akt1/2 pathways and not p38 involved in SAS activities, we used the following inhibitors: farnesyl transferase inhibitor (Fti), which inhibits p21ras activity, geldanamycin (GA, inhibitor of c-raf-1), PD98059 (MEK inhibitor), LY294002 (PI3K inhibitor), SB203580 (p38 inhibitor) and RO318220 (PI3K-activated protein, aPKC). Figure 2 (b) and (c) shows that incubation of PC12 cells in the presence of either one of the inhibitors detailed below, except SB318220 and RO318220, abrogates the neural PC12 differentiation induced by SAS. These results indicate that both the p21ras-ERK1/2 and p21ras-PI3K pathways are necessary for PC12 differentiation induced by SAS.

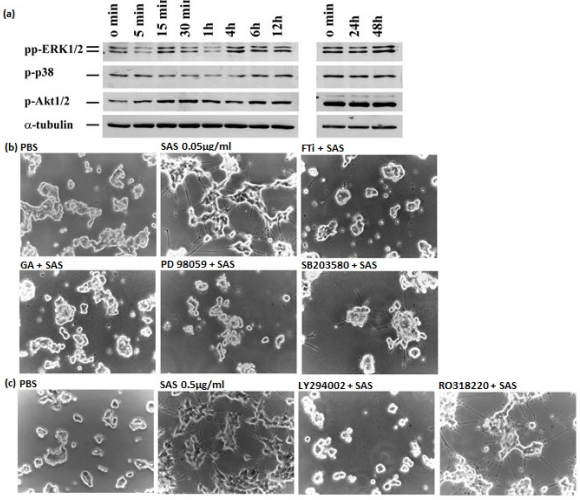


Figure 2: (a) SAS elevates ppERK1/2 and pAkt1/2 in a time dependent manner, while reduces pp38 expression. (b) and (c) inhibitors of ras-ERK1/2 and PI3K pathways abrogates SAS-induced PC12 differentiation. Different inhibitors [farnesyl transferase inhibitor (Fti, ras inhibitor), geldanamycin (GA, inhibitor of c-raf-1), PD98059 (MEK inhibitor), LY294002 (PI3K inhibitor), SB203580 (p38 inhibitor), Ro318220 (aPKC inhibitor)] were added in order to confirm protein involvement in SAS ability.

Role of Nitric Oxide and p21waf in SAS-induced PC12 cells differentiation – It is known that the activation of p21ras-c-raf-1-ERK1/2 cascade induces nitric oxide (NO) production, and that NO has a potential role in the regulation of p21waf protein expression, and PC12 differentiation and survival. We therefore analyzed the role of NOS activity and p21waf involvement in SAS differentiating ability. As shown in Figure 3 (a) addition of SAS at 0.5µg/ml with an active NOS inhibitor to PC12 cells abolishes their differentiation. Figure 3 (b), demonstrate that SAS treatment induce iNOS expression in PC12 cells in late stimulation time (6-12h). The role of p21waf up-regulation by SAS in PC12 cells differentiation was assessed by showing that transfection of anti-sense p21waf to these cells abrogated the differentiating capabilities of SAS (Fig. 3 (c)). SAS up-regulates p21waf expression compared to levels in untreated cells after 24h (Fig. 3 (d)). L-NAME, but not D-NAME, inhibited the accumulation of p21waf, as well as treatment of PC12 cells with either p21ras inhibitor or MEK inhibitor abrogates SAS-induced PC12 differentiation (Fig. 3 (d)). Suggesting, that SAS induces p21waf expression via ras-ERK1/2-iNOS signaling.

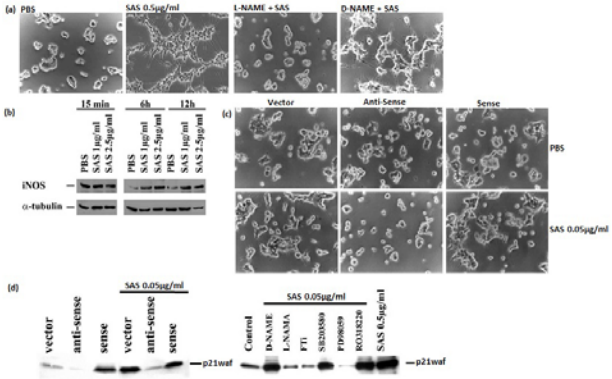


Figure 3: iNOS and p21waf are obligated in SAS ability to induce PC12 differentiation. (a) SAS or SAS in combination of L-NAME (active inhibitor of NOS) or D-NAME (inactive inhibitor of NOS) inhibitors were applied on PC12 cells and incubated for 48h. Inhibitors were applied 2h before SAS addition. At the end incubation time cells were observed in a phase contrast microscope (b) SAS induces iNOS expression. Blot represents data from one of three performed experiments. (c) p21waf involvement in SAS ability to induce PC12 differentiation. Transfected PC12 cells with stable vectors were treated SAS. At the end of incubation time cells were observed in a phase contrast microscope . Cells were collected, lysed and Western blot analysis for detection of p21waf expression was performed (bottom panel). Each assay carried out three times. (d) p21waf expression in PC12 cells treated with SAS and different inhibitors for 24h. Treated cell with SAS 0.5μg/ml alone or in combination of inhibitors [farnesyl transferase inhibitor (Fti, ras inhibitor), geldanamycin (GA, inhibitor of c-raf-1), PD98059 (MEK inhibitor), SB203580 (p38 inhibitor), Ro318220 (aPKC inhibitor)] were incubated, the cells were collected, lysed and Western blot analysis for p21waf expression was done.

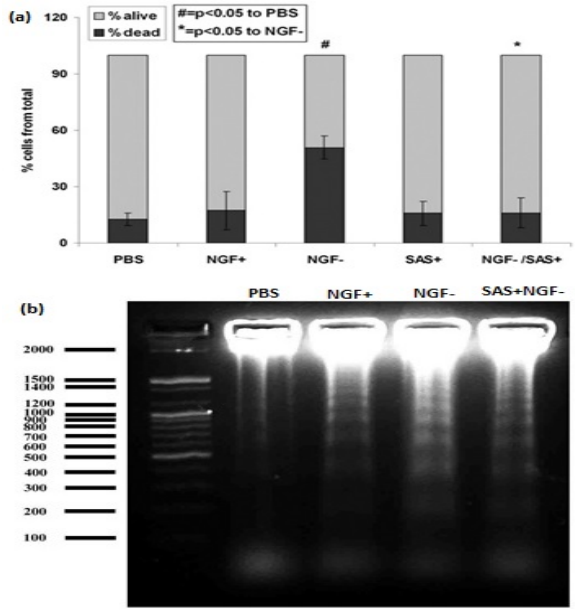


Figure 4: (a) PC12 cells were treated with NGF and incubated in growth factor starvation. Additionally, plates with PC12 cells were treated with NGF (samples were called NGF+) or SAS (samples were called SAS+) or PBS (samples were called PBS). At the end of incubation time hovered and attached cells were collected and stained with trypan blue dye (b) SAS reduces intensity of DNA ladder in NGF deprived PC12 cells. Cells were treated as described above, at the end of incubation time cells were collected and DNA was purified as described.

SAS decreases amount of dead NGF-differentiated PC12 cells in neurotrophic deprivation system – Based on the ability of SAS to activate the p21ras-ERK1/2 pathway, to activate Akt1/2, to up-regulate p21waf and to elevate expression of iNOS, all of those have been known to promote the survival signals in ongoing neuron cells [9], we analyzed SAS ability to prevent cell death of differentiated PC12 cells following withdrawal of trophic support. As shown in Figure 4 (a), basal amount of dead scored NGF-differentiated PC12 cells are 15% after 120h of incubation time (sample called 'NGF+') and 10% in undifferentiated cells (sample called 'PBS'). NGF depravation from NGF-differentiated PC12 cells for 2 days increases the amount of dead cells to 50% (sample called 'NGF-'), whereas addition of SAS at 0.5μg/ml to depraved cells decreases percent of dead cells to basal level (sample called 'NGF-/SAS+'). Additionally intensity of DNA ladder in depraved neuronal cells twice lowers then treated by SAS (Fig. 4 (b)).

III. DISCUSSION

In the present work we investigated the properties of the novel tellurium compound, SAS, in neuronal differentiation and prevention of cell death. The SAS mechanism of action is through p21ras protein, which active cascades induce iNOS and p21waf expression. Importantly activation of crutial cascades implicates on reduction neuronal cell death.

Neurodegeneration diseases are characterized by neural abnormalities that result largely from progressive loss of neurons in different parts of brain. The neurons damaged areas appear to be in pre-apoptotic state for an extended period of time prior to being irreversibly damaged. Thus, both the anti apoptotic and neurotrophic agents are suitable candidates to prevent delayed cell death and/or restore neural function.

Available treatment for neurodegenerative diseases is mainly symptomatic instead of halting or reversing degenerative processes affecting the disease. New methods being proposed to treat degenerative disorders of the central nervous system include cell implantation and trophic-factor strategies. Some works show direct tissue transplantation whereas others use stem cells to create tissue that will successfully function as neurons after implantation into the brain. In order to accomplish this, the right combination of growth factors and cell culture conditions must be found that will bring undifferentiated cells in a culture dish to a point where they are committed to becoming neurons [10] Also, neurotrophic compounds or compounds that trigger neurotrophic factors may prove to be beneficial in a variety of neurodegenerative disorders by preventing neural death and may also substantially promote the

survival and function of neuronal grafts used for the treatment of these conditions.

Indeed, stem cell implantation methodology is more advanced but expansive and very complicated. Most clinically used drugs do not elevate neurotropic factors [11]. And, the trophic-factor approach which is currently recommended has problems with simple and practical means of delivery to the brain in a safe and long-term manner, limiting the undesirable adverse effects [12].

Here we focused on ability of novel small tellurium compound to induce neuronal differentiation to PC12 cells. SAS is non toxic compound and induces outgrowth of neurites in PC12, which is a fundamental morphological change associated with neural development and differentiation [13][14], in a broad concentration range (Fig. 1), which contributes superior potential for SAS in clinical research. SAS-differentiated PC12 expresses neuronal markers such as neurofilament M. The ability tellurium compound to induce PC12 transformation involves p21ras activity (Fig. 2b). Another tellurium compound, AS101, had been shown to differentiate PC12 cells through cys118 of p21ras [15]. Thiol formation on a critical cysteine is apparently responsible for p21ras activation. In fact, others have found that redox agents modulate binding of GTP to p21ras immunoprecipitates. Because Cys-118 resides on a loop that has contact with the guanine nucleotide, it has been suggested that modification of this cysteine may directly affect the bound GTP/GDP ratio of p21ras, thus changing its activity, as occurring following S-nitrosylation by nitric oxide [3]. SAS, like AS101 has unique mechanism of action, which mediated by tellurium ability to regulate the redox status of cysteines by thiol formation [16][17][18][19]. Consuming that SAS may activate ras directly through cys118. Moreover, SAS induces nitric oxide productions which in back may contribute to strengthening the ras activation.

Ras actives c-raf-1, which transmits the signal to MEK1/2. MEK1/2 activates ERK1/2 via phosphorylation of a –Thr-Glu-Tyr- motif in the activation loop [20] Inhibition of c-raf-1 or MEK1/2 activation abrogates SAS-induced PC12 differentiation. Other cascade activated by ras is PI3K-Akt1/2, and not aPKC, is also involved in SAS ability to induce PC12 differentiation, since addition of PI3K inhibitor delete SAS-induced differentiation (Fig. 2c) and Akt1/2 phosphorylation (data not shown).

NO in cell is produced by nitric oxide synthase (NOS) [21]. It has been postulated that NOS levels are induced during differentiation of PC12 cells by NGF. The pathway by which NO assists in PC12 differentiation seems to be vary. It was already reported that NO triggers p21ras activation and recruitment of its effectors, c-raf-1 and PI3K [22], and that NO enhances neurite outgrowth through a cGMP [23]. Inhibition of NOS by the active inhibitor L-NAME, abolishes the ability of SAS to induce PC12 differentiation, which includes neurite outgrowth (Fig. 3) and the expression of neurofilament M (data not shown). The amount of secreted NO was not detectable in SAS induced differentiated PC12 cells at 48h. Consuming that SAS-stimulated PC12 may produce low endogenous amounts of NO.

The production of NO in response to NGF serves as a signal for growth arrest [24]. Blocking NO production prevents arrest of the cell cycle arrest and thereby prevents differentiation of PC12 cells. Here we show that SAS induces cyclin-dependent kinase, p21waf, production after 24 hours. Both cascades p21ras/ERK1/2 and p21ras/PI3K are involved in SAS-induced p21waf production (Fig. 4d). Additionally, elimination of mRNA levels of p21waf inhibits SAS induced differentiation of PC12 cells (Fig. 4c). EGF is well known to induce PC12 proliferation like NGF differentiation. Also it was postulated that EGF activate transient ERK1/2, whereas for differentiation with NGF ERK should be activated twice [25]. SAS induces double ERK phosphorylation, thus SAS may mimic NGF signal. Moreover, we may suggest that the second ERK1/2 activation induced by SAS may be due to NO production in late stimulation time. Thus, NO, at physiological levels, may act as a second messenger, following stimulation of PC12 cells by SAS, to enhance expression of p21waf.

NGF withdrawal from postmitotic PC12 cells led to sustained activation of the JNK and p38 enzymes and to inhibition of ERKs [26]. The dynamic balance between the NGF-activated ERK and stress-activated JNK-p38 pathways may be important in determining whether a cell survives or undergoes apoptosis. Inhibition MAP kinase, p38, is not deleted SAS ability to induce PC12 differentiation, and even more, SAS reduces p-p38 levels in PC12 cells. Moreover, SAS may promote the p21ras-ERK1/2 and p21ras-PI3K cascades activation, and iNOS and p21waf expression, thus trigger survival signaling. Furthermore, SAS reduces number of dead cells in ongoing PC12 cells in neurotropic support withdrawal model.

The growing recognition of the role of apoptotic pathways in various human pathologic states indicates the need of identifying their targeting therapeutic potential by finding effective means of controlling their activation. The challenge is to find alternative and new approaches to supersede existing strategies of cell death inactivation. Development of non-toxic small compounds, like SAS, with potential activate

survival signals and induce neuronal stem cells differentiation, represents a new class of multipotent neuroprotective strategies in neurodegenerative disorders.

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