Inhibition of Human Neuroblastoma Cell Proliferation by N-acetyl-L-cysteine as a Result of Increased Sulfane Sulfur Level

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Abstract. *Background/Aim: In various cancer cells, the level of sulfane sulfur-containing compounds is decreased compared to normal cells. In the present study the effect of N-acetyl-L-cysteine (NAC), which acts as a precursor of H_2S synthesis, on the human neuroblastoma SH-SY5Y cell proliferation, the activity of 3-mercaptopyruvate sulfurtransferase (MPST), and the level of sulfane sulfur were investigated. Materials and Methods: SH-SY5Y cells were treated with NAC, while untreated cells were used as the control. The toxicity of NAC on the cells was studied by the LDH cytotoxicity assay; cell proliferation was examined by the MTT method. MPST activity and sulfane sulfur level were also analyzed in the NAC-treated cells. Results: The addition of NAC to the medium, in non-cytotoxic concentrations, resulted in inhibition of the SH-SY5Y cell proliferation after 48 h of culture. The MPST activity and the level of sulfane sulfur-containing compounds were also elevated under the same culture conditions. Conclusion: The antiproliferative activity of NAC in the SH-SY5Y cells was associated with an increase in the MPST activity and consequently with an increase in the intracellular level of sulfane sulfur in these cells.*

In mammalian cells, sulfur exists under physiological conditions in various forms, including free sulfide (H_2S, HS^- or S^{2-}), acid labile sulfide (in the form of iron–sulfur complexes), and bound sulfane sulfur (such as persulfides, polysulfides, thiosulfate, polythionates, thiosulfonates, elemental sulfur) (1).

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According to Toohey *et al*. (2), tumors with inherently high growth rates may have lower activity of the sulfurtransferases, the enzymes participate in H_2S and bound sulfane sulfur production, than normal tissue or tumors with low growth rate. A recent study by Rajpal *et al*. (1) showed that the levels of H_2S and bound sulfane sulfur are also reduced in cardiovascular diseases. H_2S is known to be able to exert both pro- or anti-proliferative effects, even in the same tumor cells, depending on the type (rapid- or slow-releasing H_2S donors) and concentrations of the tested compounds; further studies are needed to clarify these effects (3). Our previous research demonstrated that increasing the level of sulfane sulfur in human astrocytoma U373 cells *via* the administration of L-cysteine precursors, such as N-acetyl-L-cysteine (NAC) (4) and ribose-cysteine (5), had an inhibiting effect on their proliferation.

NAC performs various biological functions: it scavenges free radicals, reduces protein disulfides, and acts as a precursor of glutathione and H_2S synthesis (3, 6, 7). NAC is commonly used as a precursor of L-cysteine, but the molecular mechanisms of NAC antioxidative activity are still uncertain (8). Ezerina *et al*. (8) have recently suggested that sulfane sulfur species in the mitochondria are the key mediators of the antioxidative and cytoprotective effects provided by NAC.

Three enzymes are involved in the H_2S production (cystathionine β-synthase - CBS, γ-cystathionase - CTH, 3-mercaptopyruvate sulfurtransferase - MPST) (9, 10), but only one of them - MPST - is present in both the cytoplasm and mitochondria (11). CBS and CTH are exclusively cytoplasmic enzymes (12). MPST produces H_2S from 3-mercaptopyruvate, which is generated from L-cysteine and α-ketoglutarate by cysteine aminotransferase in the presence of the thioredoxin and dihydrolipoic acid (13). MPST, with redox-sensitive thiol groups in the catalytic center, serves as a local antioxidant protein (11), is involved in L-cysteine catabolism, cyanide detoxification (10), cellular bioenergetics (14), and supplies sulfur from persulfide to thiolation of tRNA (12). Kimura *et al*. (12) have shown that

in the brain, MPST (but not CBS) produces persulfurated species involved in redox homeostasis. The levels of bound sulfane sulfur in MPST-knockout brains were lower than approximately 50% in wild-type brains, but in CBS-knockout they did not differ statistically (12).

In the human neuroblastoma SH-SY5Y cells, the specific activity of MPST is higher than the activity of other sulfurtransferases (15), and the level of L-cysteine is negligible (16). Therefore, in the present study, the SH-SY5Y cells were treated with NAC to determine whether the activity of MPST and the production of sulfate sulfur would be altered, and moreover, how it would affect the proliferation of these cells. With respect to MPST, the Km for 3-mercaptopyruvate is over 7 mM, and the Km of cysteine aminotransferase for L-cysteine is 22 mM (both concentrations are far higher than the ambient levels of free L-cysteine in cells) (7). Therefore, it was hypothesized that increasing cellular L-cysteine levels should increase the generation of H_2S and bound sulfane sulfur by MPST in the NAC-treated SH-SY5Y cells.

Materials and Methods

Sources of chemicals. N-acetyl-L-cysteine, albumin, N-ethylmaleimide, lactate dehydrogenase (LDH), Folin–Ciocialteau reagent, NADH, and 1,4-dithio-bis-(2-nitrobenzoic acid) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Potassium cyanide was obtained from Merck (Darmstadt, Germany), and sodium 3-mercaptopyruvate from Flucka Chemie GmbH (Buchs, Switzerland). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin, and penicillin/streptomycin were obtained from HyClone Laboratories (Logan, UT, USA). The Cytotoxicity Detection Kit (LDH) was obtained from Roche Applied Science (Penzberg, Germany). All the other chemicals were of reagent grade and purchased from common commercial suppliers.

Cell culture. The human neuroblastoma cell line SH-SY5Y was purchased from ECACC (Salisbury, UK) and cultivated in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, in T75 flasks at a seeding density of $2×10^6$ cells per flask at 37°C in a humidified atmosphere with 5% CO_2. The cells were passaged every 2-3 days after reaching 80-90% confluence and were then used for subsequent experimentation.

Cell homogenization. The SH-SY5Y cells were suspended in 0.1 M phosphate buffer pH 7.5 ($1×10^6$ cells/0.04 ml of the buffer), and sonicated 3×5 sec at 4°C (Bandelin Sonoplus GM 70). After centrifugation at 4,500 g at 4°C for 10 min, the supernatant was used for the determination of protein concentration, the activity of MPST, and levels of sulfane sulfur.

Cytotoxicity LDH assay. The effects of NAC on membrane permeability in the SH-SY5Y cell line were determined using the Cytotoxicity Detection Kit. Cells were treated with various concentrations of NAC (0.25, 0.5, 1.0, 1.5, 2.0, and 5 mM) for 48 h, while cells incubated without NAC served as negative controls. Triton X-100-treated cells (1% Triton X-100) that give the

maximum loss of lactate dehydrogenase (LDH) were used as the positive control and assumed to be 100% dead. The release of LDH from the cell into the culture supernatant was quantified according to the manufacturer's instructions as described previously (5). The absorbance of the samples was measured at 490/690 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA).

Cell proliferation. Cell proliferation was assessed using the MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide] (Sigma-Aldrich Corp.) method. First, $1.5×10^3$ cells/well were seeded in a 96-multiwell dish and allowed to attach for 24 h. Subsequently, the culture medium was removed and the cells were treated for 48 h with various concentrations of NAC as described above. Subsequently, MTT (5 mg/ml) was added to each well and incubated for 4 h in a humidified atmosphere at 37°C with 5% CO_2. The resulting formazan crystals were dissolved with 1 ml of acidified isopropanol (0.05 N HCl in absolute isopropanol), and the absorbance of the obtained solution was measured at 570 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments Inc).

Enzyme activity assay. The MPST activity was assayed according to the method of Valentine and Frankelfeld (17), with some modifications as described by Wróbel *et al.* (18). The enzyme activity was expressed as nmoles of pyruvate produced during 1 min incubation at 37°C per 1 mg of protein.

Determination of sulfane sulfur and protein levels. Sulfane sulfur was determined by the method of Wood (19), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion, and protein was determined by the method of Lowry *et al.* (20) using crystalline bovine serum albumin as a standard.

Statistical analysis. The results were expressed as the means±standard deviation (SD). All the experiments were repeated at least three times. Statistical analyses were performed using the Student's *t*-test. All *p*-values <0.05 were considered to indicate a statistically significant difference.

Results

Evaluation of NAC cytotoxicity on the SH-SY5Y cells. The cytotoxic effect of NAC (0.25, 0.5, 1.0, 1.5, 2.0, and 5 mM) on the SH-SY5Y cells was studied using LDH assay. The SH-SY5Y cells released LDH in a concentration-dependent manner. The cytotoxicity of NAC at 0.25-2 mM concentrations was less than 5%, whereas, at 5 mM, it was elevated to 31% (Figure 1). Therefore, for further analysis, the SH-SY5Y cells were treated with 1.0, 1.5, and 2 mM NAC.

Effect of NAC on the SH-SY5Y cell proliferation. As shown in Figure 2, NAC treatment significantly inhibited the proliferation of the SH-SY5Y cells in a dose-dependent manner. Specifically, the proliferation of the SH-SY5Y cells was significantly decreased by 12% and 16% at 1.5 mM and 2 mM NAC, respectively, after 48 h of treatment.
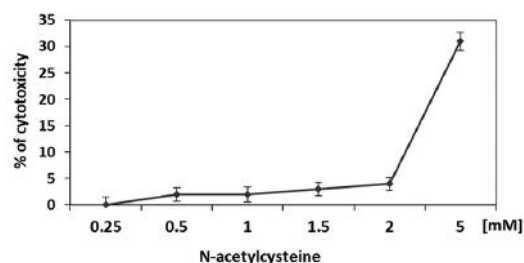
Figure 1. *Effect of N-acetyl-L-cysteine (NAC) on the lactate dehydrogenase (LDH) release by SH-SY5Y cells. The SH-SY5Y cells were incubated for 48 h with various concentrations of NAC. The results are presented as the mean±SD of three independent experiments performed in five replications.*
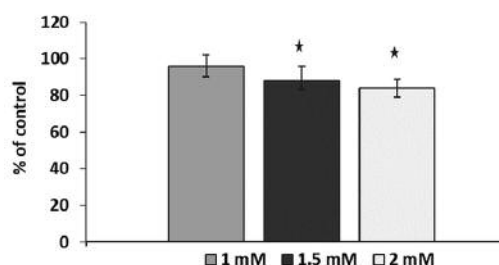

Figure 2. *Proliferation of N-acetyl-L-cysteine-treated SH-SY5Y cells. The cells were incubated with NAC for 48 h and cell proliferation was examined using MTT method. The results are expressed as the mean±SD of three independent experiments performed in seven replications. *p<0.05 vs. control.*

Figure 3. *Activity of 3-mercaptopyruvate sulfurtransferase (MPST) and sulfane sulfur level in N-acetyl-L-cysteine (NAC)-treated SH-SY5Y cells. The cells were treated with various concentrations of NAC (1.5 mM, 2.0 mM) for 48 h. The activity of MPST determined in the control SH-SY5Y cells equaled 745 ± 83 nmole x mg protein^{-1} x min^{-1}. The intracellular level of sulfane sulfur determined in the control SH-SY5Y cells equaled 107 ± 11 nmole x mg protein^{-1}. The results are expressed as the mean±SD of three independent experiments; *p<0.05 vs. control.*

Figure 4. *The possible mechanism of inhibition of human SH-SY5Y cell proliferation by N-acetyl-L-cysteine (NAC). MPST, 3-mercaptopyruvate sulfurtransferase.*

Effect of NAC on MPST activity and sulfane sulfur level in the SH-SY5Y cells. The results presented in Figure 3 show changes in the activity of MPST and the sulfane sulfur level in SH-SY5Y cells in the presence of NAC. A significantly increased activity of MPST as well as sulfane sulfur level was observed in SH-SY5Y cells cultured for 48 h with NAC as compared to the control cells. For 1.5 and 2 mM NAC, the MPST activity was increased by about 24% and 25%, respectively. It was associated with an increase in the level of sulfane sulfur in these cells by about 56% and 51% (for 1.5 and 2 mM NAC, respectively) (Figure 3).

Discussion

The cellular oxidative stress is associated with many diseases, such as neoplastic diseases (21). Thus, the understanding of the mechanisms leading to reduction of reactive oxygen species level might be helpful in cancer treatment.

The human neuroblastoma SH-SY5Y cells have a low metabolic rate and can adapt their metabolism to available substrates (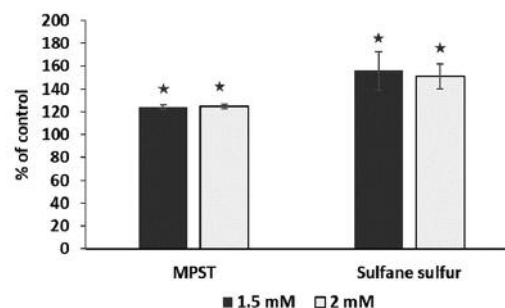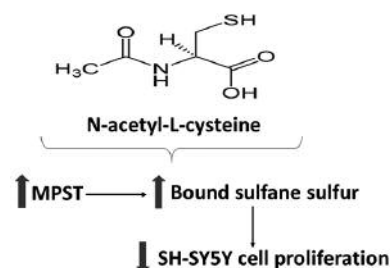22, 23). Our previous study (16) showed that the level of L-cysteine (a precursor of both reduced glutathione and bound sulfane sulfur) in the SH-SY5Y cells was undetectable. L-cysteine administered as a pure chemical is more reactive, tending to oxidize spontaneously to L-cystine, is less bioavailable and more prone to evoking side effects than NAC, which is deacetylated in cells (7). Therefore, we added NAC to the SH-SY5Y cells.

In this study we showed that NAC administered to the SH-SY5Y cells in non-cytotoxic concentrations – 1.0, 1.5, and 2 mM [cytotoxicity lower than 5% (Figure 1)] affects the inhibition of their proliferation (Figure 2). De Miranda Ramos *et al*. (24) have also demonstrated that NAC significantly decreased the proliferation of the control SH-SY5Y cells after 72 h of culture, but the concentrations of NAC were much higher (2.5, 5, 10 mM) than those used in the present study. In our results, 5 mM NAC had the highest cytotoxic effects on the SH-SY5Y cells (about 30% cytotoxicity) during 48 h of culture (Figure 1).

NAC, which reduces oxidative stress, has been extensively studied as an anticancer agent. Previous studies have shown that NAC reduces the proliferation of cancer cells, such as astrocytoma (4), ovarian (25, 26), breast (25, 27), prostate (28), bladder (29) and endometrial adenocarcinoma (26).

However, the molecular mechanisms of NAC antioxidative activity are not fully understood. Recently, Ezerina *et al*. (8) showed that in human lung acenocarcinoma cells, NAC-derived L-cysteine is desulfurated to generate H_2S, which in turn is oxidized to sulfane sulfur species, predominantly within the mitochondria. This confirms our results that the inhibition of the SH-SY5Y cell proliferation (Figure 2) is closely related to the increase in sulfane sulfur level in neuroblastoma cells (Figure 3). Furthermore, Faten's latest research showed (30) that proliferation of human carcinoma cell lines (liver HepG2, colon HCT116, breast MCF-7, prostatic PC3) was significantly inhibited by sulfur nanoparticles. Thus, all these studies confirm that sulfane sulfur can play an important role in the proliferation of cancer cells.

Due to the fact that sulfane sulfur levels were increased in the NAC-treated SH-SY5Y cells, we also examined the activity of MPST, a cytosolic and mitochondrial enzyme involved in the formation of sulfane sulfur-containing compounds (Figure 4). We showed that NAC clearly affects the increase in MPST activity in the SH-SY5Y cells (Figure 3). Tain *et al*. (6) reported that NAC therapy increased MPST protein level in rat kidneys, which was associated with H_2S production. The concentration of intracellular L-cysteine is much higher in the mitochondria, as compared to the cytoplasm (31), which suggest that most of the bound sulfane sulfur generated by MPST in the SH-SY5Y cells occurs in mitochondria.

Conclusion

It has been previously demonstrated that increasing the level of sulfane sulfur in human astrocytoma U373 cells *via* administration of L-cysteine precursors, such as NAC, had an inhibiting effect on their proliferation (4). Herein, the use of NAC in human neuroblastoma SH-SY5Y cells, which are known to have negligible intracellular L-cysteine levels, led to increased activity of the cytoplasmic and mitochondrial enzyme MPST that produces sulfane sulfur. The intracellular sulfane sulfur level, which is decreased in various cancer cells compared to normal cells, was increased as well and cell proliferation was inhibited. Thus, the antioxidant properties of NAC in the SH-SY5Y cells seem to be associated with an increase in the pool of sulfane sulfur-containing compounds suggesting that the elevation of bound sulfane sufur levels in cancer cells may be of therapeutic significance.

References

1 Rajpal S, Katikaneni P, Deshotels M, Pardue S, Glawe J, Shen X, Akkus N, Modi K, Bhandari R, Dominic P, Reddy P, Kolluru GK and Kevil CG: Total sulfane sulfur bioavailability reflects ethnic and gender disparities in cardiovascular disease. Redox Biol *15*: 480-489, 2018.

2 Toohey JI: Sulphane sulphur in biological systems: a possible regulatory role. Biochem J *264*: 625-632, 1989.

3 Yang CT, Chen L, Xu S, Day JJ, Li X and Xian M: Recent development of hydrogen sulfide releasing/stimulating reagents and their potential applications in cancer and glycometabolic disorders. Front Pharmacol 8: 664, 2017.

4 Jurkowska H and Wróbel M: N-acetyl-L-cysteine as a source of sulfane sulfur in astrocytoma and astrocyte cultures: correlations with cell proliferation. Amino Acids *34*: 231-237, 2008.

5 Jurkowska H, Uchacz T, Roberts J and Wróbel M: Potential therapeutic advantage of ribose-cysteine in the inhibition of astrocytoma cell proliferation. Amino Acids *41*: 131-139, 2011.

6 Tain YL, Hsu CN, Lee CT, Lin YJ and Tsai CC: N-Acetylcysteine prevents programmed hypertension in male rat offspring born to suramin-treated mothers. Biol Reprod 95: 1-8, 2016.

7 DiNicolantonio JJ, OKeefe JH and McCarty MF: Boosting endogenous production of vasoprotective hydrogen sulfide *via* supplementation with taurine and N-acetylcysteine: a novel way to promote cardiovascular health. Open Heart 4: e000600, 2017.

8 Ezeriņa D, Takano Y, Hanaoka K, Urano Y and Dick TP: N-acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H_2S and sulfane sulfur production. Cell Chem Biol *25*: 447-459.e4, 2018.

9 Filipovic MR, Zivanovic J, Alvarez B and Banerjee R: Chemical biology of H_2S signaling through persulfidation. Chem Rev *118*: 1253-1337, 2018.

10 Rose P, Moore PK and Zhu YZ: H_2S biosynthesis and catabolism: new insights from molecular studies. Cell Mol Life Sci *74*: 1391-1412, 2017.

11 Nagahara N: Multiple role of 3-mercaptopyruvate sulfurtransferase: antioxidative function, H_2S and polysulfide production and possible SO_x production. Br J Pharmacol *175*: 577-589, 2018.

12 Kimura Y, Koike S, Shibuya N, Lefer D, Ogasawara Y and Kimura H: 3-Mercaptopyruvate sulfurtransferase produces potential redox regulators cysteine- and glutathione-persulfide (Cys-SSH and GSSH) together with signaling molecules H_2S_2, H_2S_3 and H_2S. Sci Rep 7: 10459, 2017.

13 Shibuya N, Tanaka M, Yoshida M, Ogasawara Y, Togawa T, Ishii K and Kimura H: 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain. Antioxid Redox Signal *11*: 703-714, 2009.

14 Modis K, Coletta C, Erdelyi K, Papapetropoulos A and Szabo C: Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransfrase maintains mitochondrial electron flow and supports cellular bioenergetics. FASEB J *27*: 601-611, 2013.

15 Jurkowska H, Placha W, Nagahara N and Wróbel M: The expression and activity of cystathionine-γ-lyase and 3-mercaptopyruvate sulfurtransferase in human neoplastic cell lines. Amino Acids *41*: 151-158, 2011.

16 Jurkowska H, Wróbel M, Kaczor-Kamińska M and Jasek-Gajda E: A possible mechanism of inhibition of U87MG and SH-SY5Y cancer cell proliferation by diallyl trisulfide and other aspects of its activity. Amino Acids *49*: 1855-1866, 2017.

17 Valentine WN and Frankenfeld JK: 3-Mercaptopyuruvate sulfurtransferase (EC 2.8.1.2): A simple assay adapted to human blood cells. Clin Chim Acta *51*: 205-210, 1974.

18 Wróbel M, Jurkowska H, Sliwa L and Srebro Z: Sulfurtransferases and cyanide detoxification in mouse liver, kidney, and brain. Toxicol Mech Methods *14*: 331-337, 2004.

19 Wood L: Sulfane sulfur. Methods Enzymol *143*: 25-29, 1987.

20 Lowry OH, Rosebrough NJ, Farr L and Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem *193*: 265-275, 1951.

21 Carini F, Mazzola M, Rappa F, Jurjus A, Geagea AG, Al Kattar S, Bou-Assi T, Jurjus R, Damiani P, Leone A and Tomasello G: Colorectal carcinogenesis: Role of oxidative stress and antioxidants. Anticancer Res *37*: 4759-4766, 2017.

22 Xun Z, Lee DY, Lim J, Canaria CA, Barnebey A, Yanonne SM and McMurray CT: Retinoic acid-induced differentiation increases the rate of oxygen consumption and enhances the spare respiratory capacity of mitochondria in SH-SY5Y cells. Mech Ageing Dev *133*: 176-85, 2012.

23 Kim J, Han J, Jang Y, Kim SJ, Lee MJ, Ryu MJ, Kweon GR and Heo JY: High-capacity glycolytic and mitochondrial oxidative metabolisms mediate the growth ability of glioblastoma. Int J Oncol *47*: 1009-1016, 2015.

24 de Miranda Ramos V, Zanotto-Filho A, de Bittencourt Pasquali MA, Klafke K, Gasparotto J, Dunkley P, Gelain DP and Moreira JCF: NRF2 mediates neuroblastoma proliferation and resistance to retinoic acid cytotoxicity in a model of *in vitro* neuronal differentiation. Mol Neurobiol *53*: 6124-6135, 2016.

25 Zhang X, Wang YN, Zhu JJ, Liu XX, You H, Gong MY, Zou M, Cheng WH and Zhu JH: N-acetylcysteine negatively regulates Notch3 and its malignant signaling. Oncotarget *7*: 30855-30866, 2016.

26 Kapur A, Felder M, Fass L, Kaur J, Czarnecki A, Rathi K, Zeng S, Osowski KK, Howell C, Xiong MP, Whelan RJ and Patankar MS: Modulation of oxidative stress and subsequent induction of apoptosis and endoplasmic reticulum stress allows citral to decrease cancer cell proliferation. Sci Rep *6*: 27530, 2016.

27 Monti D, Sotgia F, Whitaker-Menezes D, Tuluc M, Birbe R, Berger A, Lazar M, Cotzia P, Draganova-Tacheva R, Lin Z, Domingo-Vidal M, Newberg A, Lisanti MP and Martinez-Outschoorn U: Pilot study demonstrating metabolic and anti-proliferative effects of *in vivo* anti-oxidant supplementation with N-acetylcysteine in breast cancer. Semin Oncol *44*: 226-232, 2017.

28 Supabphol A and Supabphol R: Antimetastatic potential of N-acetylcysteine on human prostate cancer cells. J Med Assoc Thai *12*: S56-S62, 2012.

29 Kawakami S, Kageyama Y, Fujii Y, Kihara K and Oshima H: Inhibitory effect of N-acetylcysteine on invasion and MMP-9 production of T24 human bladder cancer cells. Anticancer Res *21*: 213-219, 2001.

30 Faten Z, Mustafa H and Muayad ALD: Synthesis of nano sulfur particles and their antitumor activity. J Microb Biochem Technol *10*: 59-68, 2018.

31 Mitidieri E, Tramontano T, Gurgone D, Citi V, Calderone V, Brancaleone V, Katsouda A, Nagahara N, Papapetropoulos A, Cirino G, d'Emmanuele di Villa Bianca R and Sorrentino R: Mercaptopyruvate acts as endogenous vasodilator independently of 3-mercaptopyruvate sulfurtransferase activity. Nitric Oxide *75*: 53-59, 2018.