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Interference of Arsenic Trioxide on Magnesium Dependent Polymerization of Microtubule Proteins

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Prevention of microtubule polymerization is considered as one of the promising approaches towards inhibition of cell proliferation, especially in treatment of malignancies. Arsenic trioxide, As_2O_3, is being successfully used in the treatment of human lymphoma, while the mechanism of its therapeutic function is still under investigation. Experiments were designed to determine if indeed As_2O_3 interferes with polymerization of nanotube microtubule. Microtubules were extracted from sheep brain and their interaction with arsenic trioxide was examined by spectrometery. Electrical conductometry of 2 mM $MgSO_4$ solution containing various concentrations of As_2O_3 was studied in order to determine their possible interaction. Transmission electron microscopy was used to show microtubule structure in the presence of arsenic trioxide. Fluorometric characteristics of tubulin dimer were examined in presence of varying concentrations of arsenic trioxide. It is concluded that arsenic trioxide interacts with Mg^{2+} ion around GTPase site of β-tubulin, resulting enhancement of depolymerization of the microtubule polymer.

Keywords: Microtubule polymerization, Arsenic trioxide, Anticancer drug, Magnesium ion

INTRODUCTION

Microtubules in physiological condition exist in two forms, tubulin dimer and polymer formed from tubulin subunits with dimensions 25 nm diameter and their length can vary from tens of nanometers to hundreds of microns [1]. The nucleating proteins including gamma tubulin participate in the nucleation process of tubulin polymerization [2]. Multi functional behavior of microtubule proteins has attracted a great deal of attention among researchers in the last decades. Tubulins are shown to participate in several vital processes in different cells such as mitosis [3,4], signal transduction [5], molecular transportation especially in nerve cells [6], and mediation of regulation [7] in addition to their function as cytoskeleton proteins [3,8]. Because of the importance of tubulin in cell proliferation in diseases such as cancer [9], an extensive amount of research has been dedicated towards the control of its polymerization.

To this end, a wide variety of chemicals have been examined for inhibition of tubulin polymerization. Cholchicine and vinblastine and small molecules such as nitrogen mustard have been widely used as anticancer. However, all these anticancer agents bind not only to tubulin but interrupt many important biological processes which depend on microtubule functions. Newly extracted compounds have been reported effective on taxol resistant tumor cell lines

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[10].

Small-molecules which bind to tubulin but do not interfere with transmembrane pumps or with the function of microtubules in neural axons are preferred in clinical practices [11]. In this regard, it is quite interesting to note that arsenic trioxide treatment has been demonstrated effective in many clinical trails involving human lymphoma [12]. Arsenic trioxide has an old history in oriental medicine prescriptions for cancer therapy [13]. As_2O_3 is a toxin which binds to sulfhydril groups of cysteine containing proteins, such as mitochondrial enzymes; succinic dehydrogenase [14]. There are several evidences suggesting that arsenic trioxide binds to microtubule proteins. In spite of the fact that arsenic trioxide is a small and simple molecule, the mechanism of its interaction with tubulin has not yet been fully resolved [11,15]. Apparently, its complex behavior is due to the dynamic behavior of the tubulin polymerization. Polymerization of tubulin occurs in three distinct steps involving tubulin dimers, nucleating proteins, kinesin and the tau protein. Microtubule associated proteins play an important role in enforcing microtubule polymers stability and are thus very effective in inhibition of microtubule polymerization [16]. It was assumed that As_2O_3 may interfere with microtubule polymerization, therefore, experiments were designed to determine its effectiveness on tubulin dimers and microtubule associated proteins.

EXPERIMENTAL

Materials

Arsenic trioxide and PIPES [Piperazine-1,4-bis(2-ethanesulphonic acid)] were purchased from Merck, Germany and GTP (Guanosine-5'-triphosphate), ATP (Adenosine-5'-triphosphate), EGTA [Ethylene bis(oxyethylenenitrilo) tetraacetic acid] and Magnesium Sulfate from Sigma, USA, and phosphocellulose p11, from Whatman, UK.

Equipments

Turbidometry was conducted by UV-Vis spectrophotometer, on a Varian, Cary Bio100, Australia. Conformational changes were monitored by fluorescence spectrophotometery, using a Varian, model FP-6200, Australia. Mg^{2+} interaction with arsenic trioxide was assessed by conductometery, Metrohm model 644, Switzerland. Electron micrographs were taken by a transmission electron microscope, model 902A, Zeiss, Germany.

Purification of Microtubule Protein from Sheep Brain

Sheep brain microtubule protein was purified by a two cycle assembly/disassembly using PEM buffer (containing 100 mM PIPES, pH 6.9, 2 mM $MgSO_4$ and 1 mM EGTA) and GDP 0.2 mM (17). The cold homogenized centrifuge pellet was frozen as drops in liquid nitrogen and stored at -70 °C. Tubulin concentration was 2.5 mg ml^{-1}.

Tubulin Dimer Purification by Phosphocellulose Column

Tubulin dimer was separated from microtubule associated proteins (MAPs) *via* chromatography on a 15 cm × 1.7 cm phosphocellulose column [18]. Fractions were collected on a fraction collector every 2 min at 4 °C.

Tubulin Assembly

Tubulin concentration was determined by Bradford method at 595 nm [19] and at 280 nm [20] *via* UV-Vis spectrophotometer. Tubulin assembly was performed in PEM buffer (pH 6.9) and PMG buffer (containing 80 mM PIPES, pH 6.9, 1 mM EGTA, 2 mM $MgSO_4$ and 3.4 M glycerin). Tubulin assembly was started by adding GTP in appropriate concentrations at 37 °C and 25 °C and measuring the turbidity at 350 nm for 30 min. Following the increase in turbidity, optical density was measured at 350 nm with a spectrophotometer at 37 °C for 30 min.

Tubulin was polymerized in the presence of various concentrations of As_2O_3 (20 mM aqueous stock solution) for 30 min at 25 °C and 37 °C.

To study the effect of arsenic trioxide on microtubule polymerization at different concentrations, arsenic trioxide was added to the tubulin solution at constant concentration of GTP. To investigate if arsenic trioxide would interfere with GTP activity in polymerization process, various concentrations of GTP were added to protein samples in the presence of constant concentration of As_2O_3 (2.5 mM) at 37 °C.

To examine if the tubulin polymer interaction with arsenic

trioxide blocks the GTPase activity, polymerization was allowed to progress up to the maximum of elongation phase and negligible amounts of arsenic trioxide was then added to the solution so as not to change the temperature or the concentration of the tubulin solution.

Dialysis

In order to omit GTP from tubulin solution and study the As_2O_3-tubulin interaction in the absence of GTP interference, microtubule protein was dialysed (cutoff 10 kDa) at 4 °C for 4 h in PEM buffer.

Spectrofluorimetry

Fluorimetric characteristic of tubulin dimer at 0.5 mM As_2O_3 was investigated by Varian, FP-6200 spectrofluorimeter. The measurement was conducted by excitation at 380 nm, emission at 400-550 nm for ANS (8-anilino-1-naphthalensulfunic acid) [21,22].

Conductometry

To study the possible interaction of As_2O_3 with microtubules in the presence of Mg^{2+}, coductometric experiments were performed at different concentrations of arsenic trioxide at constant concentration of Mg^{2+} as $MgSO_4$.

Electron Microscopy

Samples for transmission electron microscopy were prepared by negative staining using uranyl acetate on carbon coated copper grid [23] and observed at 65000 × magnification for normal microtubule and 12000 × for 5 mM As_2O_3.

RESULTS

Tubulin Polymerization Assay

Figure 1A shows that addition of arsenic trioxide to tubulin in the presence of GTP exhibits a decrease in polymerization and an increase in lag time at 25 and 37 °C. The results demonstrate a decrease in the extent of polymerization and an increase in nucleation phase at 37 °C and 25 °C (see Fig. 1). Figure 2A demonstrates the effect of various concentrations of GTP in the range 0.35-1 mM in polymerization solution in the presence of 2.5 mM arsenic trioxide.

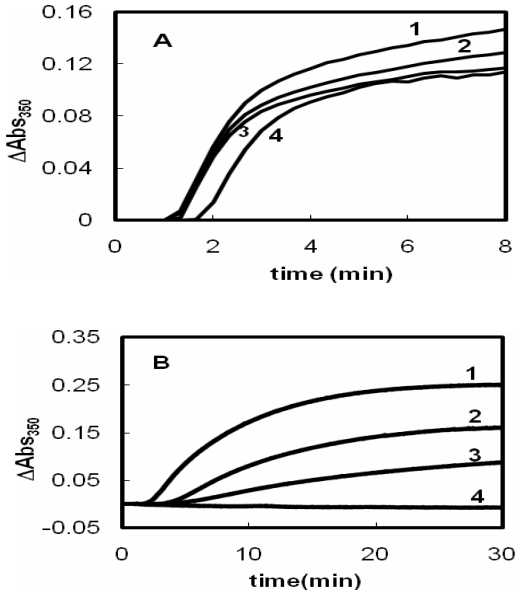

Fig. 1. Effect of various concentrations of As_2O_3 on tubulin Polymerization in 1 mM GTP. 1) 0 mM (normal), 2) 1 mM, 3) 2.5 mM, 4) 5 mM, A) 37 °C, B) 25 °C.

In order to find out quantitatively the possible effect of GTP on three phases of tubulin assembly, an experiment was conducted at different concentrations of GTP in the absence of As_2O_3. The results showed that different concentrations of GTP did not change the time of nucleation significantly, but the extent of polymerization was decreased upon decreasing the GTP concentration (see Fig. 2B). The effect of various concentrations of arsenic trioxide (1-5 mM) at constant concentration of GTP on tubulin polymerization at 37 °C was tested. Similarly tubulin polymerization was examined at different concentrations of GTP but at constant concentration of As_2O_3.

Addition of 1-5 mM arsenic trioxide to tubulin solution under assembly condition, 280 s after the start of polymerization, showed a sharp decrease in absorbance. The slope of decrement increased with increasing the As_2O_3 concentration (see Fig. 3). It is known that tubulin polymerization is completed at the end of elongation phase.

Irreversiblity Assay

To determine the reversibility of tubulin-As_2O_3 interaction, repolymerization of depolymerized tubulin in the presence of

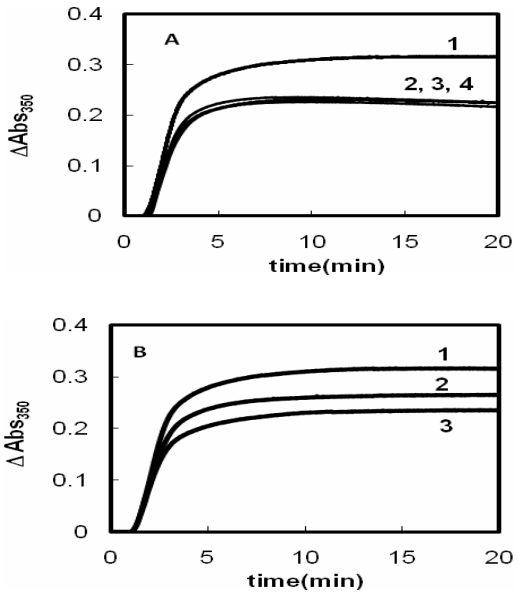

Fig. 2. Effect of various concentrations of GTP (0.35-1 mM) on tubulin polymerization at 37 °C. A) At constant concentration of As_2O_3 (2.5 mM). 1) 0 mM As_2O_3+ GTP1 mM, 2) As_2O_3+1 mM GTP, 3) As_2O_3+0.5 mM GTP, 4) As_2O_3+0.35 mM GTP. B) In the absence of As_2O_3, 1) GTP1 mM, 2) GTP0.5 mM, 3) GTP0.35 mM.

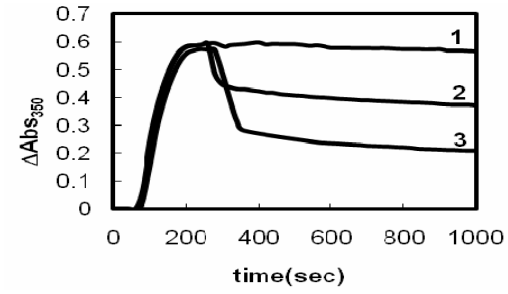

Fig. 3. Comparison of equilibrium state of tubulin polymerization after late addition of different concentrations of As_2O_3 to tubulin solution at 280 s after incubation at 37 °C. 1) As_2O_3 1 mM, 2) As_2O_3 3 mM, 3) As_2O_3 5 mM.

arsenic trioxide was conducted. Microtubule proteins are affected by As_2O_3 at 1 mM GTP (see Fig. 4). To answer the question whether arsenic trioxide interacts with tubulin close

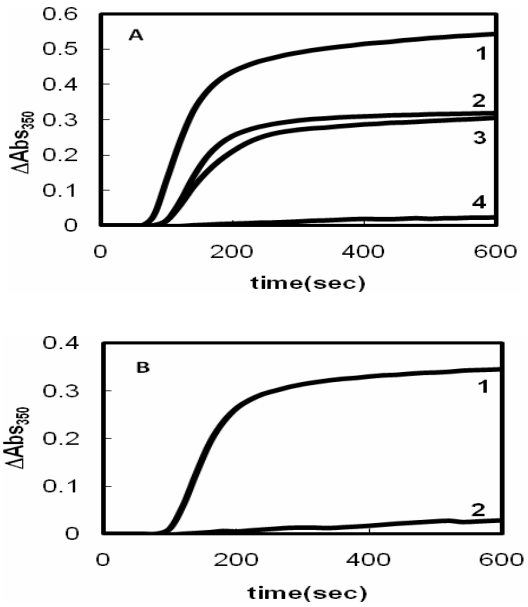

Fig. 4. A) Polymerization of dialyzed tubulin. A) 1) 0 mM As_2O_3 before dialysis, 2) 0 mM As_2O_3 after dialysis, 3) As_2O_3 5 mM before dialysis, 4) As_2O_3 5 mM after dialysis, B) Polymerization of tubulin at 37 °C in 5 mM As_2O_3. 1) at zero time, 2) pretreatment with 5 mM As_2O_3 for 4 h at 4 °C.

to the GTP site or with a site which affects the alpha or beta tubulin structure, GTP concentration was decreased to as low as possible by dialysis of tubulin polymer in PEM buffer for 4 h at 4 °C. Repolymerization of tubulin samples at 37 °C was conducted in the presence and absence of arsenic trioxide (see Fig. 4A).

MAPs Isolation

MAPs free tubulin was prepared by separating tubulin from MAPs, and tubulin dimers were removed on a phosphocellulose column (see Fig. 5A). MAPs free tubulin was mixed with arsenic trioxide and then polymerized (see Fig. 5B).

Conductometry Assay

To determine if arsenic trioxide interacts with magnesium ions and causes depletion of Mg^{2+} in tubulin solution, conductometric tests were done at varying concentrations of As_2O_3 at constant concentration of $MgSO_4$. It was seen that

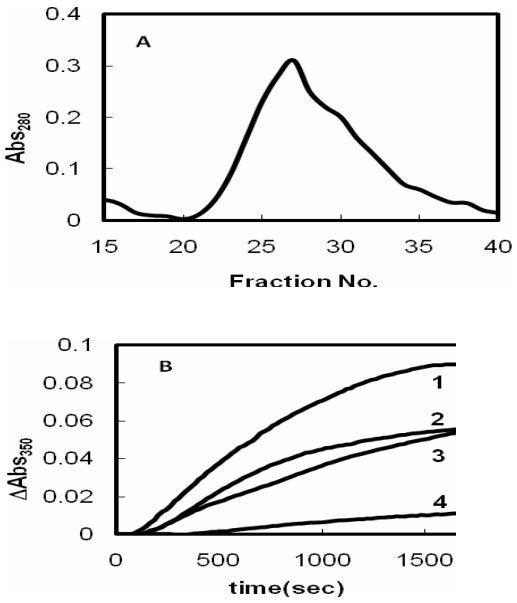

Fig. 5. A) Purification of Tubulin dimer from MAPs by phosphocellulose column chromatography. Tubulin dimer was separated in fractions 25 and 26, B) Polymerization of MAPs free Tubulin dimer at various concentrations of arsenic trioxide, 1) 0 mM, 2) 0.5 mM, 3) 1 mM, 4) 5 mM.

conductivity of the mixture decreased upon increasing the As_2O_3 concentration (see Fig. 6).

Spectrofluorimetry

To observe the relationship between the conformational change and tubulin activity, fluorescence spectrophotometry was conducted both in the absence and presence of ANS (50 µm) at 0.5 mM arsenic trioxide. As can be seen, there is a decrease in the ANS emissions (see Fig. 7) in the presence of As_2O_3.

Electron Microscopy

Electron photomicrographs show respectively the normal structure of microtubule protein polymer (see Fig. 8) at 37 °C and 1 mM GTP and that which has been treated by arsenic trioxide (see Fig. 9). As can be observed in the case of As_2O_3 treated sample the assembled microtubules are shorter, thinner and bent like the GDP tubulin rings [24,25]. The width of these microtubule polymers relative to the normal ones is

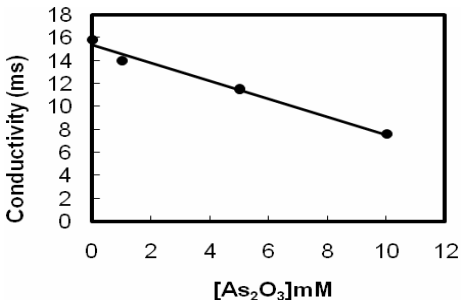

Fig. 6. Effect of different concentrations of As_2O_3 on conductivity at constant concentration of magnesium sulfate (2 mM).

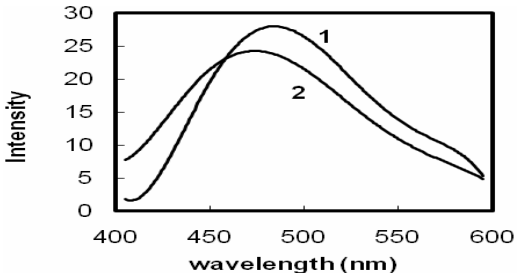

Fig. 7. Emission spectrum of tubulin dimer (2µM) in the presence of ANS (50 µM) after 7 and 10 min incubation in the absence (1) and presence (2) of 0.5 mM As_2O_3.

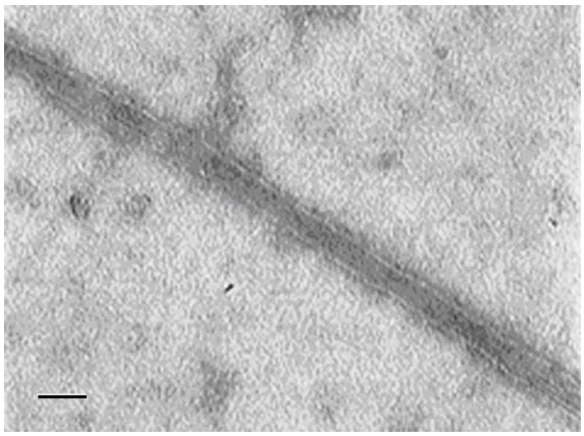

Fig. 8. Microtubule protein polymerized in normal condition, 1 mM GTP and 37 °C in absence of As_2O_3, Bar = 140 nm.

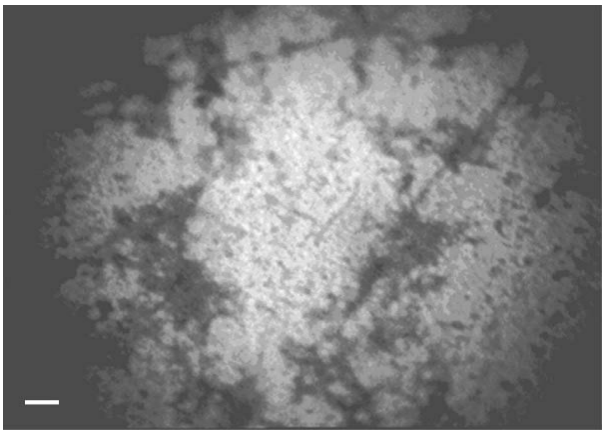

Fig. 9. Microtubule protein polymerized in presence of 5 mM As_2O_3, Bar = 500 nm.

about 4:1 indicating 3 to 4 protofilaments participating in construction of an abnormal polymer.

DISCUSSION

The role of microtubule protein in cell life is greatly dependent upon tubulin polymerization and depolymerization. Several molecules such as tau protein, kinesin, GTP, ligands and even Mg^{2+} cooperate in polymerization process. Antimitotic agents such as cholchicine and taxol have the ability to prevent tubulin polymerization by binding to tubulin subunits. Arsenic trioxide by non-bonded electrons on oxygen atom and a partial positive charge on arsenic atom inhibit tubulin polymerization and prevent cell proliferation. It is a unique molecule compared to large organic molecules such as cholchicine. Arsenic trioxide is used as a drug in cancer therapy but the mechanism of action is not yet fully known. Yong Ming Li [12] reported that cystein 12 and 213 on β-tubulin interact with As_2O_3. The two amino acids are in the vicinity of GTPase site on β-tubulin which play a key role in tubulin polymerization and depolymerization process. On the other hand, one of the key factors in stabilizing tubulin conformation is the concentration of magnesium ions. Changing ionic concentration of the tubulin solution leads to change in the ionic environment around tubulin and ends up abnormal nanopolymers such as protofibrils (see Fig. 9).

When As_2O_3 is added to polymer solution at the end of elongation phase, a fast decrease in turbidity is observed depending upon the As_2O_3 concentration (see Fig. 3). The rate of decrement demonstrated a fast decrease much higher than expected normal depolymerization. Therefore, highly interactive ionic compounds would strongly interfere with the depolymerization process. The rate of depolymerization of microtubule polymer with As_2O_3 indicated a different mechanism usually taken by the protein without As_2O_3. Magnesium ion which is necessary for tubulin polymerization and stability was suspected to interact with As_2O_3.

Conductometric data of As_2O_3 and $MgSO_4$ demonstrated a notable interaction between the two compounds (see Fig. 6). Dialysis data demonstrated an irreversible interaction between As_2O_3 and tubulin (see Fig. 4). It is suggested that As_2O_3 interacts strongly with Mg^{2+} thereby changing the conformation of tubulin dimer. Electron microscopy of tubulin polymers in the presence of As_2O_3 confirmed the extremely effect of arsenic trioxide on microtubule polymerization (see Fig. 9). Since fluorometric observation indicated a small protein conformational change in the presence of As_2O_3 (see Fig. 7), it is concluded that As_2O_3 interacts with Mg^{2+} ions around the negatively charged microtubule polymers to destabilize polymer formation. Therefore, the small conformational change in tubulin causes depolymerization and hydrophobic interaction between segments of protofilament observed in electron micrograph (see Fig. 9). Magnesium ions around active GTPase site and two negative charges in nearby cysteins 12 and 213, rapidly interact with As_2O_3 to change the conformation of active site, leading to catastrophic depolymerization (see Fig. 3).

Since the in vitro concentration of Mg^{2+} ion in microtubule solution is much higher than in cytosol (26, 27), investigation of As_2O_3 interaction with Mg^{2+} ions in the cell would be necessary to unravel the antimitotic mechanism of arsenic trioxide. It means that As_2O_3 would interact with many different cellular components other than microtubule which enhance cell death. Therefore, to affect the polymerization of microtubule in vitro, higher concentration of As_2O_3 was necessary to observe the share of microtubule-As_2O_3 interaction in cell death process. Based on our *in vitro* data, a small conformational change at a vital position on β-subunit leads to a plug-off mechanism.

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