Aluminium Induced Endoplasmic Reticulum Stress Mediated Cell Death in SH-SY5Y Neuroblastoma Cell Line Is Independent of p53

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Abstract

Aluminium (Al) is the third most abundant element in the earth’s crust and its compounds are used in the form of house hold utensils, medicines and in antiperspirant etc. Increasing number of evidences suggest the involvement of Al³⁺ ions in a variety of neurodegenerative disorders including Alzheimer’s disease. Here, we have attempted to investigate the role of Al in endoplasmic reticulum stress and the regulation of p53 during neuronal apoptosis using neuroblastoma cell line. We observed that Al caused oxidative stress by increasing ROS production and intracellular calcium levels together with depletion of intracellular GSH levels. We also studied modulation of key pro- and anti-apoptotic proteins and found significant alterations in the levels of Nrf2, NQO1, pAKT, p21, Bax, Bcl2, Aβ1-40 and Cyt c together with increase in endoplasmic reticulum (ER) stress related proteins like CHOP and caspase 12. However, with respect to the role of p53, we observed downregulation of its transcript as well as protein levels while analysis of its ubiquitination status revealed no significant changes. Not only did Al increase the activities of caspase 9, caspase 12 and caspase 3, but, by the use of peptide inhibitors of specific and pan-caspases, we observed significant protection against neuronal cell death upon inhibition of caspase 12, demonstrating the prominent role of endoplasmic reticulum stress generated responses in Al toxicity. Overall our findings suggest that Al induces ER stress and ROS generation which compromises the antioxidant defenses of neuronal cells thereby promoting neuronal apoptosis in p53 independent pathway.

Introduction

The distinguishing features of neurodegenerative disorders include loss of neurons in the brain or spinal cord which, over a long period of time, result in the loss of a particular neuronal subtype or indiscriminate loss of neuronal populations. There have been reports in that Alzheimer’s disease and Huntington’s disease there is a loss of neurons [1,2], while in Parkinson’s disease there is a specific and inadequate loss of dopaminergic neurons in the substantia nigra [3]. All these conditions disclose exclusive neuronal pathologies the precise mechanisms for neuronal loss are complex, making the identification of effective treatments indistinct.

Al which is the third most abundant element in the earth crust is not essential for organisms and no biological function has been assigned to it, however, its accumulation in tissues and organs has been reported to result in their dysfunction and toxicity [4]. Al compounds are being used in many industrial as well as house hold applications like water treatment, drugs and utensils, etc [5]. Though dietary intake of Al from food is small, the use of Al-containing antacids may provide doses of 50–1000 mg/day [6]. Studies have demonstrated that degenerating neurons in Alzheimer’s disease show high local Al concentrations [4], further its level in the brains of untreated DES patients have exceeded 25 µg/g in brain tissue [7]. Al can enter the central nervous system following systemic administration, causes behavioral impairments and neurolipofuscinogenesis [8].

Experimental evidences, both in vitro and in vivo, support that Al causes oxidative stress [9–11] though it is devoid of redox capacity in biological systems. Several lines of evidence suggest that apoptosis is the major mode of cell death induced by Al [12,13]. A study by [14] indicated association of Al induced apoptosis with potential role of p53 signaling in Neuro 2a cells. Although mitochondria may play a central role in stress induced neuronal apoptosis by the activation of p53 [15], growing evidence now suggests that endoplasmic reticulum (ER) may also regulate neuronal apoptosis in stress conditions [16].

Perturbation of ER homeostasis affects protein folding and causes ER stress which is also implicated in many neurological disorders. ER stress can result in apoptosis [17] through activation of caspase-12. Expression of CHOP/Gadd153 is also associated with ER stress and recent micro-array studies revealed that...
CHOP/Gadd153 is one of highest inducible genes during ER stress [18].

Neurodegenerative disorders, like Alzheimer’s disease, exhibit characteristics of neuronal cell death however, their link with Al toxicity with respect to molecular mechanisms is still controversial. In the present study, we have attempted to explore the role of p53, if any, during oxidative and endoplasmic reticulum stress caused by Al(mal)₃ (aluminium maltolate) in human neuroblastoma cell line. We have employed Al(mal)₃ because of its electroneutral complex and provides significant amount of free aqueous Al at physiological pH and is soluble and stable from pH 3-10 [19]. We have demonstrated that Al(mal)₃ induces apoptosis in neuroblastoma cells through ER stress pathway. Further, Al(mal)₃ induced p53 dysregulation and it was not related to its post translational modification, but rather involves transcriptional disruption. This is the first report which shows that Al may induce p53-independent apoptosis in human neuron like cell line.

Materials and Methods

Materials

Aluminium chloride hexahydrate, Maltol, Tox 7 kit, (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dye and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). DMEM/F12 cell culture medium, trypsin–EDTA, fetal bovine serum (FBS) and 100X antibiotic and antimycotic solution were purchased from (Gibco, USA). Caspase 12 and p53 antibodies were purchased from Abcam (Cambridge, UK), while Nrf2, NQO1, pAKT, p21, Bax and Bcl2 were procured from BioLegend (USA) and Akt antibodies were purchased from Abcam (Cambridge, UK), while CHOP/Gadd153 and ubiquitin antibodies were purchased from BioVision Inc. (Mountain View, CA) while pan-caspase inhibitor (QVD-OPh) was procured from Abcam. All other chemicals were obtained from Merck unless otherwise mentioned.

Cell Culture

SH-SY5Y human neuroblastoma cell line was purchased from National Centre for Cell Sciences (NCCS) Pune, India, and cultured in DMEM F-12 Hams (1:1) supplemented with 10% fetal bovine serum, 0.2% sodium bicarbonate (Merck) and 1% antibiotic and antymycotic solution. Cultures were maintained at 37°C and 5% CO₂ under high humid atmosphere. Medium was changed twice weekly and the cultures were split at a ratio of 1:5 once a week.

Preparation of Al(mal)₃

Al(mal)₃ was prepared from aluminium chloride hexahydrate and maltol (3-hydroxy-2-methyl-4H-pyran-4-one) according to the method described by Berthold et al. [20]. Briefly, for 10–15 g of complex, 15.5 g (122.8 mM) of maltol and 9.9 g (40.9 mM) of AlCl₃.6H₂O were dissolved in approximately 160 ml of deionized water accompanied by mild heating to facilitate dissolution. When most or all of the particulate material had dissolved, the pH was adjusted to 8.3. Subsequently, the mixture was heated to a temperature of 65°C that produced a finely divided precipitate, the formation of which was enhanced by stirring the solution. After cooling, the off-white crystals obtained were filtered, washed several times with acetone, and dried overnight in vacuum-desiccator. Yields of 75–85% of the theoretical 16.5 g of product were obtained.

MTT Assay

MTT assay provides an indication of mitochondrial integrity and activity, which is interpreted as a measure of percent cell viability. MTT assay was done as per the method of Mosmann [21]. Briefly, cells were seeded in 96-well tissue culture plates (10,000 cells/well) in complete DMEM F-12 medium, followed by incubation in 5% CO₂-95% atmosphere for 24 h at 37°C. Cells were exposed to different concentrations of Al(mal)₃ (100 µM, 200 µM, 400 µM, 500 µM and 600 µM in a final volume of 100 µl media) followed by addition of MTT (10 µl per well of 5 mg/ml stock solution) 5 h prior to completion of incubation periods. Media was completely removed from each well and DMSO (200 µl) was added for solubilization of formazan crystals. After 10 min, absorbance was measured at 550 nm and relative percentage cell viability was calculated taking absorbance of control as 100 percent.

LDH Activity-based Cytotoxicity Assay

Cells were seeded as described above for the MTT assay. The assay was performed as per the method used by Yang et al. [22] using TOX7 kit (Sigma) for estimation of live, apoptotic and necrotic populations. Briefly, after the treatment schedule, the floating apoptotic cells were collected from each well and medium was centrifuged at (240 x g) at 4°C for 5 min. The LDH content from the pellets gave an index of apoptotic cell death (Ap) while that obtained from the medium gave an indication of necrotic population (Np). The enzymatic analysis from adherent cells was used as an index for live population (Lp). For total LDH, 1/10 volume of LDH Assay Lysis Solution was added to separate set of wells and plate was returned to incubator for 45 min to release LDH from adherent as well as floating cells. Plate was centrifuged at 250 x g for 5 min to pellet debris and aliquot transferred to clean flat-bottom plate. The enzymatic analysis of this supernatant aliquot gave total cellular LDH (Tp). For interference from media, blank measurements were calculated by performing enzymatic analysis on cell-free media (Blk). The percentage of apoptotic and necrotic cell death was calculated as follows. % Live population = (Lp-Blk)/Tp-Blk)*100, % Necrotic population = (Np- Blk)/Tp-Blk)*100, % Apoptotic population = (Ap- Blk)/Tp-Blk)*100.

Table 1. List of primer sequences used in real time PCR.

| Gene | Forward | Reverse |
|------|---------|---------|
| Bcl-2 | F- 5’-GTGGAGAGACCTTTCCAGGGA-3’ | R- 5’-AGGCCACCCAGGGTGTGATGCAA-3’ |
| Bax  | F- 5’-GGGCCACACAGCTCTGAGCAGA-3’ | R- 5’-GCCACCGTGCCCTGCTCCAAGAT-3’ |
| p53  | F- 5’-CGCTGGCACCACCATGAGC-3’ | R- 5’-CTGGAGCTTCCAGTAGTGAATG-3’ |
| CHOP | F- 5’-AGACCTCGAGAAAACAGCATG-3’ | R- 5’-TCTCCATGACGCTGCCGTTT-3’ |
| GAPDH | F- 5’-GTCAGCGGTATTTTGTCGATTG-3’ | R- 5’-TGGAGGGAGATCTGCTGGAGAT-3’ |

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Figure 1. Al(mal)$_3$ induced morphological changes in neuroblastoma cells. Phase-contrast image (20X objective) of the monolayers showing Al(mal)$_3$ induced cell loss and morphological changes in neuroblastoma cells at different concentration of Al(mal)$_3$ in cell culture plates. doi:10.1371/journal.pone.0098409.g001

Figure 2. Al(mal)$_3$ induced both apoptotic and necrotic cell death. Viability of neuroblastoma cells (10,000 cells/well) was evaluated by MTT assay in 96-well plates at 24 h and 48 h exposure to (A) concentrations of Al(mal)$_3$ varying between 100 μM–600 μM and (B) Maltol alone at 3x concentration corresponding to each of Al(mal)$_3$ concentration. (C) Live, Apoptotic and necrotic populations estimated using LDH activity-based cytotoxicity assay and (D) Fluorescence microscopy of Annexin V/PI staining of neuroblastoma cells 24 h post Al(mal)$_3$ exposure (100 μM–600 μM) under 20X objective. The data are represented as means ±SE of three independent experiments. *P<0.05 vs. control. doi:10.1371/journal.pone.0098409.g002
Annexin V and PI Staining

Annexin V/PI staining was carried out using Annexin V/PI staining kit provided by BioLegend following manufacturer’s instructions. Briefly, cells were plated in 48 well tissue culture plate and treated with different concentration of Al(mal)₃ for 24 h. Cells were then washed with PBS followed by incubation with prepared solution of annexin V/PI in annexin V binding buffer for 15 mins in dark at room temperature. Images were captured using Nikon eclipse Ti-S fluorescence microscope (Nikon Instruments Inc., Melville, NY) under 20X objective.

Determination of Total ROS

Intracellular ROS generation was estimated by the method of Wan et al. [23] using 2',7'-dichlorofluorescein diacetate (DCFH-DA) dye by measuring the conversion of non-fluorescent DCFH-DA to fluorescent dichlorofluorescein (DCF) within the cell using SYNERGY-HT multi-well reader (Bio-Tek, Winooski, USA).

Measurement of GSH Activity

Cells were treated with the fluorescent probe Cell Tracker Green CMFDA (Molecular Probes) solubilised in DMSO (Sigma) to 10 mM concentration and used at a final concentration of 1 μM. As in case of ROS measurement, cells were treated with CMFDA for 30 min in black 96-well tissue culture plate prior to treatment. Fluorescence quantitation was done at 517 nm with excitation at 492 nm. The level of reduced glutathione (GSH) in control cells was set to 1.

Measurement of Intracellular Calcium

Calcium Green 1-AM dye was used at a final concentration of 5 μM to measure intracellular calcium levels and treatment of cells with dye and Al(mal)₃ was carried out similar to ROS measurement, except that fluorescence was recorded at 531 nm with excitation at 506 nm. The level of intracellular calcium in control cells was set to 1.

Real-time PCR

RNA was isolated from control and Al(mal)₃ treated cells using RNaseqous kit (Ambion Inc., Austin, TX, USA) following the manufacturer’s protocol. RevertAid First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany) was used to synthesize cDNA from 100 ng of RNA. Real-time PCR was performed using Power SYBR green PCR master mix (Applied Biosystems, Foster City, USA) on a Step One Plus Real-Time PCR System (Applied Biosystems, Foster City, USA).

Figure 3. Al(mal)₃ compromised antioxidant defenses of neuroblastoma cells. (A) ROS generation was assessed in terms of relative fluorescence units using 10 μM DCFH-DA in neuroblastoma cells after 1 h, 3 h and 6 h exposure to Al(mal)₃ in black-bottomed 96-well plates. (B) Fluorescence micrographs of ROS generation at (100 μM–600 μM) Al(mal)₃ concentrations in neuroblastoma cells observed at 20X objective after 1 h treatment. (C) GSH levels were assessed in terms of relative fluorescence units using 5 μM CMFDA dye at 1 h, 3 h, 6 h and 24 h of Al(mal)₃ exposure. After conjugation with GSH, CMFDA is hydrolyzed to the fluorescent 5-chloromethyl-fluorescein (CMF) by cellular esterases. (D) Western blot analysis of Nrf2, NQO1 and pAKT at 24 h of Al(mal)₃ treatment (100 μM–600 μM). Band intensities were calculated by densitometry and change in protein expression (Al-treated) was calculated with respect to controls and expressed as fold change in graph. Results were normalized to β-actin. The data are represented as means ±SE of three independent experiments. *P<0.05 vs. control.

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Figure 4. Al(mal)_3 altered the levels of key apoptotic markers. (A) Ratio of Bax and Bcl2 was determined using western blot analysis of protein obtained after 24 h exposure to Al(mal)_3 (100 μM–600 μM). Band intensities were calculated by densitometry and change in protein expression (Al-treated) was calculated with respect to controls and expressed as fold change in graph. Results were normalized to β-actin and then the ratio was obtained. (B) Relative quantification (RQ) of mRNA expression of Bax and Bcl2 were determined by real time PCR, endogenous control used was GAPDH. (C) Caspase 9 and Caspase 3 activity was assessed in cell lysates of the SH-SY5Y following treatment with Al(mal)_3 (100 μM–600 μM) by spectrophotometric detection of the chromophore p-nitroaniline (pNA) formed after cleavage from the labeled substrate LEHD-pNA and DEVD-pNA.
Caspase Activity

Cells seeded in 75 cm² flask were treated with Al(mal)₃ and cytotoxic lysates were extracted and enzymatic activities of caspase-3, -9, and -12 were measured using Caspase-3 Colorimetric Assay Kit, Caspase-9 Colorimetric Assay Kit and Caspase-12 Fluorometric Assay Kit respectively (BioVision) according to manufacturer's instructions. The absorbance was measured at 405 nm for caspase-3 and caspase-9, while for caspase-12 fluorescence was recorded at 505 nm emission with 400 nm excitation filter in a SYNERGY-HT multi-well plate reader (Bio-Tek).

Statistical Analysis

All experiments were performed for a minimum of three times and results have been presented as mean ± SE. Statistical analysis was performed using SPSS 14.0 statistical package (SPSS Inc., Chicago, IL, USA). Statistical significance of the results was determined using one-way ANOVA by Tukey’s multiple comparison test. Differences were considered statistically significant at P < 0.05.

Results

Al(mal)₃ Induces Morphological Changes in SH-SY5Y Cells

We exposed neuroblastoma cells to different concentrations of Al(mal)₃ (100 μM to 600 μM) for 24 h in 6-well tissue culture plates. The cell morphology was assessed using phase contrast microscopy under 20X objective (Figure 1) which clearly revealed cell loss from the monolayer as well as shrinkage and deformation of cell bodies at 400 μM and higher concentrations of Al(mal)₃.

Al(mal)₃ Causes Both Apoptotic and Necrotic Cell Death

We exposed neuroblastoma cells to different concentrations of Al(mal)₃ (100 μM to 600 μM) for 24 h and 48 h. A 3x concentration of maltol alone was also included as control for each concentration of Al(mal)₃. MTT assay results showed that at 100 μM and 200 μM concentrations, cell viability reduced to 98.3% and 74.5% respectively at 24 h which further dropped to 33.58% and 21.51% at 48 h (Figure 2A). Thus, significant cell death could be observed at 400 μM, 500 μM and 600 μM concentrations of Al(mal)₃ at 24 h. At a concentration of 400 μM, only 54.6% cells were viable at 24 h and no significant cell death was observed upon treatment with its respective maltol control (1200 μM maltol; Figure 2B), hence, we selected 24 h time period for further experiments.

The percentage of live, apoptotic and necrotic populations estimated using Lactate dehydrogenase (LDH) assay revealed that significant apoptotic and necrotic cell death occurred upon exposure to 400 μM, 500 μM and 600 μM concentration of Al(mal)₃ (Figure 2C). At 400 μM concentration, out of the approximate 50% population undergoing cell death, 31.6% cells
Figure 5. Al(mal)₃ induced ER stress in neuroblastoma cells. (A) Intracellular calcium levels were determined in terms of arbitrary fluorescence units using 5 μM calcium green-1 AM dye at time periods ranging from 30 mins to 300 mins of Al(mal)₃ exposure (100 μM–600 μM). Upon binding calcium ions, an increase in fluorescence of the respective dye is observed. (B) Relative quantification (RQ) of CHOP mRNA was done by Real time PCR following Al(mal)₃ treatment (100 μM–600 μM) for 24 h. GAPDH was used as endogenous control. (C) Western blot analysis of CHOP and caspase 12 at 24 h of Al(mal)₃ treatment (100 μM–600 μM). Band intensities were calculated by densitometry and change in protein expression (Al-treated) was calculated with respect to controls and expressed as fold change in graph. Results were normalized to β-actin. (D) Western blot analysis of Aβ(1–40) following 24 h of Al(mal)₃ treatment (100 μM–600 μM) showed oligomers formation at different concentrations of Al(mal)₃. (E) Enzymatic activity of caspase 12 was assessed in cell lysates of the SH-SY5Y following treatment with Al(mal)₃ (100 μM–600 μM) by the detection of cleavage of substrate ATAD-AFC using fluorimeter (Ex/Em 400/505). The data are represented as means ±SE of three independent experiments.*P<0.05 vs. control; †P<0.05 vs. Al(mal)₃ treatment.
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were apoptotic as against 16.19% necrotic population. However, at higher concentrations of Al(mal)$_3$, that is at 500 μM and 600 μM, necrosis was found to be predominant. This was further evaluated by fluorescence microscopy of annexin V/PI staining which confirmed that at 500 μM and 600 μM concentrations Al(mal)$_3$ induced necrosis, while, at 400 μM, significant apoptosis was being induced (Figure 2D).

**Al(mal)$_3$ Induces Oxidative Stress and Disturbs the Antioxidant Defenses within Neuroblastoma Cells**

ROS generation was assessed at 1 h, 3 h and 6 h after treatment with Al(mal)$_3$ (100 μM–600 μM) concentrations. Significant elevation in ROS levels could be observed at all the tested time intervals and doses, however, maximum ROS levels were detected at 500 μM and 600 μM during the first hour (Figure 3A). Fluorescence micrographs of DCFH-DA stained cells further confirmed the above fluorometric findings (Figure 3B).

**GSH levels** estimated at 1 h, 3 h, 6 h and 24 h after treatment with Al(mal)$_3$ revealed maximum levels at 100 μM, 200 μM and 400 μM Al(mal)$_3$ at 3 and 6 h (Figure 3C).

We next assessed protein expression of oxidative stress and cell survival related proteins by western blotting and found that except at 100 μM concentration, the levels of Nrf2 and its downstream molecule NQO1 were significantly suppressed at higher Al(mal)$_3$ concentrations. p-AKT (phosphorylated Akt), which is a cell survival protein, also showed a similar expression pattern (Figure 3D). The data suggests Al evokes apoptosis of SH-SY5Y cells by disruption of survival pathways, and this disruption involves a free radical component.

**Al(mal)$_3$ Induces Expression of Apoptosis Related Proteins**

We assessed protein and mRNA expression of apoptosis related proteins by western blotting and real time PCR. Bax, a pro-

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**Figure 6.** Al(mal)$_3$ induced apoptosis in p53 independent manner. (A) Western blot analysis of p53 and p21 at 24 h of Al(mal)$_3$ treatment (100 μM–600 μM). Band intensities were calculated by densitometry and change in protein expression (Al(mal)$_3$ treated) was calculated with respect to controls and expressed as fold change in graph. Results were normalized to β-actin. (B) Status of p53 ubiquitination at different concentrations of Al(mal)$_3$ (100 μM–600 μM) at 24 h. Total cell lysates were immunoprecipitated with anti-ubiquitin antibody followed by immunoblotting with anti-p53 antibody, p53-null cell line HL60 were used as negative control. (C) Relative quantification (RQ) of p53 mRNA expression was determined by real time PCR following Al(mal)$_3$ treatment (100 μM–600 μM) at 24 h. GAPDH was used as endogenous control. The data are represented as means ±SE of three independent experiments. *P<0.05 vs. control.

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apoptotic protein significantly increased at 200 μM concentration (Figure 4A) and its mRNA expression also showed a similar pattern (Figure 4B). Bcl2, which is an anti-apoptotic protein, was down regulated at all the concentrations of Al(mal)₃ (Figure 4A), though slight upregulation of its mRNA could be observed at 100 μM Al(mal)₃ concentration (Figure 4B).

A significant increase in caspase 9 enzymatic activity was observed at 100 μM, 200 μM and 400 μM concentration of Al(mal)₃ with respect to control (Figure 4C), while that of caspase-3 was observed at 200 μM and 400 μM concentration (Figure 4C). Western blot analysis of cytochrome c (Cyt c), an intermediate in the apoptotic process, revealed considerable increase at all the concentrations of Al(mal)₃ (Figure 4D). The data further confirms that at lower concentrations, Al(mal)₃ induces apoptotic cell death in neuroblastoma cell line.

**Al(mal)₃ Perturbs the Calcium Fluxes and Induces ER Stress within the Neuroblastoma Cells**

Perturbation of intracellular calcium levels is implicated in ER stress which was estimated using calcium green-1 AM dye at various time intervals ranging from 30 min to 300 min after exposure to Al(mal)₃. Fluorometric estimation revealed that cytosolic calcium increased as the Al(mal)₃ concentration increased and maximum levels were recorded for 200 μM at all the tested time periods (Figure 5A).

ER stress related protein CHOP/GADD153 is highly expressed during ER stress. Its m-RNA was upregulated at all the

**Figure 7. Treatment with pan caspase inhibitor and caspase 12 inhibitor rescues SH-SY5Y cells from Al toxicity.** SH-SY5Y cells were incubated with 400 μM Al(mal)₃ or with 400 μM Al(mal)₃ and peptide inhibitors of pan caspases (QVD-oph), caspase 12 (Z-ATAD), caspase 9 (Z-LEHD) and caspase 3 (Z-DEVD) for 24 h. Cell viability was determined by MTT reduction (A) and live, apoptotic and necrotic cell populations were assessed by estimation of LDH released (B) as per the protocol mentioned under Materials and Methods. The data are represented as means ±SE of three independent experiments. *P<0.05 vs. control; **P<0.05 vs. Al(mal)₃ treatment.

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concentrations of Al(mal)$_3$ except 100 mM (Figure 5B). Besides this, its protein expression pattern revealed significant increase at all the concentrations of Al(mal)$_3$ and maximum levels were observed at 400 mM concentration (Figure 5C). While significant cleavage of pro-caspases 12 into active cleaved bands was observed at 200 mM and 400 mM, its enzymatic activity was also significantly increased (Figure 5C and Figure 5E). ER is also the site of amyloid beta (Aβ) generation. Western blot analysis revealed that Al(mal)$_3$ treatment also enhanced the protein expression as well as the oligomeric forms of Aβ1-40 (Figure 5E). The data suggests that Al(mal)$_3$ induced neuroblastoma insufficiencies involve dysregulation in ER.

**Al(mal)$_3$ Induces Apoptosis in p53-independent Manner**

In order to further delineate the mechanism implicated in Al induced neurotoxicity, we planned to explore the involvement of p53 and its associated pathway. However, we observed that p53 protein expression level was down-regulated at all the concentrations of Al(mal)$_3$ except 100 μM (Figure 6A). Also p21 protein, which is the downstream effector molecule of p53 pathway, was also reduced at all the concentrations of Al(mal)$_3$ (Figure 6A). So, we next analyzed the ubiquitination status of p53 to ascertain whether its levels are being post-translationally suppressed. However, no significant poly-ubiquitination could be observed, and only bands corresponding to mono-ubiquitinated p53 could be seen (Figure 6B). Hence, we studied the transcriptional status of p53 gene and observed decreased levels of p53 mRNA in Al(mal)$_3$ treated cells as compared to control (Figure 6D) indicating that p53 is transcriptionally suppressed in response to Al treatment and plays no positive role in mediating Al induced apoptosis of SH-SY5Y human neuroblastoma cells.

**Caspases Inhibition Rescues Cells from Al-induced Apoptosis**

Since we observed that caspase 9, caspase 3 (Figure 4) as well as caspase 12 (Figure 5) were involved in promoting apoptosis in SH-SY5Y cells, we sought to determine if inhibition of any of the caspases would rescue SH-SY5Y cells from apoptosis. We determined viability of SH-SY5Y cells challenged with 400 μM concentration of Al(mal)$_3$ upon caspase inhibition. Compared to 400 μM concentration of Al(mal)$_3$, maximum protection was accorded by pan-caspase inhibition, which increased cell viability to about 30% as determined by MTT assay (Figure 7A) while decreasing apoptotic mode of cell death by 16% as indicated by LDH assay (Figure 7B). This was followed by ER specific caspase-12 inhibitor which significantly increased cell viability up to 21% measured by MTT assay and decreased apoptosis up to 14% as indicate by LDH assay. Caspase-3 and caspase-9 inhibitors increased cell viability up to 7% and 13% respectively and decreased apoptosis by only 5% and 9% (Figure 7A and 7B). The results reveal the prominent role played by ER specific caspase 12 in apoptotic signalling cascade evoked by Al(mal)$_3$ treatment.

**Discussion**

The role of environmental factors in ageing, neurodegenerative disorders and neuronal apoptosis is not conclusive. In the present study, we have attempted to investigate the role of Al, which is still controversial with respect to its relation with Alzheimer’s and other neurodegenerative disorders. We have chosen human neuroblastoma SH-SY5Y, which is a relatively homogeneous neuroblast like cell line that has been extensively used as model of neurons since these cells possess many biochemical and functional
properties of neurons. SH-SY5Y cell line is being widely used in experimental neurological studies involving functional analysis of neuronal differentiation, metabolism, neurodegenerative and neuro-adaptive processes, neurotoxicity, and neuro-protection [26,27]. Therefore, SH-SY5Y cell line is a good in-vitro neuronal model system to evaluate toxicity of Al. Our results revealed that Al(mal)3-induced cytotoxicity in time and dose dependent manner, which was supported by decreased levels of pAKT protein, an important component of cell survival pathway. From the results of Lactate dehydrogenase assay and Annexin/PI staining, apoptosis was observed to be the main mode of cell death at 200 μM and 400 μM concentrations, which, at higher concentrations, transformed into necrosis (Figure 2C and 2D). Significant activation of caspase 9 and caspase 3 activities further confirm apoptosis as the major mode of cell death at lower concentrations of Al(mal)3. Further the role of specific caspases in Al induced cell death was confirmed by the use of peptide inhibitors of caspases. Pan-caspase inhibitor revealed significant protection against Al-induced cell death (Figure 7A). Following pan-caspase, maximum protection was accorded by caspase12 inhibitor (Figure 7A). These results convey that both endoplasmic reticulum and mitochondria specific caspases are involved in Al-induced cell death and ER specific caspase 12 may play key role in observed cell death.

Apoptosis, while on one hand, is fundamental to normal development and maintenance of tissue homeostasis, on the other, is also a process by which physiologically normal cells may die under neurotoxic conditions [28]. Al has been shown to cause oxidative stress and promote apoptosis through mitochondrial intrinsic pathway [29], yet another study has shown it to be p53 mediated in Neuro2a cells [14]. Our results revealed concentration-dependent increase in cytosolic cytochrome C, while pro-apoptotic protein Bax, which is responsible for disruption of Mitochondrial membrane potential (MMP), increased at 100 μM and 200 μM concentrations but decreased at higher concentrations. The observed effect may be due to necrosis being the main form of cell death at higher concentrations. Bcl2, an anti-apoptotic protein, was down regulated at both mRNA and protein levels as the Al concentration increased. These observations suggest that Al induced perturbation of cellular Bax/Bcl2 ratio which promoted the initiation of apoptotic events.

Glutathione (GSH) is an abundant antioxidant in cells that prevents oxidation of cellular macromolecules from the action of reactive oxygen species [30]. In the present study we observed that initially during the first 6 h, as the Al(mal)3 concentration increased from 100 μM to 400 μM, the GSH levels also increased, however, at higher concentrations of 500 μM and 600 μM, its levels were considerably low. Nevertheless, at 24 h, the GSH levels were significantly reduced when compared with earlier time periods for all concentrations. The possible reason for the increased levels of GSH at the initial time periods may be in part due to the activation of certain pathway which maintains the intracellular GSH pool. A study by [31] suggested that maintenance of GSH is a novel physiological role of the IKKβ-NFκβ signalling cascade to prevent oxidative damage and preserve the functional integrity of the cells. NFκβ activates in response to oxidative stress insult upon Al-sulphate treatment in human brain cells [32].

The possibility of compromised cellular antioxidant defenses was confirmed by decreased Nrf2 levels, which regulates the protective mechanism within the cells against oxidative stress by the transcription of antioxidants enzymes [33]. A number of studies have revealed that pathological conditions exacerbated due to oxidative burden often involve diminished survival responses [34–37]. In addition, a number of neurological insufficiencies have been linked to impaired Nrf2-signaling that enhances the susceptibility to oxidative stress [30–40]. We observed significant reduction in protein levels of Nrf2, and its regulated downstream target NQO1, which confirms that Al exposure causes dysregulation of Nrf2 pathway, aggravating free radical generation by compromising cell’s potential to counteract the oxidative stress. Nrf2 stability is a critical determinant of its functional capacity. While Keap1 is said to perform a key role in suppressing cytosolic Nrf2 levels, many other mechanisms co-exist which may influence Nrf2 activity. A recent study has related the weakening of cellular antioxidant defenses to stress-mediated Akt-deactivation leading to compromised Nrf2 stability [41]. The observed down-modulation of Akt phosphorylation due to Al exposure in SH-SY5Y cells may explain for the diminishing Nrf2 and NQO1 levels.

Tumor suppressor gene TP53 activates during genotoxic stress and promotes cell cycle arrest by the activation of p21 [42]. Wild-type p53 is required for the normal function of the p53 protein. It is well reported that SH-SY5Y cells harbor wild-type p53 [43,44]. Several studies employing single-strand conformational polymorphism analysis to detect p53 mutations have shown that p53 is rarely mutated in neuroblastoma tumors and cell lines [45,46,43]. Further, studies have shown intact p53 signaling and activation of p53 in terms of nuclear localization of its protein in response to drug and irradiation treatment in SH-SY5Y cells [47–49,43,44]. Johnson et al. [14] demonstrated increased transcript levels of p53 during Al induced toxicity in Neuro 2a cells, while, on the other hand, a study by Daniela et al. [50] demonstrated that oxidative stress mediates DNA damage-induced apoptosis through p53-independent pathway in Alzheimer patient’s fibroblast cells. Our results showed that p53 protein level was up-regulated only at 100 μM concentrations while at rest of the concentrations, as Al induced its cytotoxic effects, p53 level decreased in comparison to control, p21, the downstream molecule of p53 pathway, was also down regulated at all the concentrations of Al(mal)3 which suggest the inactivation of p53 pathway. In order to explore the reason for decline in p53 protein, we evaluated p53 ubiquitination. However, no significant poly-ubiquitination could be observed, except for bands corresponding to monoubiquitinated p53. Hence, we next estimated the mRNA status of p53 gene and observed that its transcript was down regulated at all the concentration of Al(mal)3. These findings collectively indicate that the observed downregulation of p53 protein in Al(mal)3 treated cells may not be post-translationally regulated, rather, transcriptional down-regulation is responsible for the observed effect. Our results show that the apoptotic pathway induced by Al(mal)3 in human neuroblastoma cells is p53-independent.

Endoplasmic reticulum stress is implicated in many neurological disorders [51]. CHOP/GADD153, sensor of endoplasmic reticulum stress, is highly up-regulated during ER stress [52]. Increase in intracellular calcium and activated caspase-12 are also markers of ER stress [53]. We observed caspase 12 activation, enhanced intracellular calcium levels together with increased mRNA and protein levels of CHOP/GADD153 that reached their maximum at 400 μM Al(mal)3 concentration. Previous studies have reported that ER is also the site of Aβ generation which is a hallmark of Alzheimer’s disease [54,55]. While, on one hand, reports have shown that Aβ induces Ca2+ release from endoplasmic reticulum (ER) stores [56], others report that influx of Ca2+ through calcium channels of the plasma membrane or through release from ER stores increases Aβ generation [57] by alteration in the metabolism and production of Aβ [58]. Whichever, an intricate relationship between Ca2+ dysfunction and Alzheimer’s disease does exist [59]. In our study, we have observed an association between increased intracellular calcium and Aβ[1–40] levels in response to Al(mal)3...
concentration as low as 200 μM reaching to their maximum at 400 μM where apoptosis is observed as the main mode of cell death. Further, p53 independent apoptotic pathway entailing largely endoplasmic reticulum stress is implicated in Al(mal)₃ mediated cell death in human neuroblastoma cells (Figure 8). Increase in intracelular calcium levels and amyloid β explicate the association of Al with ageing disorders like Alzheimer’s that may help to understand the insight of Al induced neurotoxicity and the molecular mechanism involved in neuronal apoptosis.

Author Contributions
Conceived and designed the experiments: AAM SHMR AP MA IA AKV. Performed the experiments: SHMR AP. Analyzed the data: SHMR AP AAM IA. Contributed reagents/materials/analysis tools: AAM KV IA MA. Wrote the paper: SHMR AP AAM.

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