Neurotoxic effect of salsolinol through oxidative stress induction and Nrf2-Keap1 signalling regulation

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ABSTRACT

Parkinson’s disease (PD) is commonly characterized by motor movement deterioration and cognitive impairment. A dopamine-derived endogenous neurotoxin, namely salsolinol was found contributing to pathogenesis of the disease. However, the precise molecular mechanisms of the neurotoxin remain unexplored. Hence, this study aims to evaluate the effect of salsolinol on SH-SY5Y neuronal cells, focusing on oxidative stress-associated apoptotic cell death. Salsolinol was added to SH-SY5Y cells for determination of cell proliferation and viability using cell growth curve and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays respectively. Cell cycle analysis was performed to measure cell cycle phase distribution while presence of apoptotic cell death was confirmed with Hoechst stain. Additionally, 2',7'-dichlorofluorescein diacetate assay was carried out to investigate generation of reactive oxygen species (ROS) and enzyme-linked immunosorbent assay was performed to determine the expression of superoxide dismutase (SOD), NF-E2 related factor 2 (Nrf2) and kelch-like ECH-associated protein 1 (Keap1) proteins. Results reveal that SH-SY5Y cells, when treated with salsolinol (0-100 µM) for 24, 48 and 72 hours elicited neurotoxicity. The reduction and inhibition of cell growth and induction of apoptosis were coincided with enhanced ROS production and increased SOD, Nrf2 and Keap1 proteins expression. Thus, it is suggested that salsolinol can induce oxidative stress-associated apoptotic cell death via SOD, Nrf2 and Keap1 activation.

Keywords: Parkinson’s disease, Salsolinol, Apoptosis, Oxidative stress, Nrf2-Keap1 pathway

INTRODUCTION

Parkinson’s disease (PD) represents one of the most common and progressive age-related degenerative neurological disorders. It is predominantly affecting people over the age of 60 years. The worldwide prevalence of PD is increasing. About 4 million of people was diagnosed with the disease in year 2005 and the number is predicted to increase to approximately 9 million by year 2030 [1]. Today, the definitive cause of PD still remains poorly understood. However, numerous studies have implicated that ageing, genetic susceptibilities, neurotoxins and environmental exposures to heavy metal or pesticides are the aetiological factors for the onset of PD [2].

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) is an endogenous catechol isoquinoline that can either be synthesized from Pictet-Spengler condensation of dopamine with aldehyde or catalysed by (R)-salsolinol synthase to produce (R)-salsolinol from dopamine and acetaldehyde. Alternatively, formation of the racemic mixture of salsolinol occurs via condensation between dopamine and pyruvate [3]. There are increasing evidences indicate that salsolinol promotes neuronal degeneration in substantia nigra pars compacta of brain, leading to PD. Salsolinol has been shown to reduce the enzyme activities of mitochondrial complex-1 and complex-2 in electron transport chain [4], inhibit type A monoamine oxidase and tyrosine hydroxylase [5-7], increase reactive oxygen species (ROS) production as well as evoke apoptosis in neural stem cells by activating caspase 3 and suppressing...
phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway [8,9]. However, little is known about the molecular mechanism underlying the neurotoxic effects of salsolinol, particularly in the induction of oxidative stress-associated apoptotic cell death.

Several antioxidant defence systems comprising of superoxide dismutase (SOD), catalase and glutathione peroxidase have been well documented to play pivotal roles in the protection of cellular macromolecules from oxidative damage [10-12]. NF-E2 related factor 2 kelch-like ECH-associated protein 1 (Nrf2-Keap1) signalling pathway also provides important antioxidant response [13,14]. Post mortem analysis of brain tissues from PD patients demonstrated that Nrf2 could be activated as a neuroprotective response against ROS [15,16].

The correlation between neurotoxic effect of salsolinol and Nrf2-Keap1 antioxidant defence mechanism has not been previously addressed. Hence, this study aims to elucidate the neurotoxic and oxidative stress inducing effects of salsolinol on human dopaminergic neurons; and the role of Nrf2-Keap1 signalling pathway in the cell death induced by salsolinol.

**EXPERIMENTAL SECTION**

**Neuronal cell culture**
The SH-SY5Y human neuroblastoma cell line was obtained from American Type Cell Culture (ATCC® CRL-2266) and maintained in complete growth medium of Dulbecco’s Modified Eagle’s Medium (Gibco Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, MO, USA), 1% penicillin-streptomycin antibiotics (Gibco Life Technologies, CA, USA) and 0.1% gentamicin (Gibco Life Technologies, CA, USA). The cells were incubated in a humidified 5% CO$_2$ atmosphere at 37°C.

**Cell growth curve assay**
The proliferation of SH-SY5Y cells were analysed through plotting the cell growth curve. SH-SY5Y cells were seeded into 24 well-plate (SPL Life Sciences, Gyeonggi-do, Korea) at the density of 1 x 10$^5$ cells/well. The cells were then treated with salsolinol (Sigma-Aldrich, MO, USA) in increasing concentrations of 0, 6.25, 12.5, 25, 50 and 100 µM for 24, 48, 72 and 96 hours. At each time point, the cells were trypsinised, collected and centrifuged at 1500 rpm for 5 minutes. Later, the supernatant was discarded and the cells pellet was re-suspended with 500 µL of phosphate buffered saline (PBS) (Bio Basic, Ontario, Canada). Then, an equal volume of 0.4% trypan blue dye (Gibco Life Technologies, CA, USA) and cell suspension was mixed and loaded into a haemocytometer (Marienfeld, Lunda-Königshofen, Germany) for cell counting under an inverted microscope. The number of viable cells was used to plot the cell growth curve.

**3-[4,5-Dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay**
MTT assay was conducted to determine the cell viability. SH-SY5Y cells were first seeded into 96-well plates (TPP Techno Plastic Products, Trasadingen, Switzerland) at a density of 5 x 10$^4$ cells/well. Then, the cells were exposed to the various concentrations of salsolinol (Sigma-Aldrich, MO, USA) in increasing concentrations of 0, 6.25, 12.5, 25, 50 and 100 µM for 24, 48, 72 and 96 hours. After 4 hours incubation, the medium was removed and replaced with 100 µL of DMSO (Friedemann Schmidt, WA, USA) to dissolve purple formazan crystal formed. The absorbance was then measured by using a microplate reader (DynexOpsys MR 24100, VA, USA) at wavelength of 570 nm.

**Cell cycle analysis**
Cell cycle analysis was done by quantitatively measure the DNA content in sub G1, G0/G1, S and G2/M phases. A total of 0.7 x 10$^5$ cells were seeded into cell culture dishes (SPL Life Sciences, Gyeonggi-do, Korea). Then, various concentrations of salsolinol were added to the cells. After 48 hours, the cells were harvested, washed with PBS and centrifuged followed by fixation with 70% ethanol in PBS for overnight at 4°C. Subsequently, the cells were centrifuged at 3000 rpm and 4°C for 5 minutes. The supernatant was discarded and the cells were washed twice with PBS. After that, the cell pellet was re-suspended with DNA staining solution containing ribonuclease A (Novagen, WI, USA) and propidium iodide (Calbiochem, Darmstadt, Germany) in PBS. Finally, flow cytometry was performed (BD FACSCalibur flow cytometer, BD Biosciences, NJ,USA) and analysis was done by Cell Quest Pro software.

**Hoechst staining**
Hoechst stain was performed to observe apoptosis in the cells. SH-SY5Y cells were grown in 24-well plates at a density of 1 x 10$^5$ cells/well. Following salsolinol treatment at 50 and 100 µM for 72 hours, old medium was removed and the cells were washed with PBS. Then, the cells were fixed with 4% formaldehyde (Sigma-Aldrich, MO, USA) for 20 minutes at 4°C. After washing with PBS, the freshly prepared Hoechst stain (Sigma-Aldrich, MO,
USA) was added into each well and incubated in the dark for 10 minutes. Subsequently, the staining solution was removed and PBS was added into the wells. Finally, the plates were observed under Nikon Eclipse Ti-S fluorescence microscope (Nikon Corporation, Tokyo, Japan) and images were captured using Nikon Microscope Imaging software at total magnification of 200×.

**2’,7’-Dichlorofluorescein diacetate (DCFH-DA) assay**

The production of intracellular ROS was measured using DCFH-DA fluorescent probe. SH-SY5Y cells were seeded into 12-well plates at a density of 1.5 × 10^5 cells/well. Upon completion of treatment with different concentrations of salsolinol for 24, 48 and 72 hours, the cells were collected and centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded and the cells were re-suspended with 500 μL of PBS. One hundred μL of the cell suspension was later transferred into a 96-well black plate. Following that, DCFH-DA working solution (40 μM) (Sigma-Aldrich, MO, USA) was added into each well while fluorescence readings were taken immediately at 0, 10, 20 and 30 minutes by using a microplate reader (Infinite® 200, Tecan, Männedorf, Switzerland) at fluorescence excitation of 485 nm and emission of 535 nm. Blank consisting PBS and DCFH-DA was included. Lastly, the fluorescence reading of each sample was normalised according to the respective cell concentration. The treated groups were then compared to the untreated cells which served as control.

**Enzyme-linked immunosorbent assay (ELISA)**

Protein expressions in cells were detected by ELISA. SH-SY5Y cells (5.5 × 10^4 cells/well) were seeded into 96-well plates and treated with increasing concentrations of salsolinol for 24, 48 and 72 hours. The cells were first fixed with methanol at 4°C for 20 minutes and rinsed 3 times with 0.1% Triton X-100 in Tris-buffered saline (TBST) for 5 minutes. Then, the cells were incubated with 100 μL of 0.6% hydrogen peroxide (H_2O_2)(Calbiochem, Darmstadt, Germany) in Tris-buffered saline (TBS) for 30 minutes prior to 3 washes with TBST. This was followed by blocking with 3% bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA) in TBS for 1 hour at room temperature. Later, the cells were incubated with anti-Nrf2,anti-Keap1 and anti-SOD 1 primary antibodies(diluted 1:300 in 3% BSA) (Santa Cruz Biotechnology, Texas, USA)for overnight at 4°C. Then, the primary antibodies were decanted followed by 3 washes with TBST. This was then proceeded with 1 hour incubation of secondary horseradish peroxidase-linked antibody (anti-rabbit IgG, diluted 1:500 in 3% BSA) (Cell Signalling Technology, USA). Later, 3 rinse with TBST were performed. After that,3, 3’, 5’, 5’-tremethylbenzidine substrate solution (Sigma-Aldrich, MO, USA) was added to the cells followed by addition of sulphuric acid (500 mM) to stop the reaction. The colorimetric measurement was then obtained with a microplate reader (DynexOpsys MR 24100) at 490 nm.

The cell density was quantified by using crystal violet whole-cell staining method. The solution in 96-wells plate was discarded and the cells underwent 3 washes with distilled water for 5 minutes each. After the plate has been dried thoroughly, 0.04% crystal violet was dissolved in 4% ethanol and 100 μL of the solution was added into each well for 30 minutes incubation at room temperature. Then, the plate was rinsed 3 times with running tap water to remove the excess crystal violet solution. The plate was then dried with a dryer. One hundred microliter of 1% sodium dodecyl sulfate(Calbiochem, Darmstadt, Germany) was later added into each well and the plate was tapped gently. Finally, the absorbance values were taken at 595 nm with microplate reader (DynexOpsys MR 24100).Relative SOD1, Nrf2 and Keap1 protein expressions were calculated as [absorbance value at 490nm/absorbance value at 595nm].

**Statistical analysis**

In this study, 3 independent experiments were performed in triplicates. All the data were assessed by using Student’s t-test to compare the treated groups with untreated control. The statistical analysis was done by using Microsoft Excel 2013. The results were presented as mean ± standard deviation and P value with < 0.05 was considered statistical significance.

**RESULTS AND DISCUSSION**

At present, the aetiology of PD still remains uncertain. Salsolinol, as a potent endogenous neurotoxin in human brain, might be contributing to the progress of the neurodegeneration in PD. Hence, this study aims to investigate the underlying mechanisms involved in the neurotoxic effects of salsolinol. The present results show that salsolinol was toxic against SH-SY5Y cells by decreasing the cell proliferation, causing cell cycle alteration, inducing apoptotic cell death, increasing ROS production as well as modifying several protein expressions.

Figure 1 shows the effects of salsolinol on SH-SY5Y cell proliferation. The untreated cells proliferated exponentially with the normal growth pattern of lag and log phases. Following that, treatment with salsolinol at 6.25 and 12.5 μM were observed to have growth curves similar to that of the untreated cells, but with a profound reduction in proliferation rate. Nevertheless, no growth was observed in the cells treated with 25 μM salsolinol for 4 days, whereas 50 and 100 μM salsolinol inhibited the growth rate of SH-SY5Y cells to different extent. Significant
reductions in cell proliferation were first observed at 48 hours following treatment with 12.5 and 100 µM salsolinol. The SH-SY5Y cell proliferation decreased by 81.68% upon exposure to 100 µM salsolinol while the number of viable cells after treatment with 12.5 µM salsolinol reduced by 27.12% when compared to the control. Moreover, the treatment with 100 µM salsolinol for 72 hours resulted in significant decline of the growth of SH-SY5Y cells to about 90.76% of control. Other than that, the salsolinol administration in other doses and length of treatment did not significantly alter the cell proliferation when compared to the control.

Figure 1: Effect of salsolinol on the proliferation of SH-SY5Y cells. Cells treated with various concentration of salsolinol for 24, 48, 72 and 96 hours were harvested for viable cell count using trypan blue exclusion method. Data was expressed as mean ± SD (n = 3). The symbol * and ** denotes significant difference between control and treatment groups at p < 0.05 and p < 0.01 respectively.

Figure 2 shows that salsolinol decreased the SH-SY5Y cell viability in a dose-dependent manner. A significant reduction in viability was observed in cells treated with 100 µM salsolinol for 24, 48 and 72 hours. The treatment with 100 µM salsolinol for 24 hours had decreased cell viability by 13.53% while 48 hours treatment caused a 38.90% reduction in cell viability. Moreover, 50.96% reduction in cell viability was detected after 72 hours of treatment. In contrary, no significant reduction in cell viability was observed following treatment with lower concentrations of salsolinol.
In the present study, salsolinol showed to decrease SH-SY5Y cell proliferation in both dose- and time-dependent manner. MTT assay further revealed that the viability of SH-SY5Y cells reduced significantly upon exposure to salsolinol. These results are in concordance with a previous study made by Wanpen et al. (2004), showing an anti-proliferative effect on SH-SY5Y cells when salsolinol was administered at higher concentration (100 - 500 µM) [8]. Taken together, salsolinol showed to possess cytotoxic effect towards neuronal cells. We noted that the decreased SH-SY5Y cell viability after salsolinol treatment was more noticeable in the cell growth curve detected by using the trypan blue exclusion method compared to the MTT assay. Result from the trypan blue exclusion method showed about 80% cell death while MTT assay showed about 50% of cell death after 72 hours of salsolinol treatment at 100 µM. The discrepancy might be due to the different sensitivity of the assays.

Figure 3 shows the distribution of cells in the cell cycle. SH-SY5Y cells treated with salsolinol showed elevating sub G1 population and this effect was accompanied by a concomitant decline in G0/G1 and S populations. However, the salsolinol administration did not affect the G2/M phase of the cell cycle. After 48 hours of treatment with 100 µM salsolinol, the percentage of cells in the sub G1 phase increased significantly (to about threefold) while a decrease was observed in S phase when compared to the control. Furthermore, 25 and 50 µM of salsolinol caused a significant decrease in the percentage of G0/G1 phase in SH-SY5Y cells.

Salsolinol was shown to alter the cell cycle profile. It increased the percentage of cell in sub G1 phase and decreased the G0/G1 and S phases. The increased sub G1 phase indicates the presence of apoptotic cells. This was further confirmed by the Hoechst staining results, which shows the characteristic apoptotic features in the salsolinol-treated SH-SY5Y cells (Figure 4). Control SH-SY5Y cells displayed distinctive neuronal shape with branching of short and fine neurites outgrowth(Figure 4A). The synaptic connection could also be seen between the neurons. However, the SH-SY5Y cells lost their normal morphologies following salsolinol treatment at 50 and 100 µM (Figure 4B and 4C). No network of neurites could be seen under the bright field microscopy. Furthermore, the cells treated with salsolinol showed cell shrinkage and loss of elongated neuronal shape, resulting in round and irregular morphologies. Fluorescence microscopic images of salsolinol-treated SH-SY5Y cells demonstrate the typical apoptotic features of chromatin condensation and nuclear fragmentation(Figure 4B1 and 4C1). On the other hand, control cells showed a homogenous staining of the nucleus (Figure 4A1). Together, these results suggest that exposure to salsolinol caused apoptotic cell death; and this contributed to the reduction in number of cells that entering into the G0/G1 phase and hence, reducing the cell proliferation over the treatment period.
Figure 3: Representative DNA histogram of SH-SY5Y cells treated with salsolinol. Cells were treated with various concentrations of salsolinol for 48 hours and stained with propidium iodide. Cell cycle analysis was performed by using flow cytometry. Data was expressed as mean ± SD (n = 3). The symbol *, ** and *** denotes significant difference between control and treatment groups at p < 0.05, p < 0.01 and p < 0.001 respectively.

Figure 4: Bright field and fluorescence microscopic images of SH-SY5Y cells after exposure to salsolinol for 72 hours. (Magnification: 200X). A, B and C are bright-field images while A1, B1, and C1 are corresponded to the bright field images respectively, showing cells stained with Hoechst 33258. At control (A), SH-SY5Y cells show normal morphology of network of neurites. For 50 µM (B) and 100 µM (C) salsolinol-treated groups, SH-SY5Y cells are smaller in size (shrinkage) and some floated dead cells are noted. Hoechst-stained control cells (A1) show homogenous nuclear staining. At 50 µM of salsolinol (B1), nuclear fragmentation (arrow) was seen and at 100µM (C1), nuclear condensation (dotted arrow) was observed.
Figure 5 illustrates ROS production in SH-SY5Y cells that were treated with various concentrations of salsolinol for 24, 48 and 72 hours. Significant decrease in ROS level was detected after the cells were treated with 6.25 µM of salsolinol for 24 and 48 hours. Following 24 hours of treatment with 6.25 µM salsolinol, there was a slight decrease of ROS level to 94.25%. However, when the cells were treated with 12.5 µM salsolinol, ROS level was increased by 11.67%. Similar pattern was observed after salsolinol treatment for 48 hours, whereby the percentage of ROS decreased to 80.86% at 6.25 µM, followed by an increase to 131.51% at 12.5 µM. Meanwhile, a significant three-fold increase of ROS production was recorded after treatment with 100 µM of salsolinol. Significant increase in the percentage of ROS was also observed in the cells that were treated for 72 hours.

Salsolinol showed to modulate ROS production in SH-SY5Y cells. The elevated ROS level in cells after exposure to high concentrations of salsolinol suggests that salsolinol induced oxidative stress-associated cell death. High level of ROS are often associated with oxidative stress, leading to decreased intracellular glutathione and increased apoptotic cell death [8,10,17,18]. In the present study, the intracellular ROS production dropped significantly upon treatment with low concentration (6.25 µM) of salsolinol. At low concentration, salsolinol might trigger the activation of antioxidant defence mechanisms which eliminate the ROS generated [19], thus effectively attenuates increase in the ROS level.

SOD, Nrf-2 and Keap1 protein expressions in SH-SY5Y cells after salsolinol treatment were shown in Figure 6. SOD1 protein expressions were reduced at all concentrations tested after 24 hours of treatment with salsolinol. Likewise, slight decrease in SOD1 expression was also noted following the salsolinol administration for 48 hours at various concentrations, except for 6.25 µM. However, the decreases in the protein expression were not statistically significant when compared to the control. In contrast, increased SOD1 was noted when the cells were treated with salsolinol for 72 hours. A significant increase of the protein expression was detected at the concentration of 50 µM. About two-fold increase in Nrf2 protein expression was found in SH-SY5Y cells that exposed to 100 µM salsolinol for 24, 48 and 72 hours. Salsolinol treatment at 100 µM also caused significant increase in Keap1 protein expression by 36.13%, 49.74% and 63.08% at 24, 48 and 72 hours respectively. The 50 µM salsolinol administration for 24 and 48 hours increased the Keap1 protein expression by 18.17% and 31.25% respectively.
The pathogenesis of neurodegenerative diseases has been linked to the occurrence of oxidative damage, where there is excessive generation of ROS but relatively inadequate antioxidant response [11]. Herein, the activities of endogenous antioxidant enzymes that implicated in the induction of oxidative stress-associated apoptotic cell death was examined. SOD is a scavenging enzyme which plays a key role in the defence against oxidative stress [20]. SOD catalyses dismutation of superoxide anion radical into \( \text{H}_2\text{O}_2 \) and oxygen, thereby protecting the cells from oxidative free radicals damage [21]. In the present study, the expression of SOD1 was increased in SH-SY5Y cells after exposure to salsolinol. This suggests that SOD1 might be generated and utilised by SH-SY5Y cells in response to the ROS formed.

Nrf2 is a key transcription factor that maintains cellular redox homeostasis by regulating various antioxidant and detoxifying genes. Under normal physiological condition, Nrf2 activity is constantly repressed by a cytoplasmic protein, known as Keap1, which mediates its polyubiquitination and subsequent proteasomal degradation [22]. However, this Keap1-dependent Nrf2 degradation can be attenuated when the cells are exposed to ROS or electrophiles [23,24]. In the present study, both Keap1 and Nrf2 proteins were upregulated by the salsolinol induction. Normally oxidative stress build-up in cells will inactivate Keap1 for the activation of Nrf2 [14,24]. However, in the present study, salsolinol administration that caused excessive ROS formation was found to elevate the Keap1 protein. These might be related to the multitude of molecular mechanisms underlying the regulation of Nrf2. Besides Keap1-dependent cytosolic stabilization, ubiquitination and proteasomal degradation, Nrf2 can be activated independently through different pathways [24]. Mitogen-activated protein kinases, protein kinase c, PI3K/Akt pathway, glycogen synthase kinase 3β and casein kinase 2 could also promote the translocation of Nrf2 into nucleus [22,24,25]. Furthermore, activation of Nrf2 could be triggered via interaction with microRNAs, p62, p21 and caveolin-1 [24,25]. Therefore, it is possible that Nrf2 was activated through the Keap-1 independent mechanism as a compensatory response to mitigate cellular oxidative damage. Nonetheless, further investigations on
the involvement of Keap-1 independent pathways could be performed in future. On top of that, glutathione that normally protects neurons from cell death by restoring the antioxidant defence is often found to be depleted in PD patients [8, 25-27]. Hence, further investigation on glutathione or enzyme activity of glutathione peroxidase could be performed to validate the oxidative stress inducing effect of salsolinol on the SH-SYSY cells. In addition, expression of NAD(P)H: quinone acceptor oxidoreductase 1 and glutamate-cysteine ligase that are regulated by the Nrf2-Keap1 pathway could be investigated.

Activation of the antioxidant pathway usually protects cells against apoptosis and cell death through reduction of oxidative stress. However, despite the increased expressions of SOD, Nrf2 and Keap1, increased ROS and evident cell death were still noted in the present study. One of the possible explanations for this observation is that some of the components of the antioxidant pathway might possess both anti-oxidant and pro-oxidant roles. For example, SOD1 might act as a primary antioxidant enzyme by increasing its compensatory actions in response to the elevated ROS formation or alternatively, participate as an effective pro-oxidant that stimulated the production of radical oxidants [20]. Based on the results obtained from the present study, SOD was more likely to have the pro-oxidant role as increase of its expression lead to apoptosis and cell death. Another possible explanation is that the antioxidant mechanism provoked in the current study might not be sufficient to counteract with the ROS generated and hence finally failed to protect the cells against the oxidative stress-mediated cell death.

In conclusion, salsolinol induced ROS generation, altered cell cycle progression, induced apoptosis, reduced cell viability and inhibited neuronal cell proliferation. Furthermore, it increased the protein levels of SOD1, Keap1 and Nrf2. This study provides insights about how salsolinol, a naturally occurring endogenous toxin, exerted neurotoxic effects. It provides a natural occurring endogenous toxin, exerted neurotoxic effects.

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