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Long-term treatment of farnesyltransferase inhibitor FTI-277 induces neurotoxicity of hippocampal neurons from rat embryo in a ROS-dependent manner

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ABSTRACT

Despite the well established anti-cancer effect of farnesyltransferase inhibitor FTI-277, the neurotoxic effects of the agent are not yet clearly defined at the molecular and cellular levels. Here, we report the neurotoxic effects of FTI-277 and the involvement of reactive oxygen species (ROS) in FTI-induced neurotoxicity. Although there is no significant effect of FTI-277 for 2 days, long-term treatment of FTI-277 for 4 days induced dramatic reduction in outgrowth, maturation and branching of neuritis and considerable cytoxicity in a dose- and time-dependent manner in primary cultured rat embryo hippocampal neurons. Interestingly, FTI-277 for 4 days dramatically decreased expression of synapsin I, a crucial molecule involved in the neuronal growth and plasticity, and increased a cytotoxic G-protein RhoB of which ectopic expression induced the neurotoxicity in hippocampal neurons. Moreover, treatment with FTI-277 dramatically increased intracellular levels of ROS, which was sustained for 4 days; while blockage of ROS rescued FTI-277-induced neurotoxicity as well as both decrease of synapsin I and increase of RhoB. Taken together, these results provide the molecular insights for the mechanisms which might be of use aiming for avoiding neurotoxic side effects by FTI agent for a drug development for a clinical use.

1. Introduction

Farnesyltransferase (FTase) is a prenylating enzyme of oncogenic $p21^{Ras}$ for translocation to the membrane region and subsequent activation of downstream effectors for its transforming activation in human cancers [1]. Therefore, development of FTase inhibitors (FTIs) has been the therapeutic target for cancer treatment. Although initially developed to target oncogenic Ras for cancer therapy, FTIs have now been acknowledged to act by more complex mechanisms that may extend beyond Ras [2–6]. However, FTI-associated neurotoxicity and the mechanistic link between ROS generation and neurotoxicity are not yet defined.

Reactive oxygen species (ROS) are generated by many cytotoxic stressors, such as anticancer agents and cytokines, thereby playing a critical role in the cytotoxicity of cancer cells [7]. ROS induce oxidative stress, leading to activation of JNK, p53 and caspase-3 [8–10]. Previous reports have stated that FTI agents induce apoptosis of cancer cells via ROS generation [11], but until now, no study has investigated ROS generation by FTIs in primary neuronal cells.

In the current study, we identified the neurotoxic effects of FTI-277 on rat embryo primary hippocampal neurons. FTI-277, a potent anticancer agent [12–14], induced neurodegeneracy of hippocampal neurons. Furthermore, FTI-277 dramatically decreased the level of synapsin I protein and increased the level of cytotoxic G-protein RhoB. Notably, these neurotoxic effects were significantly blocked by prior treatment of the ROS scavenger NAC, implying that ROS are critical mediators for the neurotoxic effects by FTI-277. These findings are beneficial for not only understanding the neurotoxic effects by FTIs, but also for providing the rationale to eliminate the neurotoxic side effects of FTI agents, resulting in improved drug development.

2. Methods

2.1. Materials

FTI-277, purchased from Merck–Calbiochem (Darmstadt, Germany), was dissolved in dimethyl sulphoxide (DMSO) as a 10 mM solution. Antibodies against anti-MAP2 (mouse monoclonal), synapsin I (rabbit polyclonal) and β-tubulin-III (rabbit polyclonal) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Mouse monoclonal antibody against cyclin D1 was purchased from BD Pharmingen (San Diego, CA, USA). Antibodies against RhoB

Abbreviations: FTI, farnesyltransferase inhibitor; DIV, days *in vitro*; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; SRB, sulphorhodamine B.

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(rabbit polyclonal), synaptophysin (rabbit polyclonal) and FTase-α (rabbit polyclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sulphorhodamine B (SRB) and *N*-acetyl-cysteine (NAC) were purchased from Sigma–Aldrich. Dihydroethidium (DHE) was obtained from Invitrogen Molecular Probes (Eugene, OR, USA). Enhanced chemiluminescence reagent was purchased from Millipore (Bedford, MA, USA). Protease inhibitor cocktail was purchased from Roche (Nutley, NJ, USA). Fluorescent mounting medium (S3023) was obtained from Dako (Glostrup, Denmark). Cover glass (No. 1, 22 mm^2) was obtained from Corning Life Sciences (Lowell, MA, USA). Lipofectamine LTX was obtained from Invitrogen (Carlsbad, CA, USA). Anti-mouse IgG-FITC (F0257), anti-rabbit IgG (whole molecule)-TRITC (T6778) antibodies, paraformaldehyde and PLL solution (10×, 1 g/L, P8920) were obtained from Sigma–Aldrich.

2.2. Primary hippocampal neuronal cell culture

Hippocampal neuronal cell cultures containing few glial cells were prepared from Sprague–Dawley (SD) rat embryo E-18 (Koatech, Pyungtaek, Korea) as previously described [15]. Briefly, the necks of rat embryos were cut with scissors on a clean bench. The hippocampus was obtained by removing the cerebral cortex and meninges using a sterile technique. It was then minced and dissociated into individual cells using 0.25% trypsin in Hanks balanced salt solution (HBSS) at 37 °C for 15 min, and then triturated with a fire-polished Pasteur pipette. The resultant hippocampal neuronal cells were plated at a density of 800 cells/mm^2 onto 0.1 mg/mL poly-L Lysine (PLL)-coated glass slides in 6-well plates in neurobasal media (Invitrogen) containing B27 (Invitrogen), 2 mM L-glutamax (Invitrogen) and 12.5 μM L-glutamic acid (Sigma–Aldrich). Cultures were maintained at 37 °C under 5% CO_2. At 3 days after plating the cells, the media were exchanged with neurobasal media containing B27 and glutamax without glutamate, and incubated before treatment with FTI-277.

2.3. SRB assay

After each treatment, cells were fixed at room temperature (RT) for 30 min with 4% paraformaldehyde (PFA) for 15 min. The fixed cells were then washed with distilled water and stained with 0.4% (w/v) SRB in 0.1% (v/v) acetic acid solution at RT for 30 min. SRB dye bound to the cell mass was solubilised with 10 mM Tris (pH 10.5) and quantified using a spectrophotometer at 530 nm.

2.4. Western blot analysis

After each treatment, cells were washed twice with phosphate-buffered saline (PBS) and lysed in M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA) containing protease inhibitor cocktails (1 tablet/50 mL M-PER solution). The proteins in whole-cell extracts were then centrifuged at 13,000 × rpm, and the amount of protein in the supernatant was quantified using Bradford reagent and analysed by Western blotting as described previously [10].

2.5. Preparation of EGFP-RhoB-expressing plasmid

The EGFP gene was subcloned by PCR from the pEGFP-C1 vector with primers SacI-EGFP-5′ (5′-GCA*GAGCTCG*TTTAGTGAACCGT-3′) and SacI-EGFP-3′ (5′-AGCA*GAGCTC*CTTGTACAGCTCGTCCAGT-3′) and digested with SacI. The EGFP expression vector was prepared from pcDNA-DEST53. The GFP gene was removed by digestion of pcDNA-DEST53 with SacI. The EGFP fragment and vector fragment were then ligated to generate pcDNA-EGFP-DEST53. The EGFP-RhoB expression plasmid was prepared as follows: RhoB cDNA was amplified by PCR from a human kidney cDNA library (Clontech, Mountain View, CA, USA) using the RhoB-specific primers BamH1-RhoB-5′ (5′-AGCA*GGATCC*ATGGCGGCCATCCGCAAG-3′) and EcoR1-RhoB-3′ (5′AGCA*GAATCC*TCATAGCACCTTGCAGCA-3′), and inserted into the pENTR-3C vector (Invitrogen). Then, pENTR-3C-RhoB and pcDNA-EGFP-DEST53 were ligated with an LR Clonase II enzyme reaction to generate the pcDNA3-EGFP-RhoB expression plasmid for fluorescence microscopy. The hippocampal neuronal cells at 7 days *in vitro* (DIV) were transfected with pcDNA3-EGFP-only or pcDNA3-EGFP-RhoB for 4 days, and then fixed and immunostained with anti-β-tubulin III antibody.

2.6. Confocal fluorescent immunocytochemistry

After FTI-277 treatment, cells were fixed with 4% PFA in PBS for 15 min at RT. Then, they were permeabilised with 0.1% Trition X-100 in PBS for 15 min at RT and blocked with 6% bovine serum albumin (BSA) in PBS for 45 min at RT. Cells were probed for 1 h with 1.5% BSA–PBS containing each antibody. Then, cells were washed with PBS and stained with goat anti-mouse IgG-FITC or goat anti-rabbit IgG-TRITC antibodies, washed and analysed by confocal microscopy. Images were captured with an LSM 510 microscope using a 63 × 1.4NA Apochromat objective (Carl Zeiss, Wetzlar, Germany).

3. Results

3.1. FTI-277 induces neurotoxic effects on hippocampal neuronal cells from rat embryos

We investigated the neurotoxic effect of FTI-277 on hippocampal neuronal cells because primary hippocampal neuronal cells from rat embryos were well characterised by neurite development and maturation [16]. We first tested the effect of FTI-277 on the neuronal cells in the early stages of neurite outgrowth. To this end, we treated the cells at 2 DIV with 20 μM of FTI-277 for 4 days. Notably, treatment with FTI-277 substantially inhibited the neurite extension compared to the control, indicating that FTI-277 inhibits outgrowth of the neuronal cells in the early stages of neurite outgrowth (Suppl. Fig. 1).

We next investigated the neurotoxic effects of FTI-277 by analysing neurite maturation. At 7 DIV after plating, cells were treated with varying doses of FTI-277 and maintained for an additional 4 days (Fig. 1A). To quantify the relative cell mass including neuritis, the cells were fixed and the cell mass was quantified with a SRB assay (Fig. 1B). Treatment with FTI-277 decreased the cell mass in a dose-dependent manner, especially dramatically at 20 μM (by about 50% compared to the control). These results clearly showed that FTI-277 induces neurotoxicity of primary hippocampal neuronal cells during both neurite outgrowth and neurite maturation.

3.2. FTI-277 induces degeneracy of hippocampal neuronal cells from rat embryos

We next examined the cellular effects of FTI-277 at 20 μM to understand the molecular mechanisms responsible for the neurotoxicity. Cells at 7 DIV were treated with 20 μM of FTI-277 for 2 or 4 days. Consequently, in DMSO-treated control cells, the neurites were well established into full maturation. FTI-277 did not induced significant changes for 2 days in accordance with the previous report by Pooler et al. [17]. However, this agent induced the dramatic degeneracy of the treated cells for 4 days when the neurites of FTI-277-treated cells were thinner and scantier than those of the control. The cell lysates were assessed with Western blot analysis. As expected, treatment with 20 μM of FTI-277 dramatically decreased the levels of cyclin D1 for 4 days but not for 2 days, whose

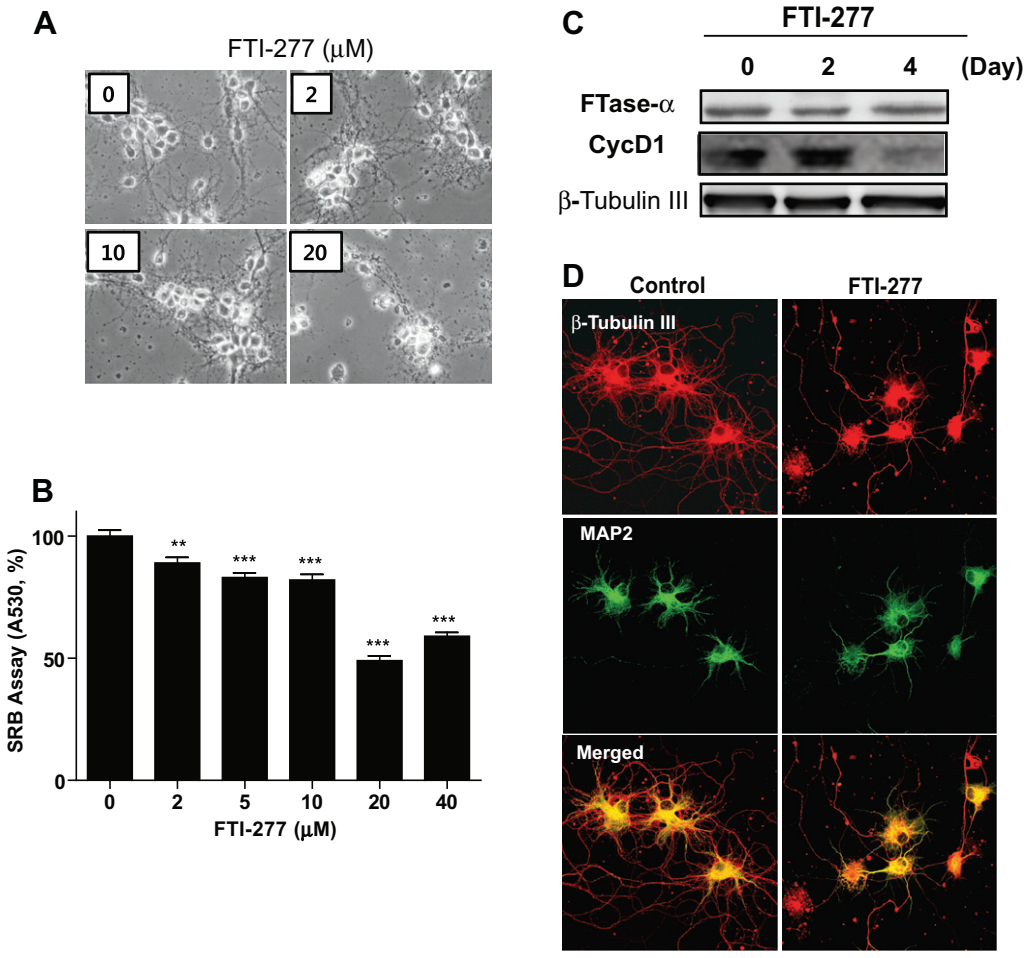

Fig. 1. Dose- and time- dependent neurotoxicity by FTI-277 in rat embryo hippocampal neuronal cells. (A) Rat embryo (E18) hippocampal neuronal cells at 7 DIV were treated with various doses of FTI-277 for 4 days and observed under a microscope. (B) The treated cells under the same conditions as (A) were fixed, and the cell mass was quantified with an SRB assay. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the untreated samples. (C) The hippocampal neuronal cells at 7 DIV were treated with 20 μM of FTI-277 for 0, 2, or 4 days and the cell lysates in the treatments were analysed with Western blotting using the indicated antibodies. (D) The hippocampal neuronal cells at 7 DIV were treated with 0 or 20 μM of FTI-277 for 4 days, fixed with 4% (v/v) formaldehyde solution, and immunostained using anti-β-tubulin III or anti-MAP2 antibody.

expression is known to be regulated by Ras-downstream signalling pathways. No change occurred in the FTase-α protein level, which is a molecular target of FTI-277 (Fig. 1C).

To verify the late neurodegeneracy by FTI-277, cells at 7 DIV were treated with 20 μM of FTI-277 for 4 days and analysed with immunocytochemistry using antibodies against the neuronal marker β-tubulin III or the dendrite-specific marker MAP2. Compared to DMSO-treated control cells, the neurites of FTI-277-treated cells were significantly thin and scarce (Fig. 1D). Furthermore, whereas the control cells exhibited the normal morphology of soma, FTI-277-treated cells showed shrinkage of soma into round shapes, implying that the agent was toxic to the cell body (Fig. 1D). Collectively, the above data indicated that FTI-277 induces neurodegenerative neurotoxic effects in rat embryo hippocampal neuronal cells for 4 days.

3.3. FTI-277 induces the neurodegeneracy of hippocampal neuronal cells via ROS generation

Previous reports have shown that FTIs induce cytotoxicity via rapid generation (in 3 h) of ROS in cancer cells [3,18,19]. Therefore, to address the neurotoxic mechanisms of FTI-277 in hippocampal neuronal cells, we first tested whether the ROS scavenger NAC protects from neurotoxicity because ROS are known to be critical mediators of the cytotoxicity of FTI in cancer cells [3]. To this end, we pretreated hippocampal neuronal cells (at 7 DIV) with 1 mM NAC for 30 min followed by the addition of 20 μM FTI-277 for 4 days. In this experiment, we observed that NAC significantly protected the neurite degeneracy (Fig. 2A, upper panel), which was supported by immunocytochemical staining using anti-MAP2 antibody (Fig. 2A, bottom panel).

In the SRB assay, prior treatment of NAC protected against FTI-277 neurotoxicity by about 25%, implying that ROS might be critical mediators of neurotoxicity by the agent (Fig. 2B). To clearly define the FTI-277-induced ROS generation in the neuronal cells, we stained the cells with 4 μM DHE after the treatments of 20 μM FTI-277 for 3 h. Compared to the control, FTI-277 dramatically increased the level of ROS (Fig. 2Ca), which was well correlated with previous reports in cancer cells [3]. Furthermore, ROS generation by FTI-277 was maintained for 4 days after treatment in the presence of the agent, revealed by staining with 4 μM DHE (Fig. 2Cb). Taken together, these data indicate that FTI-277 induced the ROS generation in hippocampal neuronal cells and that ROS might be critical mediators of FTI-277 neurotoxicity.

3.4. FTI-277 decreases synapsin I and increases the cytotoxic G-protein RhoB via ROS generation in hippocampal neuronal cells

To explain the neurodegenerative, neurotoxic effects of FTI-277 at the molecular level, we investigated whether FTI-277 affects the

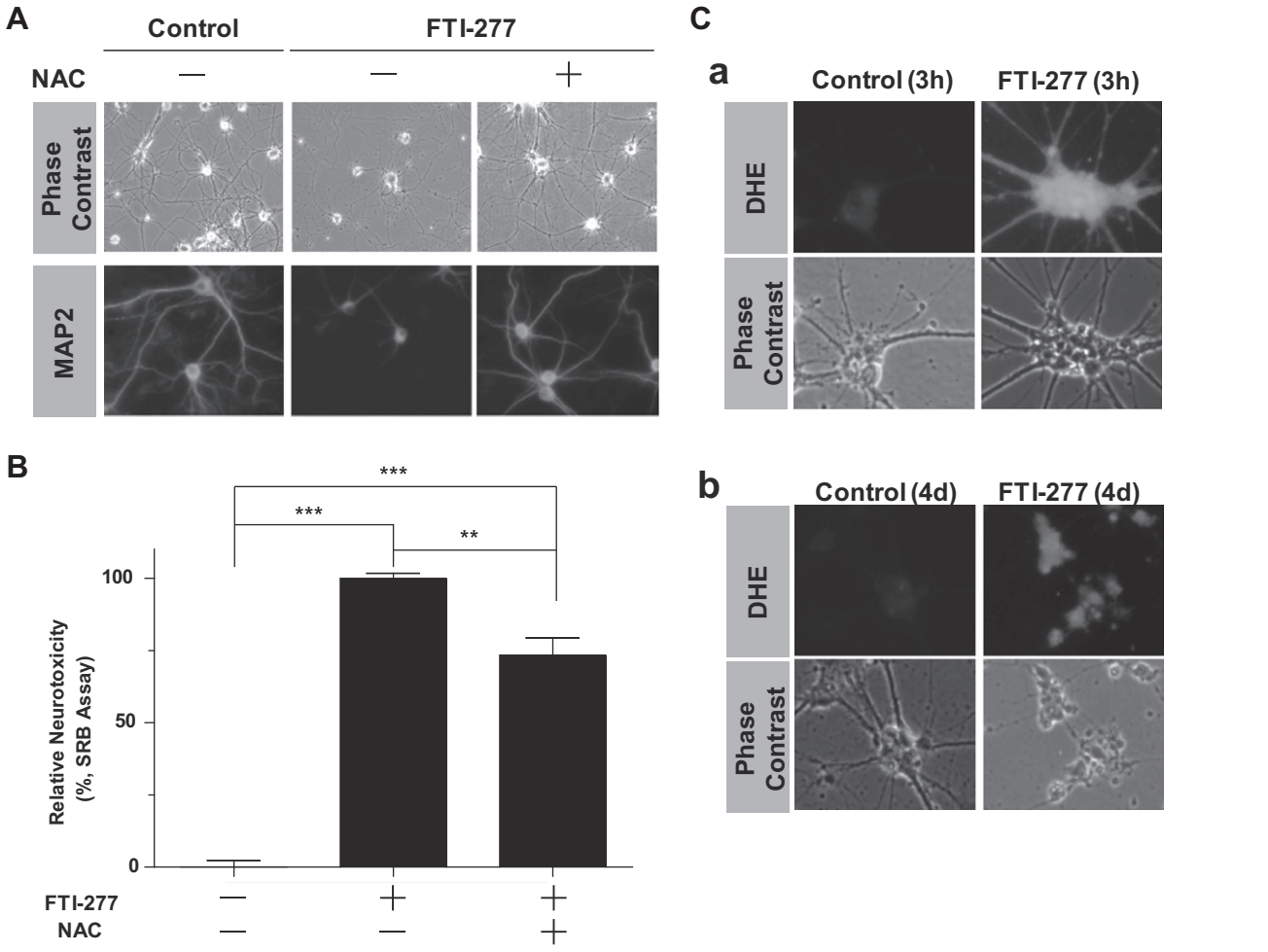

Fig. 2. FTI-277 induces the neurodegeneracy of rat embryo hippocampal neuronal cells via ROS generation. (A) The hippocampal neuronal cells at 7 DIV were pretreated with 0 or 1 mM NAC for 30 min, followed by 20 μM of FTI-277 for 4 days. The cells were then observed under a microscope (A, upper panel) or immunostained with anti-MAP2 antibody (A, bottom panel). (B) The neurotoxicity induced by 20 μM of FTI-277 only for 4 days was expressed as 100% and compared with that of the treatment in the presence of 1 mM NAC, which was pretreated 30 min before the FTI-277 treatment. The one-way ANOVA test applied to the significant effect of groups. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the untreated samples. (C) The hippocampal neuronal cells at 7 DIV were treated with 0 or 20 μM of FTI-277 for 3 h. Intracellular cellular ROS levels were then stained with 4 μM of DHE for an additional 30 min and observed under a fluorescent microscope.

level of synapsin I, which is well known to be involved in the neurite outgrowth and neuronal development of hippocampal neurons [20,21]. The neuronal cells at 7 DIV were treated with 20 μM of FTI-277 for 4 days, and the cell lysates were assessed using Western blotting. Note that treatment with FTI-277 dramatically decreased synaptophysin (Suppl. Fig. 2) and synapsin I (Fig. 3A), which correlated with the results of immunocytochemical staining against synapsin I at DIV 14–21 neurons (Fig. 3B). Considering that synapsin I is a component protein of synaptic vesicles, which play a crucial role in synaptic neurotransmission and plasticity for neuronal growth and differentiation, the decrease in synapsin I by FTIs is possibly involved in the neurotoxicity. Furthermore, FTI-277 dramatically increased cytotoxic G-protein RhoB expression. FTIs are well known to induce RhoB upregulation via the rapid generation of ROS in cancer cells for anticancer activity [3,22–24]. However, until now, no report has described the functional role of RhoB upregulation in primary neurons. In our study, when we induced the ectopic expression of GFP-only or GFP-RhoB in hippocampal neurons for 4 days, we found that ectopic expression of RhoB caused the shrinkage of soma of hippocampal neurons compared with that of RhoB-non-expressed cells (Fig. 3C). This indicated that upregulation of RhoB by FTI-277 may be involved in neuronal cell toxicity. We next investigated the effects of the ROS scavenger NAC on these neurotoxic events by FTI-277. Note that prior treatment with 1 mM NAC significantly inhibited the decrease of synapsin I and fully knocked down the increase of RhoB (Fig. 3A), implying that ROS generation might play a crucial role for the neurotoxic events by FTI-277.

4. Discussion

In the current study, we provided the neurotoxic effects of FTI-277 on rat embryo hippocampal neurons. FTI-277 inhibited the neurite outgrowth of hippocampal neurons at 2 DIV (Suppl. Fig. 1), which is an early stage of neuronal growth [16]. Long-term treatments with FTI-277 for 4 days induced a neurodegeneracy at a range of μM concentrations, especially at 20 μM (Fig. 1A). In the treatment of 20 μM for 4 days at 7 DIV, the cell mass was dramatically decreased by about 50% as revealed by SRB assay (Fig. 1B); cyclin D1, whose expression is known to be regulated by Ras, was also decreased (Fig. 1C). Moreover, neurites were severely degenerated as evidenced by immunocytochemical staining with anti-α-tubulin III (neuro-specific marker) and anti-MAP2 (dendrite-specific marker) antibody (Fig. 1D). These effects clearly show that FTI-277 induces neurotoxicity in hippocampal neurons for 4 days.

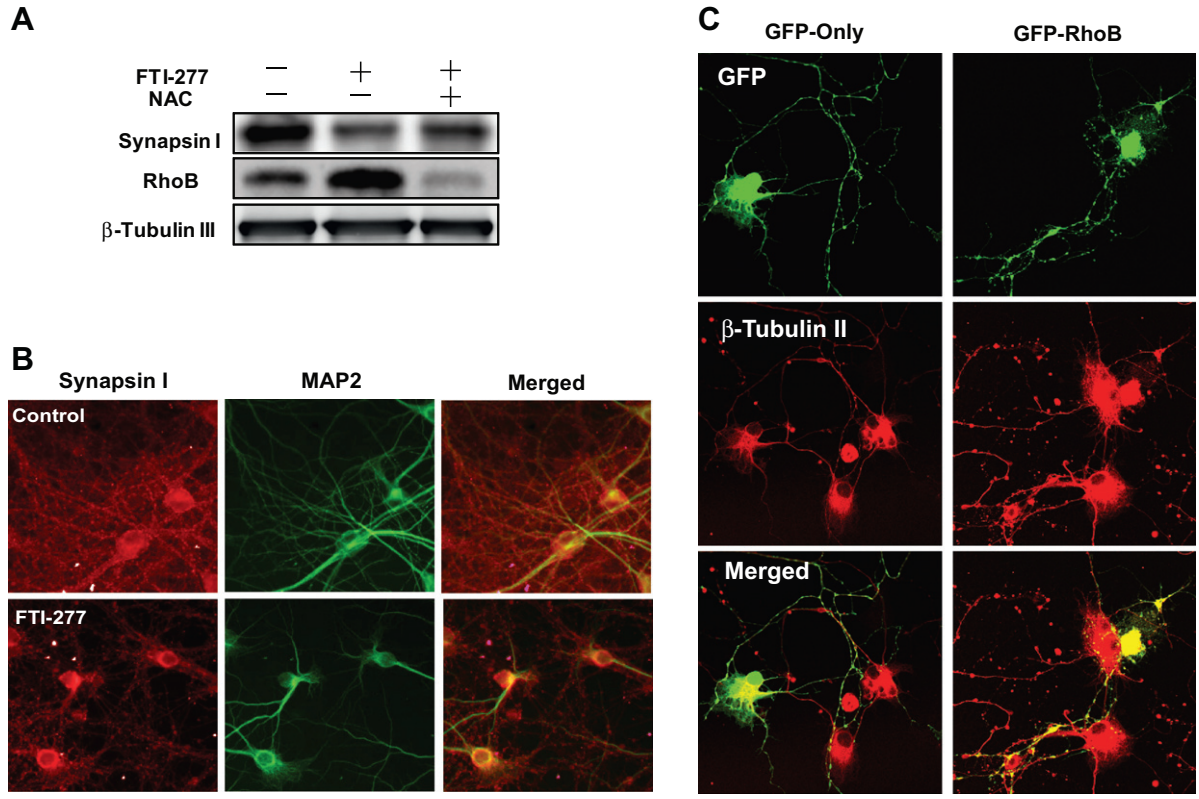

Fig. 3. FTI-277 decreases synapsin I and increases RhoB in rat embryo hippocampal neuronal cells via ROS generation. (A) The hippocampal neuronal cells at 7 DIV were pretreated with 0 or 1 mM NAC for 30 min, followed by 20 μM of FTI-277 for 4 days. The cell lysates were then analysed with Western blotting and compared with control samples. (B) The hippocampal neuronal cells at 14–21 DIV were treated with 0 or 20 μM of FTI-277 for 4 days, then immunostained with anti-synapsin I or anti-MAP2 antibody. (C) EGFP-only or EGFP-RhoB was ectopically expressed in hippocampal neuronal cells at 7 DIV using LTX lipofectamine for 4 days; the cells were then fixed and immunostained with anti-β-tubulin III antibody and observed under a fluorescent confocal microscope.

In an attempt to understand the mode of actions of FTI-277 neurotoxicity, we first investigated the functional role of ROS in the neurotoxicity of FTI-277 in hippocampal neurons because ROS are known to be rapidly generated by FTIs, causing cytotoxicity in cancer cells [3]. Note that prior treatment with the ROS scavenger NAC dramatically protected from FTI-277 neurotoxicity, indicating that ROS generation plays a crucial role in FTI-277 neurotoxicity (Fig. 2A). However, the neuroprotection of NAC from FTI-277-induced neurotoxicity was only about 25%, implying that other cytotoxic mechanisms in addition to ROS might be involved in the development of neurotoxicity by the FTI agent (Fig. 2B). When we examined whether FTI-277 induces ROS generation in the neuronal cells, we found that this agent dramatically increased the level of intracellular ROS in as early as 3 h after treatment; this was maintained for 4 days, as revealed by DHE staining (Fig. 2C).

We found that FTI-277 induces the decrease of synapsin I in the treated cells for 4 days (Fig. 3A). Synapsin I, a member of the synapsins, the most abundant phosphoproteins in synaptic vesicles, interacts with lipid and proteins in synaptic vesicles as well as with cytoskeletal proteins such as actin, thereby playing multifunctional roles, including neurite outgrowth, neuronal development and synapse formation [21,25]. Furthermore, the decrease in synapsin I is known to decrease neurite outgrowth, axonal development and synaptogenesis in hippocampal neurons [20], as well as to decrease the releasable pool of synaptic vesicles for synaptic plasticity and neurotransmission [26]. Therefore, our new finding that FTI-277 decreases synapsin I in hippocampal neurons is very promising and might explain the cause of the neurotoxicity. Additionally, we first found that FTI-277 dramatically increases RhoB expression in hippocampal neuronal cells (Fig. 3A), which was very well correlated with previous reports in cancer cells [3]. Even though the functional role of RhoB induction in neurons has not yet been clearly determined, our observations that ectopic expression of RhoB induces neurotoxicity in rat embryo hippocampal neuronal cells imply that the increase in RhoB by FTI-277 may be involved in the neurotoxicity (Fig. 3C). Additionally, prior treatment with NAC inhibited both the decrease in synapsin I and the increase in RhoB by FTI-277, indicating the functional role of ROS in the development of the neurotoxicity at the molecular level (Fig. 3A). The differential effect of ROS scavenger treatment on FTI-277-induced reduction of synaptophysin clearly indicate the involvement of alternative signal transduction pathways other than ROS signalling cascade in the neurodegenerative changes induced by FTI-277 treatment.

Nevertheless, the mode of actions of FTIs might be more complex, as described by Pan et al. (2005). However, in the current study, we clearly identified FTI-277-induced neurotoxicity in

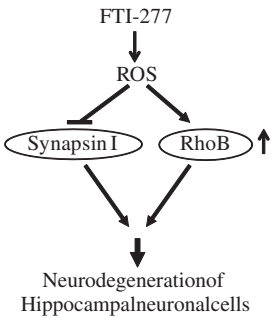

Fig. 4. Diagram of the mode of action of FTI-277 neurotoxicity in rat embryo hippocampal neuronal cells.

primary hippocampal neurons and the functional role of ROS in the neurotoxicity (Fig. 4). Considering that the hippocampus is a brain structure involved in spatial learning and memory, and that damage to hippocampal neurons generally leads to cognitive impairment such as dementia and Alzheimer's disease [27–31], damaging hippocampal neurons by FTI-277 might lead to serious cognitive disorders such as dementia. Therefore, our finding that FTI-277 induces neurotoxicity via ROS generation may shed light on how to avoid this neurotoxicity in clinical drug development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.123.

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