Cellular Cytotoxic Response Induced by Highly Purified Multi-wall Carbon Nanotube in Human Lung Cells

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Abstract

Carbon nanotubes, a promising nanomaterial with unique characteristics, have applications in a variety of fields. The cytotoxic effects of carbon nanotubes are partially due to the induction of oxidative stress; however, the detailed mechanisms of nanotube cytotoxicity and their interaction with cells remain unclear. In the present study, we focus on the acute toxicity of vapor-grown carbon fiber, HTT2800, which is one of the most highly purified multi-wall carbon nanotube (MWCNT) by high-temperature thermal treatment. We exposed human bronchial epithelial cells (BEAS-2B) to HTT2800 and measured the cellular uptake, mitochondrial function, cellular LDH release, apoptotic signaling, reactive oxygen species (ROS) generation and pro-inflammatory cytokine release. The HTT2800-exposed cells showed cellular uptake of the carbon nanotube, increased cell death, enhanced DNA damage and induced cytokine release. However, the exposed cells showed no obvious intracellular ROS generation. These cellular and molecular findings suggest that HTT2800 could cause a potentially adverse inflammatory response in BEAS-2B cells.

Keywords

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Introduction

In the present study, we used the *in vitro* acute responses of cultured human bronchial epithelial cells as a model system to study the potential inhalation health effects of HTT2800. We evaluated the toxicological potential of HTT2800 on BEAS-2B cells by studying the cellular uptake, cell viability, oxidative stress, apoptotic signaling pathway and pro-inflammatory cytokine release. Bronchial epithelial cells are targets of inhalation and play a critical role in the maintenance of mucosal integrity as mechanical barriers against various particulate [1]. *In vitro* toxicology studies have attempted to link specific particle sources with cellular responses. Cellular and animal exposure investigations on the toxicity and pathogenicity of carbon nanotubes have demonstrated biological interactions, including cell proliferation [2], oxidative stress [3], apoptosis [4] and inflammatory reactions [5]. However, the existing information on the lung toxicity of multi-wall carbon nanotubes (MWCNTs) are limited and remains inconclusive.

The BEAS-2B cell line is the non-tumorigenic SV-40 immortalized cell line closest to normal bronchial epithelium [6]. We assessed the cytotoxicity and oxidative stress in BEAS-2B cells after acute exposure to HTT2800, which is one of the most distributed highly purified carbon nanotubes. The iron catalyst is removed from HTT2800 by thermally treating the material at a temperature of 2,800°C in an argon atmosphere [7]. Only a very low concentration of the iron-based material remains in the heat-treated HTT2800. Recently, MWCNTs have been commercially supplied as an additive for Li-Ion batteries, fuel cells and capacitors (formerly known as condensers) produced by Japanese companies. The bulk volumes of manufactured MWCNTs have already reached a few hundred tons per year [8]. Investigations of potential medical applications are also being considered [9]. Therefore, it is necessary to learn more about the possible toxicity of this nanomaterial. The aim of the present study was to investigate the *in vitro* acute responses of bronchial epithelial cells after exposure to HTT2800. We focused on the

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effects of HTT2800 on cellular uptake, cell viability, production of ROS and release of typical

pro-inflammatory mediators such as cytokines.

Materials and methods

Reagents

We used the highly purified MWCNT abbreviated as HTT2800, which has been previously

described in detail [2]. HTT2800 was dispersed with 0.1% gelatin (Nacalai Tesque, Kyoto, Japan)

in phosphate buffered saline (PBS, pH 7.4) and ultrasonicated before use. Tissue culture media

and cisplatin were obtained from Sigma Aldrich (St. Louis, MO, USA). Rabbit polyclonal

antibody against caspase-3 (sc-7148) was purchased from Santa Cruz Biotechnology (Santa Cruz,

CA, USA).

Cell culture

BEAS-2B cells were purchased from American Type Culture Collection (Manassas, VA, USA).

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS,

penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown and maintained in 100-

mm culture plates (Iwaki, Tokyo, Japan) at 37°C in a 5% CO_2 incubator.

Assessment of cellular uptake of HTT2800 in BEAS-2B cells

BEAS-2B cells were incubated with HTT2800 (30 μg/ml) and stained with Hoechst 33342

(Dojindo, Kumamoto, Japan, 5 μg/ml) for 10 min at 37°C in a 5% CO_2 incubator. The stained

cells were analyzed on an IX71 fluorescence microscope (Olympus, Tokyo, Japan) with

excitation at 360 nm. Cells were visualized by light or fluorescence microscopy. For time-lapse

imaging, BEAS-2B cells were grown on a glass-bottom dish (Greiner, Frickenhausen, Germany)

for 24 h and exposed to HTT2800 (5 μg/ml) in medium containing Hoechst 33342 (1 μg/ml). The

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dish was placed on the stage of a LSM510 NLO laser-scanning confocal microscope (Zeiss, Jena, Germany) and was kept at 37°C in a 5% CO_2 incubator. Time-lapse confocal images were collected every 2 min for 16 h using a ×20 PlanFluor objective.

Alamar blue assay

To determine the viability of BEAS-2B cells exposed to different concentrations of HTT2800, we performed an Alamar blue assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cells were incubated for 24 h at 37°C in the presence or absence of HTT2800. Viable cells metabolized the dye, resulting in an increase of fluorescence by excitation/emission at 538/590 nm. Cytotoxic activity was calculated as follows: % cytotoxicity=100×(0% value-experimental value/0% value-100% value). Test media were assayed in triplicate for each treatment condition.

Lactate dehydrogenase (LDH) release assay

BEAS-2B cells were seeded onto 96-well plates at a density of $2×10^4$ cells/well and were incubated for 72 h at 37°C in the presence or absence of HTT2800. LDH activity was measured in the culture medium using an LDH cytotoxicity assay kit (BioChain Institute, Heyward, CA, USA) and expressed as a percentage versus the detergent-extracted controls (100% cytotoxicity). The red formazan product was measured at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Test media were assayed in triplicate for each treatment condition.

Determination of ROS

The ROS assay was performed according to the manufacturer's instructions. Briefly, BEAS-2B cells were seeded in 12-well plates at a density of $5×10^4$ cells/well and incubated at 37°C for 24 h. Then, the culture medium was aspirated and cells were washed with Dulbecco PBS (-) (DPBS)

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followed by the addition of 1 ml of fresh culture medium containing 10 μM carboxy-DCFDA (C-400, Molecular Probes, CA, USA) dissolved in DMSO. After cells were incubated for 15 min in a CO_2 incubator, 10 μl of test solution was added to the cells. Hydrogen peroxide (100 μM) was used as a positive control stimulus. Following exposure for 60 min, the cells were washed by DPBS once and harvested with trypsin-EDTA. Finally, the cells were suspended with 0.3 ml of 10% FBS in DPBS and passed through nylon mesh. Cells were subjected to flow cytometry (FACSCalibur™, Becton Dickinson, San Jose, CA, USA) until 20,000 events were recorded.

Western blot analysis

BEAS-2B cells were treated with HTT2800 (30 μg/ml) for the indicated time points. Cells were washed 2 times with ice-cold PBS and solubilized with whole-cell extraction buffer (20 mM HEPES (pH 7.9), 0.5% NP-40, 15% glycerol, 300 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin and 0.5 mM PMSF). The cell lysate was centrifuged at $14,000 \times g$ for 5 min, and the protein in the supernatant was quantified using Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA). Total protein was diluted 1:4 with lane marker reducing sample buffer (ThermoFisher Scientific, Waltham, MA, USA) and boiled for 5 min. The resultant protein was then separated on 10% SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (pH 7.6) for 1 h at room temperature and probed with primary rabbit anti-human caspase-3 antibody at 4°C overnight. After washing, the membrane was incubated with secondary anti-rabbit antibody (GE Healthcare, Little Chalfont, England) for 1 h at room temperature and then developed with ECL-plus chemiluminescent detection reagent (GE Healthcare, Piscataway, NJ, USA).

Assay of cytokines in culture supernatant

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Cytokines were measured with a cytometric bead array set system (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol. Briefly, BEAS-2B cells were exposed to HTT2800 (30 μg/ml) or lipopolysaccharides (LPS, 100 ng/ml) for 24 h, and then cytokine capture beads (for IL-12, TNF-α, IL-10, IL-6, IL-1β and IL-8) were added to the samples or cytokine standards in flow cytometry tubes. The mixtures were vortexed, and antibody for fluorescence detection was added to each tube. The samples were incubated at room temperature for 3 h. Beads were pelleted by centrifugation, washed once, and resuspended prior to reading with a FACSCalibur™ apparatus.

Statistical analysis

Data are presented as the mean ± SEM. Values were compared to the negative control or positive control using the Dunnett's test after performing ANOVA. We considered p values less than 0.05 to be statistically significant.

Results

Uptake of HTT2800 by BEAS-2B cells

We used light and fluorescence microscopy to study BEAS-2B cells exposed to HTT2800. The HTT2800 particles were internalized inside the cells (Fig. 1A and B). After staining the cell nuclei with Hoechst 33342, we observed an accumulation of HTT2800 around the nucleus (Fig. 1B and 2E) as well as balloon-like nuclear morphology (Fig. 2D), which is typically associated with necrotic cell death [10].

Acute toxicity of HTT2800

Cytotoxicity assays are widely used for *in vitro* toxicology studies. However, each assay has a different sensitivity [11]. Hence, we evaluated the effects of HTT2800 on the viability of BEAS-2B cells using Alamar blue dye uptake and an LDH leakage assay. Alamar blue has improved

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sensitivity and performance when compared to MTT assays [12]. As shown in Fig. 3A,

mitochondrial function studies performed using the Alamar blue assay indicated that the cell

metabolism was markedly decreased by HTT2800 treatment. There was a dose-dependent

decrease in cell viability at concentrations as low as 10 μg/ml (IC_{50}=15 μg/ml). HTT2800 toxicity

was also evaluated by the LDH leakage assay. Intracellular LDH leakage is an indicator of cell

membrane integrity and cell toxicity [11]. As shown in Fig. 3B, the LDH leakage increased in a

dose-dependent manner. This result is consistent with the results from the Alamar blue uptake

assay.

Formation of ROS

Next, we investigated whether HTT2800 causes ROS formation in BEAS-2B cells using DCF

(2',7'-dichlorofluorescin diacetate) fluorescence as a reporter of intracellular oxidant production.

No obvious DCF response was observed after a 1-h exposure to HTT2800 at concentrations

between 0.1 and 30 μg/ml (Fig. 4A).

Detection of cleaved caspase-3 after exposure to HTT2800

Caspase-3 is the most extensively studied apoptotic protein among caspase family. Caspase-3

activation was correlated with internucleosomal DNA fragmentation of the cells [13]. However,

HTT2800 did not induce common apoptotic fragmentation of caspase-3 (Fig. 4B). These results

indicate that HTT2800 triggers necrotic effects in BEAS-2B cells on the plasma membrane such

that plasma membrane is ruptured.

Inflammatory response

BEAS-2B cells secrete inflammatory mediators like cytokines upon stimulation [14]. We

examined the possibility that HTT2800 causes the release of pro-inflammatory cytokines by

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BEAS-2B cells. The amounts of both IL-6 and IL-8 in the cell supernatant were significantly increased after a 24-h incubation with HTT2800 (Fig. 5A and B). Other cytokines (IL-12, TNF-α, IL-10 and IL-1β) were not detected after exposure to HTT2800 (data not shown).

Discussion

Studies have suggested [3] that single-wall carbon nanotubes (SWCNT) are not readily taken-up by lung cells. Herzog et al. reported no uptake of SWCNT by BEAS-2B cells [3]. However, the potential uptake of MWCNT was not examined. Light or fluorescence microscopy images demonstrate that HTT2800 enters the cell and becomes concentrated around the nucleus. Carbon nanotubes are carbon cylinders composed of benzene rings. They have been used to mediate drug delivery into cells [15] [16]. These recent reports are consistent with our own results. Dai *et al*. suggested that an energy-dependent endocytosis mechanism is responsible for the uptake of carbon nanotubes [17]. Endocytosis is the process by which cells absorb molecules or substances from outside the cell by engulfing them with the cell membrane. HTT2800 might be able to cross the plasma membrane to enter the intracellular compartment. Although nanoparticles offer many advantages as drug carrier systems, there are still many limitations that must be solved. For example, we found abnormal nuclear morphology that indicates cellular toxicity in cells exposed to HTT2800. These results may be caused by direct damage to DNA. We examined whether HTT2800 exposure induced necrotic or apoptotic cell death in BEAS-2B cells. Cells undergoing necrosis typically exhibit swelling, followed by the loss of membrane integrity and the release of their cytoplasmic contents. The viability of HTT2800-exposed cells was remarkably decreased, and the LDH leakage rate was increased in a dose-dependent manner. Thus, BEAS-2B cells may undergo necrosis, in which they lose membrane integrity. Furthermore, the apoptosis markers of caspase-3 activation were absent after exposure to up to 100 μg/ml of HTT2800. We speculate that BEAS-2B cells undergoing necrosis *in vitro* do not have sufficient time or energy to activate the apoptotic machinery.

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Airway epithelial cells are at risk for damage after the inhalational of ROS found in many

environmental particulates [18]. Oxidative damage due to ROS results in damage to DNA,

proteins and lipids and the activation of cell signaling pathways associated with the loss of cell

growth regulation [19]. In the present study, BEAS-2B cells exposed to HTT2800 exhibit cellular

uptake, decreased cell viability and increased LDH leakage. However, by measuring the

production of ROS upon HTT2800 exposure, we found no obvious oxidative stress. Dick *et al.*

reported that the potential hazard is strongly dependent on the iron catalyst content [20]. In the

present study, as mentioned above, the iron in HTT2800 was removed by thermal treatment in an

argon atmosphere [7]. We conclude that highly purified HTT2800 is not a potent inducer of ROS

production in BEAS-2B cells and may also not contribute to enhanced oxidative stress under

these experimental conditions.

On the other hand, the production of IL-6 and IL-8 is an indicator of an inflammatory response

by HTT2800-exposed cells. IL-6 is linked to allergic responses involving asthma [21], while IL-8

is associated with chronic obstructive pulmonary disease (COPD) [22]. IL-6 stimulates the acute-

phase reaction, which enhances the innate immune system and protects against tissue damage

[23]. MWCNTs represent a possible health risk for pulmonary fibrosis due to their fiber-like

shape and potential for persistence in the lung [24]. MacNee *et al*. reported that IL-8 is a

chemoattractant chemokine that plays a role in the initiation of airway inflammation [25]. These

cytokine families are a group of pleiotropic mediators produced by a variety of cells and tissues in

response to inflammatory stimuli [26] [27]. In the present study, both the IL-6 and IL-8 secretion

by BEAS-2B cells treated with HTT2800 is high compared to the response to LPS, which was

used as the positive control. Our results indicate a poor correlation between the potency for

inducing cytokines and the ROS production in BEAS-2B cells. In conclusion, the acute cellular

response studies reported here demonstrate that HTT2800 induced pro-inflammatory cytokine

release in an ROS production–independent fashion in BEAS-2B cells. Akbulut et al. reported that

IL-6 and IL-8 levels were significantly elevated in COPD patients [28]. This result could be

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interpreted to mean that HTT2800 might be associated with incidences of compromised chronic

respiratory health issues. Further studies are needed to determine if this *in vitro* finding can be

extrapolated to the *in vivo* system.

Acknowledgments

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Figure legends

Fig. 1 Cellular uptake of HTT2800 in BEAS-2B cells

Cells were treated with HTT2800 (30 μg/ml) in DMEM and incubated for 12 h at 37°C in a 5% CO_2 atmosphere. At the end of the 12-h exposure, cells were washed in PBS and stained with Hoechst 33342 (0.1 mg/ml in DMEM) for 10 min at 37°C. A, HTT2800 was taken up by BEAS-2B cells at 12 h. B, Nuclei stained with Hoechst 33342. Cells were visualized by light or fluorescence microscopy. *Bar* indicates 20 μm. (A and B)

Fig.2 Morphological analysis of BEAS-2B cells

Cells were treated with vehicle or HTT2800 (30 μg/ml) in DMEM and incubated for 24 h at 37°C in a 5% CO_2 incubator. At the end of the 24-h exposure, cells were washed in PBS and then stained with Hoechst 33342 (0.1 mg/ml in DMEM) for 10 min at 37°C. A and B, Vehicle (0.1% gelatin) treatment. C and D, Treatment with HTT2800 for 24 h. Bar indicates 20 μm. E, Merged image. Bar indicates 8 μm. Cells were visualized by light or fluorescence microscopy. Arrows indicate nuclei with balloon morphology.

Fig. 3 Effect of HTT2800 on cell viability

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For the Alamar blue dye uptake assay (A), BEAS-2B cells (2×10^4 cells/well in a 96-well plate) were incubated overnight and then treated with different concentrations of HTT2800 for 24 h at 37°C in a 5% CO_2 incubator. For the LDH leakage assay (B), BEAS-2B cells were seeded onto 96-well plates at a density of 1×10^4 cells/well, incubated overnight, and then treated with different concentrations of HTT2800 for 72 h at 37°C in a 5% CO_2 incubator.

Fig. 4 ROS generation and apoptotic pathway activation

(A) Intracellular production of ROS in BEAS-2B cells treated with HTT2800. BEAS-2B cells were incubated for 1 h without HTT2800 (negative control) or with 0.1, 1, 5, 10 or 30 μg/ml of HTT2800 and stained with C-400. Fluorescent intensity was monitored by flow cytometry. The intracellular generation of ROS is expressed as the percentage of control. Hydrogen peroxide (100 μM) was used as a positive control. Bars indicate the mean ± SEM of two independent experiments. (B) The effect of HTT2800 on caspase-3 activation. BEAS-2B cells were exposed to HTT2800 (30 or 100 μg/ml) at 37°C for 24 h. Cell lysates were collected in whole-cell extraction (WCE) buffer, and 80 mg of proteins were loaded for SDS-PAGE. The apoptosis assay was detected using anti-caspase-3 antibody. Cisplatin (10 μM) was used as a positive control.

Fig. 5 IL-6 and IL-8 release after exposure of BEAS-2B cells to HTT2800

BEAS-2B cells were incubated with HTT2800 (30 μg/ml) for 24 h. Then, the supernatants were collected and both the IL-6 and IL-8 concentrations were determined using a cytometric bead array Flex set system (BD Biosciences). The release of IL-6 (A) and IL-8 (B) by BEAS-2B cells increased after exposure to HTT2800. LPS (100 ng/ml) was used as a positive control. (Bars indicate the mean ± SEM, n=3, **$p<0.01$).

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A

Light
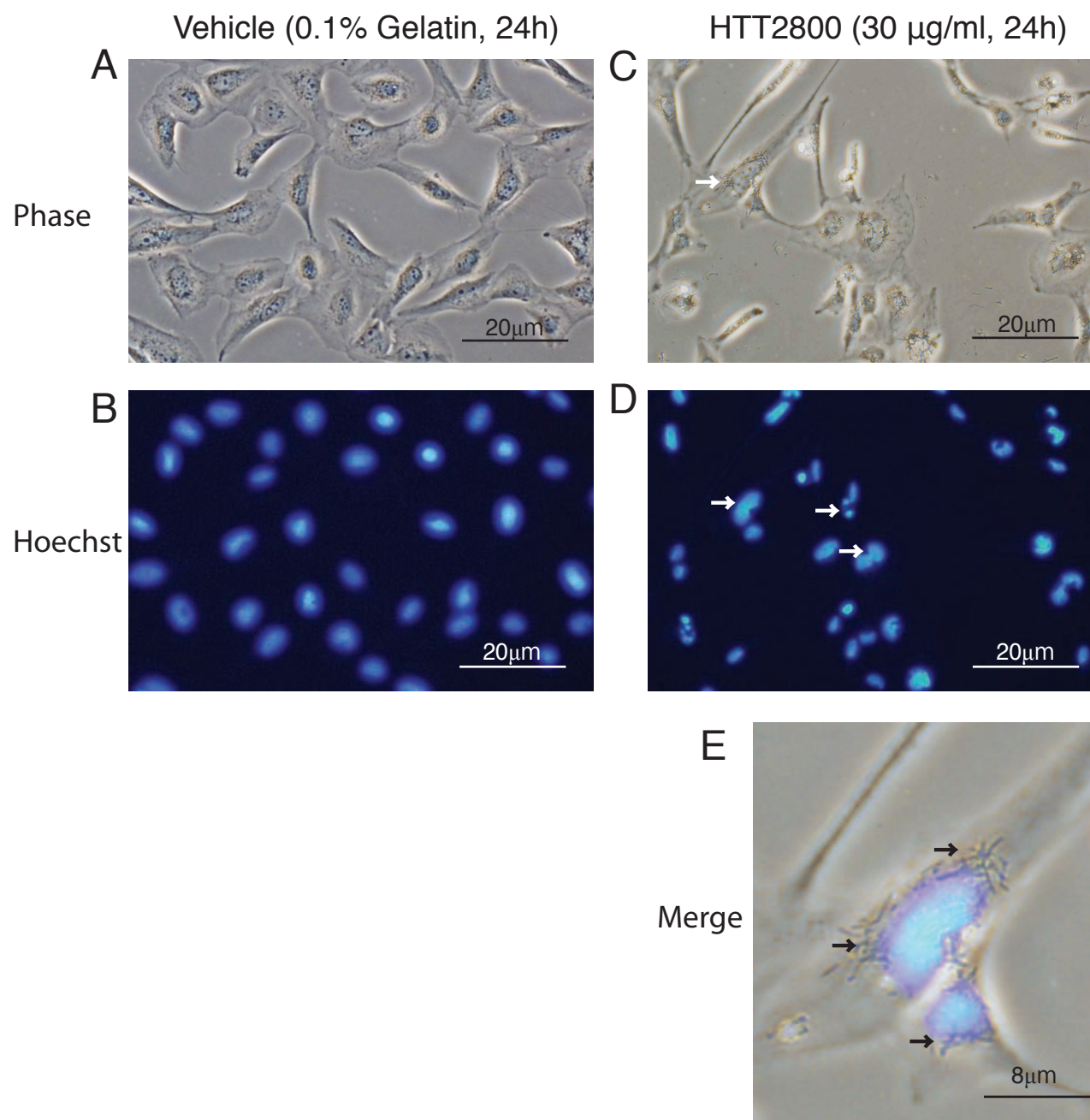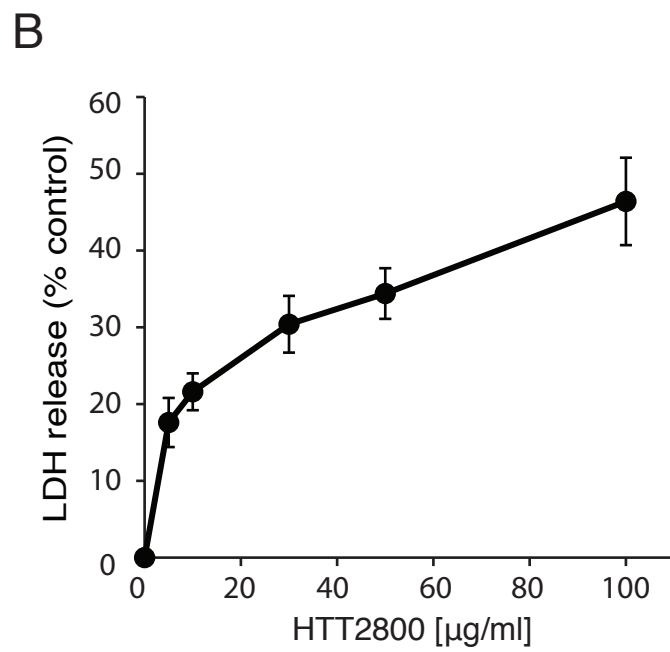

20 μm

B

Merge

20 μm

Fig.1

Fig.2

A

B

Fig.3

Fig.4

A

Percent ROS generation vs control

200

150

N.S.

100

50

0

HTT2800 [μg/ml] — 0 0.1 1 5 10 30
H₂O₂ [μM] 100 — — — — — —

B

Cisplatin

HTT2800 [μg/ml]

0   30   100

-Procaspase3

-Active form

Fig.4

A

B

Fig.5