Anticancer Activity of ZnO Nanoparticles Against Human Small-Cell Lung Cancer in an Orthotopic Mouse Model

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Running title
ZnO Nanoparticles Inhibit Growth of Small-Cell Lung Cancer

Keywords
Small-Cell Lung Cancer, Zinc Oxide, Reactive Oxygen Species, ROS-induced DNA damage, Orthotopic Lung Cancer Model

Disclosure of Potential Conflicts of Interest
R. Tanino received research funding from Nippon Boehringer Ingelheim. Y. Tsubata received honoraria for lecturing from Daiichi Sankyo, Chugai Pharmaceutical and AstraZeneca. T. Isobe received honoraria for lecturing from AstraZeneca, Pfizer and Boehringer Ingelheim.

Word count: 5216
Figures and tables: 6 figures, no tables
Abstract

Small-cell lung cancer, a highly malignant form of lung cancer, often responds to first-line treatments but relapses in most cases with resistance to further treatments. We tested ZnO nanoparticles against small-cell lung cancer and other cancer cell lines, in light of reported anticancer effects in vitro. Because of a strong safety record, ZnO nanoparticles are frequently used in biomedical research, including in cellular imaging and drug delivery, and have been used for many years in several commercial products such as skin care agents. Strikingly, ZnO nanoparticles were genotoxic against small-cell lung cancer cells, resulting in low viability, even in cells orthotopically grafted onto mouse models. However, the nanoparticles were less cytotoxic against normal lung-derived cells and did not elicit observable adverse effects after intravenous administration. ZnO nanoparticles were also found to induce highly reactive oxygen species and DNA leakage from nuclei. This study is the first comprehensive evaluation of the anticancer effects of ZnO nanoparticles in vitro and in vivo and highlights new therapeutic opportunities against small-cell lung cancer.
Introduction

Approximately 15% of all newly diagnosed cancers are lung cancer, which is the most common cancer and the leading cause of cancer death worldwide (1). Small-cell lung cancer represents about 15% of lung cancers and is the fastest progressing form (1,2). Several therapeutics, including tyrosine kinase inhibitors and immune-checkpoint inhibitors, are established treatments for non-small cell lung cancer (3,4); however, treatments against small-cell lung cancer have barely progressed (5). Accordingly, a combination therapy comprised of a platinum anticancer drug and a topoisomerase inhibitor has been used as a standard first-line regimen against the latter for over 30 years. While first-line treatments often shrink small-cell lung cancer tumors, a combination of chemotherapy and the immune-checkpoint inhibitor atezolizumab was recently shown to significantly enhance overall and progression-free survival as compared to chemotherapy alone (6). Nevertheless, second-line chemotherapy against small-cell lung cancer remains limited, although patients with high tumor mutation burden may gain the most benefit from immune-checkpoint inhibitors (7). Indeed, relapse and drug resistance to second-line therapy with a different topoisomerase inhibitor are reasonably common, resulting in low response rates (< 10%) (5). Comprehensive genomic surveys revealed that most small-cell lung cancer cells harbor mutations in RB transcriptional corepressor 1 (RB1) and tumor protein p53 (TP53), which are critical to apoptosis (8–10). These mutations elicit steady proliferation and prevent TP53-mediated apoptosis or RB1-mediated cell cycle arrest following chemotherapy (11,12). Unfortunately, other molecular mechanisms that drive drug response in small-cell lung cancer cells are poorly understood. For example, over 60 molecular inhibitors have been tested in clinical trials against small-cell lung cancers (5), even though a suitable target has not been identified. Thus, we have investigated a potentially novel anticancer drug with a different mode of action than other first-line chemotherapies, with a view to overcome the subsequent drug resistance.

Zinc oxide (ZnO) is an inorganic semiconductor compound that is typically produced as amphoteric particles that are practically insoluble in water. ZnO is inexpensive and used in cosmetic and skin care products such as sunscreens, since it absorbs ultraviolet light at wavelengths of 350-380 nm (13,14). ZnO particles, especially nanometer-scale particles, have similarly been exploited in biomedical and preclinical research, including in cellular imaging and drug delivery (15,16). For instance, particles less than 100 nm in hydrodynamic diameter were reported to be the most efficiently delivered in vivo (17). Notably, ZnO nanoparticles are also known to have an effect on many cancer cells in vitro (18), presumably because Zn^{2+} triggers reactive oxygen species (ROS) production (19,20). Ultraviolet light can also promote ZnO electrons from the valence band to the conduction band to produce photocatalytic ROS (21,22). However, Wang et al. (23) reported that ZnO protects macrophages from the cytotoxic effects of an anticancer drug, while Fujihara et al. (24,25) reported that intravenously administered ZnO nanoparticles accumulate in several tissues, particularly lung tissues, and elicit ROS-related phenomena using healthy mice. Collectively, these observations imply that ZnO may target small-cell lung cancer cells via a pathway distinct from that of current chemotherapies. Nevertheless, ZnO has not been evaluated in vivo against lung cancer. Hence, here we test ZnO against human small-cell lung cancer cells in vitro and in orthotopic mouse models.
Materials and methods

Preparation and evaluation of ZnO nanoparticles

Nitrogen-doped ZnO particles fabricated by dc arc dusty plasma were purchased from Furukawa Denshi (Fukushima, Japan). Particles (0.1 g/tube) were suspended in 1% sodium polyacrylate in PBS and homogenized for 4 h in a 2.0 mL tube by shaking with zirconia balls (3.0 g/tube) at 1,800 rpm using a CM-1000 high-speed shaker (Tokyo Rikakikai, Tokyo, Japan). The suspension was then centrifuged at 20,000 × g for 1 min to remove aggregates. The resulting supernatant was collected for use in all subsequent experiments. Particle-size distribution was measured on an LB-550 instrument (HORIBA, Kyoto, Japan). Ultraviolet-visible absorption spectra were collected on a UV-3600 system (Shimadzu, Kyoto, Japan), while photoluminescence was measured on a FluoroMax-4 (HORIBA).

Cell culture

NCI-N417 (N417), H82, H187 human small-cell lung cancer cells, BEAS-2B human bronchial epithelial cells, and MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). Normal human lung OUS-11 cells were purchased from JCRB Cell Bank (Osaka, Japan). LS174T human colon adenocarcinoma cells were kindly provided by Professor Yoshio Honma of Shimane University. N417 cells were authenticated by short tandem repeat profiling using PowerPlex 16 STR System (Promega, WI, USA). Other cell lines were not additionally authenticated after receipt. Cells were grown for no more than a month after thawing and routine mycoplasma testing was not performed. Cells were cultured at 37 °C in a 5% CO2 incubator and in RPMI-1640 medium supplemented with 10% FBS and 50 μg/mL gentamicin sulfate (N417, H82, H187, OUS-11, MCF-7, and LS174T) or BEGM medium (BEAS-2B). Q-VD-OPh was purchased from Bay Bioscience (Kobe, Japan), while 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt (Tiron) was purchased from Abcam (Cambridge, Cambridgeshire, UK). TP53 mutation status is described below. N417, pE298*; H82, c.375G>T leading to aberrant splicing; H187, p.S241C; OUS-11, unknown; BEAS-2B, wild type; MCF-7, wild type; LS174T, wild type. Information of TP53 mutation status was obtained from TP53 Cell Lines Compendium Version 3.1.

Hematoxylin and eosin staining

N417 cells treated with vehicle or ZnO were harvested and centrifuged for 5 min at 1,500 rpm in a Cytospin 4 (Life Technologies, Carlsbad, CA, USA), while OUS-11 cells were cultured and treated with vehicle or ZnO on an 8-well Falcon Culture Slide (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cells thus obtained were stained with hematoxylin and eosin, along with tissues (Sakura Finetek Japan, Tokyo, Japan).

Flow cytometry analysis of apoptosis and necrosis

N417 cells were cultured for indicated periods in a 6-well culture plate with 10 μg/mL of ZnO nanoparticles. BEAS-2B cells were cultured for 24 h in a 60-mm dish with the addition of 15 μg/mL ZnO nanoparticles. Apoptotic and necrotic cells were then quantified using MEBCYTO Apoptosis Kit (Medical & Biological Laboratories, Nagoya, Japan) in accordance with the manufacturer’s instructions. Flow cytometry was performed on a BD FACSCalibur and in BD CellQuest Pro software (Becton, Dickinson, and Company).

Immunoblotting
N417 or LS174T cells were cultured in a 60-mm culture dish, treated with vehicle or ZnO, harvested at 2500 × g for 10 min, washed with PBS, lysed in M-PER Mammalian Protein Extraction Reagent (Life Technologies) containing 1% Protease Inhibitor Cocktail (Life Technologies) and 1% Phosphatase Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan), and centrifuged at 14,000 × g for 15 min. The resulting supernatants were collected, and total protein was quantified by Coomassie Plus Bradford Protein Assay Kit (Life Technologies). Lysates were mixed with Bolt 4× LDS Sample Buffer (Life Technologies) and Bolt 10× Sample Reducing Agent, and heated at 95°C for 5 min. Equal volumes of each sample were electrophoresed on a 10% tris-glycine gel in a Bolt Mini Gel Tank (Life Technologies), transferred to a 0.2 µm ClearTrans Nitrocellulose Membrane (FUJIFILM Wako Pure Chemical, Osaka, Japan) using a Mini Blot Module (Life Technologies), and probed with primary antibodies to Cleaved PARP (Asp214) (Cell Signaling Technology, Danvers, MA, USA), Phospho-Chk2 (Thr68) (Cell Signaling Technology) and GAPDH (Cell Signaling Technology). Anti-Rabbit IgG HRP-Linked Whole Ab Donkey (GE Healthcare UK, Buckinghamshire, England) was used as the secondary antibody. Blots were visualized with ECL Select Western Blotting Detection Reagent (GE Healthcare UK).

Establishment of small-cell lung cancer cells resistant to CPT-11

N417 cells were continuously cultured in media supplemented with irinotecan hydrochloride (CPT-11; Yakult Honsha, Tokyo, Japan), a topoisomerase I inhibitor. The dose was gradually increased to 3 μM, and persistent resistance was confirmed by continuous culture in 3 μM CPT-11. These cells were designated N417/IR.

WST-8 cell viability assay

Cells were seeded at 5,000 cells per well, cultured in RPMI-1640, and treated with ZnO nanoparticles for 48 h in a flat-bottom 96-well culture plate (Sigma-Aldrich, St. Louis, MO, USA) with indicated doses of ZnO. Cell viability was determined by WST-8 assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), following the manufacturer’s instructions. Absorbance at 450 and 630 nm was measured on a Sunrise R microplate reader (Tecan Group, Männedorf, Switzerland) as unadjusted cell viability and baseline, respectively. Unadjusted cell viability was normalized to the baseline, and the average viability of untreated controls of the same cells was set as 100%.

Total glutathione

Untreated cells were seeded at 20,000 cells per well in a white 96-well plate. Reduced form (GSH) and oxidized form (GSSG) of glutathione in cells were measured as total glutathione (GSH+GSSG) using GSH/GSSG-Glo Assay (Promega) and GloMax Discover System (Promega).

GSH-GSSG ratio

Cells were suspended in HBSS with or without 5 μg/mL ZnO, and cells were incubated in 5% CO₂ at 37°C for 1 h. Cells were then washed by HBSS once and resuspended in HBSS, before being plated onto a 96-well plate. Total glutathione and GSSG were measured independently using GSH/GSSG-Glo Assay (Promega) and GloMax Discover System (Promega). GSH-GSSG ratio was calculated as GSH/GSSG = (total glutathione – GSSG) / (0.5 × GSSG).

Intracellular Zn²⁺

Cells were cultured in a 35-mm glass-bottom dish (AGC Techno Glass, Shizuoka, Japan), and were exposed to vehicle or 10 μg/mL ZnO nanoparticles for 60 min in 5% CO₂ at 37°C. Cells were washed with HBSS twice, replaced in...
HBSS containing 25 μM Zinquin ethyl ester (Dojindo Laboratories), and incubated in 5% CO₂ at 37°C for 30 min. Cells were washed with HBSS twice and replaced in HBSS. Zn²⁺-induced blue fluorescence was captured on a confocal fluorescence microscope, using the same settings for control and treated samples.

**Intracellular ROS**

Cells were cultured in a 35-mm glass-bottom dish (AGC Techno Glass). Media were then replaced with PBS containing 10 μM aminophenyl fluorescein (Goryo Chemical, Sapporo, Japan), and cells were exposed for 90 min to the vehicle or 10 μg/mL ZnO nanoparticles. ROS-induced green fluorescence was captured on a confocal fluorescence microscope, using the same settings for control and treated samples.

**Reverse transcription quantitative PCR**

Total RNA was extracted from untreated cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan). Quantitative PCR was performed using KOD SYBR qPCR Mix (Toyobo) and Thermal Cycler Dice Real Time System II (Takara Bio, Kusatsu, Japan) with incubation at 98°C for 2 min and 40 cycles of a cycling condition; 98°C for 10 s and 68°C for 30 s. The sequences for primers used are given in Supplementary Table S1. Gene expression of GAPDH was used as a standard to normalize for relative expression.

**Confocal fluorescence microscopy**

Fluorescent images were obtained on an FV1000D laser scanning confocal fluorescence microscope (Olympus, Tokyo, Japan). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) using ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA, USA).

**Intrapulmonary injection of human small-cell lung cancer cells**

Animal protocols were approved by the Committee for Animal Experimentation at Shimane University and were compliant with ethical standards required by law and with guidelines on animal experiments in Japan (IZ30-67). BALB/cAJcl-nu/nu mice > 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan). Mice were monitored at least twice a week for symptoms of illness by assessing weight, activity, and skin condition. N417 or H82 cells (1.0 × 10⁶ cells) with 50% Matrigel Matrix (Corning, Corning, NY, USA) were injected orthotopically into the left lung (26). Left and right lungs were collected intact from euthanized mice and weighed immediately. To preserve lung tissues surrounding tumors, tumor volumes were estimated from photographs as (tumor volume; mm³) = 0.5 × (major axis; mm) × (minor axis; mm)². Axis lengths were obtained from ellipses drawn around tumor surfaces. Lung tissues were then cut along the center of a tumor, and one piece was fixed in 10% neutral buffered formalin for staining with hematoxylin and eosin, while the other piece was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan) and immediately frozen at −80°C for subsequent histological analysis. MnTBAP chloride (Abcam) was dissolved to a concentration of 0.1 M in 0.1 M NaOH and diluted to 0.75 mg/mL in PBS.

**DNA damage in situ**

Embedded tissues were sectioned on a Leica CM1520 cryostat (Leica Biosystems, Wetzlar, Germany), and assayed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) to assess DNA fragmentation in situ,
using Click-it Plus TUNEL Assay for In Situ Apoptosis Detection and Alexa Fluor 594 (Life Technologies), and following the manufacturer’s protocol for tissue sections without deparaffinization.

**Statistical analysis**

Statistical significance was assessed in GraphPad PRISM version 8.00 (GraphPad Software, San Diego, CA, USA), with \( P < 0.01 \) considered statistically significant. Tests to compare two groups are listed in figure legends. ANOVA and a post-hoc analysis listed in figure legends were used to compare more than two groups.
Results

Preparation and evaluation of ZnO nanoparticles

ZnO was prepared as described to enrich for nanometer-scale particles. In PBS, the median and average hydrodynamic diameter were approximately 20 nm (Fig. 1A). As these particles are smaller than 100 nm (17), we anticipated that they would be efficiently delivered. These particles exhibited an ultraviolet absorbance peak at 362 nm, which is characteristic of ZnO (Fig. 1B). Precipitation was not observed for more than a year after preparation, suggesting high dispersion stability (Fig. 1C). Wide-range emission from blue to deep green wavelength was also observed (Fig. 1D). Similarly, agglomerated and dried ZnO nanoparticles exhibited blue or green fluorescence (Fig. 1E).

ZnO nanoparticles are cytotoxic against human cancer cell lines

Following exposure to 10 μg/mL ZnO nanoparticles, morphological changes were observed in N417 small-cell lung cancer cells, beginning at 120 min, as assessed by hematoxylin and eosin staining (Fig. 2A). Indeed, only damaged cells were observed at 48 h. In addition, viability was lower in cells exposed to more than 2.5 μg/mL, as assessed by WST-8 assay (Fig. 2B). In contrast, normal fibroblast-like OUS-11 lung cells tolerate and adhere to culture slides even after 48 h of exposure to 10 μg/mL ZnO (Fig. 2C), with viability diminishing only at 15 and 20 μg/mL (Fig. 2D). H82 and H187 small-cell lung cancer cells were also susceptible to ZnO (Fig. 2E). BEAS-2B normal cells were less sensitive to ZnO nanoparticles, as were OUS-11 cells (Fig. 2F). On the other hand, MCF-7 breast cancer cells and LS174T colon carcinoma cells also showed higher sensitivity to ZnO as compared to normal cells (Fig. 2G).

The viability of N417, H82, and LS174T cells declined in a dose-dependent manner, but OUS-11, H187, BEAS-2B, and MCF7 cell viability decreased sharply over the threshold of ZnO nanoparticles concentration. This may indicate that ZnO nanoparticles inhibit cell metabolism for N417, H82, and LS174T cells, but not other cells, before triggering cell death. Because LS174T has wildtype of TP53, TP53 mutation status is not the single determining factor of the sensitivity to ZnO nanoparticles.

In addition, we prepared other batches of ZnO nanoparticles that were also smaller than 100 nm (~70 nm) using the same method, and these nanoparticles showed consistent cytotoxicity in N417 cells (Supplementary Fig. S1).

To verify whether ZnO treatment induces intracellular Zn2+ ions and enhances the uptake of ZnO nanoparticles, we next evaluated fluorescence of Zinquin ethyl ester after ZnO treatment. The background level of fluorescence by innate Zn2+ and autofluorescence is shown (Supplementary Fig. S2). Expectedly, small-cell lung cancer cells (Fig. 2H and 2I) exhibited increased fluorescence, while no increase in fluorescence was observed in normal cells (Fig. 2J and 2K) after treatment with ZnO nanoparticles.

ZnO nanoparticles induce genotoxicity via ROS, leading to non-apoptotic cell death

N417 cells exposed to 10 μg/mL ZnO nanoparticles for indicated periods were stained with propidium iodide and annexin V to assess apoptosis and necrosis (Fig. 3A and 3B). Early apoptotic cells were comparable in abundance to untreated cells up to 12 h after exposure. However, early necrotic cells slightly increased from 12% to 17% at 3 h, although this increase was not significant. On the other hand, viable cells diminished, while dead cells significantly accumulated at 12 h. Similarly, ZnO-treated BEAS-2B cells showed an increase in apoptotic cells but not in necrotic cells (Fig. 3C). As these results did not definitively establish apoptosis or necrosis as a driver of ZnO cytotoxicity, we...
quantified cleaved PARP, an apoptotic marker, and phosphorylated CHK2, a DNA damage marker (Fig. 3D). ZnO treatment induced phosphorylated CHK2 in both N417 cells and LS174T cells, but increased cleaved PARP only in LS174T cells. On the other hand, Q-VD-OPh, an inhibitor of caspase-3, -1, -8, and -9, slightly increased the viability of control cells by inhibiting apoptosis, but did not block ZnO cytotoxicity (Fig. 3E).

As ROS is commonly associated with ZnO cytotoxicity, we evaluated total intracellular glutathione as a marker of potential antioxidant activity (Fig. 3F). Small-cell lung cancer cells have higher or identical levels of total glutathione as compared to normal cells. In addition, we assessed the GSH-GSSG ratio that is an indicator of intracellular ROS production (Fig. 3G). Unexpectedly, the GSH-GSSG ratio significantly decreased after ZnO nanoparticle treatment in both small-cell lung cancer and normal cells. This result indicates that ZnO nanoparticles induce intracellular ROS in both small-cell lung cancer and normal cells, but that normal cells are more tolerant to ZnO-induced ROS.

A key question relates to whether ROS is a cytotoxic driver of ZnO nanoparticles for small-cell lung cancer. To assess ROS production more specifically, we used aminophenyl fluorescein, which is transformed to fluorescein only by some species of highly ROS, such as hydroxyl radical, peroxynitrite, and hypochlorite. Strikingly, we observed fluorescence throughout N417 cells exposed to 10 μg/mL ZnO, including in nuclei (Fig. 3H), suggesting that ZnO nanoparticles penetrate cell membranes and generate highly ROS by interacting with cellular components. Accordingly, Tiron, a cell-permeable scavenger of highly ROS, significantly protected cells against ZnO (Fig. 3I). In contrast, significant ROS production was not observed in OUS-11 exposed to 10 μg/mL ZnO (Fig. 3J), confirming that OUS-11 cells tolerate such treatment (Fig. 2C and 2D). The results indicated that ZnO-induced ROS generation whose type varies according to the cell line, and highly ROS might be a critical factor in the cytotoxicity of ZnO nanoparticles.

To identify differences in antioxidants produced by small-cell lung cancer and normal cells, we assessed gene expression of common antioxidant genes, such as superoxide dismutase (SOD), copper chaperone for SOD (CCS), and catalase (CAT) (Supplementary Fig. S3A). Unexpectedly, small-cell lung cancer cells do not have lower expression for those genes than normal cells. We also evaluated gene expression of ZnO-related oxidative stress-response genes in a previous study (27). Although OUS-11 cells have significantly higher expression than small-cell lung cancer, BEAS-2B cells have no significant difference as compared to small-cell lung cancer cells (Supplementary Fig. S3B). To confirm whether this result indicates a relationship between normal lung and small-cell lung cancer, we assessed gene expression of ZnO-related antioxidants using a GEO DataSet from specimens of normal lung and small-cell lung cancer (Supplementary Fig. S3C). Our results show that HMOX1, AOX1, and SOD3 were significantly highly expressed in normal lung rather than small-cell lung cancer, and that expression is similar between OUS-11 and small-cell lung cancer cell lines. We also observed that ZnO evokes chromatin leakage from N417 cells (Fig. 3K), suggesting genotoxicity.

**Small-cell lung cancers resistant to CPT-11 are also sensitive to ZnO**

To assess second-line treatment value, ZnO was tested against small-cell lung cancer cells resistant to CPT-11, a topoisomerase-1 inhibitor used to treat not only small-cell lung cancer, but also colon cancer. N417 cells resistant to CPT-11 were acquired by long-term drug exposure in culture. We note that although parental N417 cells are sensitive to CPT-11 in a dose-dependent fashion (Fig. 4A), derivative cells remain viable for over 48 h even at 30 μM (Fig. 4B), confirming drug resistance. Nevertheless, the latter remain highly sensitive to ZnO nanoparticles (Fig. 4C), especially...
above 5 μg/mL. Importantly, we also detected the production of highly ROS in drug-resistant cells (Fig. 4D), just as in parental N417 cells (Fig. 3H). These results indicate that ZnO may be active not only against small-cell lung cancers that are sensitive to CPT-11, but also against resistant forms. Collectively, the data suggest that ZnO nanoparticles have measurable anticancer activity in vitro.

**Therapeutic efficacy of ZnO in an orthotopic mouse model of human small-cell lung cancer**

To assess potential clinical value, we performed an animal experiment. Mice were treated with 0.04 mg/kg ZnO nanoparticles or vehicle 22, 29 and 36 days after orthotopic injection of N417 cells (Supplementary Fig. S4A). We did not observe any adverse effects or anticancer effects on tumor cells (Supplementary Fig. S4B and S4C). Therefore, we decided to increase the concentration of ZnO NPs to 0.25 mg/kg and shorten duration of time prior to starting treatment.

Next, mice were treated with 0.25 mg/kg ZnO nanoparticles or vehicle 15, 22, and 29 days after orthotopic injection of N417 cells (Fig. 5A). Mice were sacrificed at day 36, and lungs were collected. Body weight was also measured during the experiment and was comparable between control and ZnO-treated xenografted mice (Fig. 5B). Additionally, illness, poor health, and death were not observed in either groups at the end of the experiment, implying that ZnO does not induce adverse effects at this dose.

Lung weight, a measure of tumor density, was marginally higher in control mice than in ZnO-treated mice (Fig. 5C). Similarly, tumor volumes were comparable (Fig. 5D). As shown in Figure 5E, tumors developed in all control mice, but grown tumors were observed in only two ZnO-treated mice. Tumors were absent or were tiny in all other ZnO-treated mice. There was no apparent metastasis in the lymph nodes and contralateral lung in the mice of either group. Microscopy of lung tissues stained with hematoxylin and eosin (Fig. 5F, 5G, and 5H) showed vigorous tumor growth in control mice (Fig. 5F and 5H), while necrotic areas, especially in the core of tumors, were observed in ZnO-treated mice (Fig. 5F and 5H). Hence, lung weight appears to be a better index of tumor growth than tumor volume, which is derived from the long and short axes of tumor surfaces. Adverse effects were not observed in normal lung tissues in ZnO-treated mice (Fig. 5F and 5G). These results highlight the potential anticancer activity of ZnO in vivo.

We also attempted to evaluate the therapeutic efficacy of ZnO in another orthotopic mouse model using H82 cells. Although we used the same injection method, H82 cells did not grow in the murine lung, either for the ZnO-treated group, or the vehicle-treated group (Supplementary Fig. S4D). There was no difference in body weight between the groups after treatment with 0.25 mg/kg ZnO nanoparticles or vehicle 14, 21, 28 and 35 days after orthotopic injection of H82 cells (Supplementary Fig. S4E). At least 4 injections of ZnO nanoparticles did not cause illness, poor health, and death in both groups by the end of the experiment.

**ZnO nanoparticles that accumulate in orthotopic tumors show in situ genotoxicity**

Confocal fluorescence microscopy of fresh-frozen, DAPI-stained lung tissues from biological duplicates (Fig. 6A and 6B) revealed only blue-fluorescent DAPI-stained nuclei in untreated tumors, as expected. On the other hand, several cells in tissues from ZnO-treated mice exhibited exogenous green fluorescence similar to that of dried ZnO particles (Fig. 1E), implying accumulation of ZnO particles. In addition, tumors from vehicle-treated mice were barely stained with TUNEL (vehicle, Fig. 6C and 6D), which stains damaged DNA fragments with double-strand breaks and is a marker of apoptosis or genotoxicity. On the contrary, a large number of TUNEL-stained cells were observed in ZnO-
treated mice, especially in necrotic areas (ZnO, Fig. 6C and 6D). DNA fragments leaking from the nuclear envelope were also observed, as seen in vitro (Fig. 3K).

To assess in vivo antitumor effects of ZnO-induced ROS, we tested a combination of ZnO and MnTBAP, a SOD mimetic and peroxynitrite scavenger for in vivo use. We randomly divided mice into 3 groups for vehicle, ZnO and ZnO plus MnTBAP treatment at day 7. The number of mice in vehicle or ZnO groups was 6 but was 5 for ZnO plus MnTBAP group since one mouse died before group division. Mice were treated with 0.3 mg/kg ZnO nanoparticles or vehicle 8, 15, 22, 29 days after orthotopic injection of N417 cells and were treated with 5 mg/kg MnTBAP or vehicle on 6 days per week until euthanasia (Fig. 6E). Body weight was measured during the experiment and was comparable among all 3 groups (Fig. 5B). Mice were sacrificed at day 39, and the lung weight and the lung volume were measured (Fig. 6G and 6H). Lung weight was comparable among the 3 groups (Fig. 6G) and the lung volume of the ZnO group, but not the ZnO plus MnTBAP group, tended to be higher than in the vehicle group (Fig. 6H). All mice in the vehicle group had apparent tumors in the lung, but only 2 mice had tumor masses in the ZnO group or ZnO plus MnTBAP group (Fig. 6I). In addition, in the vehicle group, a mouse had liver metastasis (Supplementary Fig. S5A) and another mouse had axillary metastasis (Supplementary Fig. S5B).
Discussion

We have prepared ZnO nanoparticles that inhibit the proliferation of small-cell lung cancer cells \textit{in vitro} by generating highly ROS. These nanoparticles are also active against various other cancer cells and against N417 cells that have acquired resistance to CPT-11, but less active against normal lung cells. Importantly, intravenously administered ZnO is also genotoxic \textit{in vivo} against orthotopic small-cell lung cancers, without any adverse effects through the end of the experiment. Therefore, ZnO is a potential novel therapeutic agent.

Compared to other inorganic compounds, such as cadmium selenide, biological research into ZnO has been growing, perhaps owing to its long history and safety record. ZnO is a II-VI semiconductor with a direct band gap of \( \sim 3.37 \text{ eV} \) (28), and its properties are thought to depend on particle size, shape, charge, and added impurities that alter semiconductivity (29–31). For potential application \textit{in vivo}, we homogenized ZnO particles synthesized by dc arc dusty plasma (32–34), considering that in intravenous injection, particle size and dispersion stability are critical to avoiding lethal adverse events such as venous thrombosis. Thus, we prepared ZnO nanoparticles without particles over 100 nm, not only to minimize the risk of venous thrombosis, but also to maximize delivery efficiency (17). Although we did not evaluate particle charges such as zeta potential, the nanoparticles we obtained had high dispersion stability and did not agglomerate over one year, as assessed visually. However, we note that neutral particles are preferable to maximize dispersion stability and delivery efficiency. Finally, we note that because of high stability, it is advantageous to use nanoparticles prepared once for many subsequent experiments, enhancing reproducibility and convenience.

The ability of normal lung-derived cells to tolerate ZnO, as assessed \textit{in vitro} by WST-8 assay, is likely owing to differences in macropinocytosis. Although we did not evaluate the uptake speed of ZnO nanoparticles, cytotoxicity should be increased by more uptake amount of ZnO nanoparticles. Indeed, various cancer cells appear to constitutively activate macropinocytosis to improve uptake of extracellular amino acids and fuel growth, metabolism, and signal transduction (35–37). Accordingly, macropinocytosis may also promote the uptake of 20-nm ZnO nanoparticles (38). The ensuing ROS toxicity after uptake is a likely driver of ZnO genotoxicity \textit{in vitro} and \textit{in vivo}, as noted in many studies (39). Of note, only intracellular highly ROS was produced \textit{in vitro} in ZnO-treated small-cell lung cancer cells, suggesting that intracellular components are essential for cytotoxic effects. In particular, lacking normal TP53 that is the typical feature of small-cell lung cancer, should be taken into account for the use of ZnO treatment to small-cell lung cancer. Setyawati et al. reported that DLD1 and SW480 cells that have mutant TP53 produce more ROS by ZnO nanoparticles, and those cells were more susceptible to the cytotoxicity than NCM460 and HCT116 cells that have functional TP53 (40). To explain the result, we speculate that the function of ATM, a protein acting upstream of TP53, may be involved in this mechanism. Lee et al. reported that ATM protein reacts to ROS and is necessary to phosphorylate TP53 (Ser 15) under ROS-induced oxidative stress. The loss of normal ATM protein resulted in deficiencies in mitochondrial function, autophagy and intracellular protein aggregation (41). In addition, damaged mitochondria also produce intracellular ROS (42). We did not detect the function of mitochondria, but WST-8 assay showed intracellular activity of NADH which correlated to mitochondrial function via overall cell metabolism. If the ROS-induced protective function of mitochondria was insufficient against increased levels of intracellular ROS, mitochondria should further increase of intracellular ROS and decrease NADH activity simultaneously. Although the functional involvement of TP53 in the reaction to ROS was not directly proven, TP53 may be mediate the protective...
role against ROS-induced oxidative stress, including highly ROS.

ZnO cannot be exposed to ultraviolet radiation after intravenous administration in this study, as this may generate photocatalytic ROS from ZnO itself. Alternatively, Joe et al. (43) reported that in the dark, Zn^{2+} ions from dissolved ZnO and taken up by bacterial metalloprotein channels might induce ROS and thus antibacterial activity. Although ZnO nanoparticles also have chemical activity to produce superoxide radical anion (44,45), we also observed not only various ROS but also increased Zn^{2+} ions in small-cell lung cancers after treatment with ZnO nanoparticles in vitro. This result suggests two possibilities for ZnO nanoparticles: either intracellular dissolving after the uptake or extracellular dissolving and Zn^{2+} uptake. From the results of decreased GSH-GSSG ratio and no increased Zinquin ethyl ester in normal cells after the ZnO treatment, both of normal and small-cell lung cancer cells may uptake ZnO nanoparticles, but highly ROS was induced only in small-cell lung cancer. This suggest that intracellular increases in highly ROS may result in increased intracellular Zn^{2+} from ZnO dissolved by acidic conditions due to highly ROS. Taken together, we proposed a scheme of ZnO-induced ROS production based on public information and our results (Supplementary Fig. S6).

An orthotopic mouse model of human small-cell lung cancer was necessary to assess the distribution, adverse effects, and cytotoxicity of intravenously administered ZnO nanoparticles. Although such models are considered the gold standard to evaluate drug delivery, treatments began 1 or 2 weeks after injection of N417 cells without confirmation of tumor growth in the lungs. If tumor size had been used as an index for treatment initialization, more consistent outcomes might have been observed. Pulmonary emphysema (25,46,47), a potential adverse effect, was not found. Nevertheless, we plan to monitor dose-dependent or long-term toxicity in tumor-bearing animals in the next phase of the project. We also highlight the potential activity of ZnO against cancer types other than small-cell lung cancer, as observed in vitro.

Although small-cell lung cancer is very responsive to initial treatment, most patients experience relapse with relatively resistant disease. Thus, we targeted small-cell lung cancer in this study, because options for second-line chemotherapy against this form are severely limited at present. Based on these results, we propose that ZnO should be tested first in patients that initially respond well to standard treatments with good tolerance, but no longer have further treatment options.

In conclusion, we have collected the first empirical evidence of the genotoxic anticancer effects of ZnO in an orthotopic mouse model of human small-cell lung cancer. The model also shows promise as a potentially useful preclinical model for testing novel small-cell lung cancer treatments.
Acknowledgments

We thank Ms. Mayumi Takechi (Shimane University, Japan) for assistance in animal experiments.
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Figure legends

Figure 1.
Characterization of ZnO nanoparticles. **A**, Particle size distribution. **B**, Ultraviolet-visible absorption spectrum. **C**, Color, and appearance. **D**, Photoluminescence spectrum at excitation wavelength 325 nm a.u., arbitrary unit. **E**, Blue (left) and green (right) fluorescence from ZnO nanoparticles dried on a slide, at excitation wavelengths 405 nm or 473 nm, respectively. Scale bars, 10 µm.

Figure 2.
ZnO nanoparticles are cytotoxic against various cancer cells. **A**, Hematoxylin and eosin staining of N417 cells treated with 10 µg/mL ZnO for indicated periods. Scale bar, 20 µm. **B**, Viability of N417 cells treated for 48 h with indicated doses of ZnO. Data are mean (SD), n = 4. *, P < 0.01; ***, P < 0.0001 by one-way ANOVA and Dunnett’s test. **C**, Hematoxylin and eosin staining of OUS-11 cells treated with 10 µg/mL ZnO nanoparticles for 48 h. Scale bars, 20 µm. **D**, Viability of OUS-11 cells exposed for 48 h to ZnO at indicated concentrations. Data are mean (SD), n = 4. Groups were compared by one-way ANOVA and Dunnett’s test. **E**, Viability of H82 and H187 human small-cell lung cancer cells treated for 48 h with ZnO at indicated concentrations. Data are mean (SD), n = 4. ***, P < 0.0001 by one-way ANOVA and Dunnett’s test. **F**, Viability of BEAS-2B cells exposed for 48 h to ZnO at indicated concentrations. Data are mean (SD), n = 4. Groups were compared by one-way ANOVA and Dunnett’s test. **G**, Viability of human MCF-7 breast cancer cells and LS174T colon adenocarcinoma cells treated for 48 h with indicated doses of ZnO. Data are mean (SD), n = 4. *, P < 0.01; **, P < 0.001; ***, P < 0.0001 by one-way ANOVA and Dunnett’s test. **H, I, J and K**, Confocal fluorescent microscopy of N417, H82, OUS-11 and BEAS-2B cells maintained in HBSS with 25 µM Zinquin ethyl ester and treated with 10 µg/mL ZnO for 60 min. Scale bars, 50 µm.

Figure 3.
ZnO nanoparticles induce non-apoptotic cell death in small-cell lung cancer cells by generating highly reactive oxygen species. **A**, Representative annexin V/propidium iodide staining of N417 cells treated with 10 µg/mL ZnO for indicated periods. Scale bar, 20 µm. **B**, Quantification of stained cells. Data are mean (SD), n = 3. ***, P < 0.0001 by two-way ANOVA and Dunnett’s test. **C**, Representative annexin V/propidium iodide staining of BEAS-2B cells treated with 15 µg/mL ZnO for 24 h. **D**, Immunoblots for cleaved PARP and phosphorylated CHK2 in N417 cells or LS174T cells treated with or without 5 µg/mL or 10 µg/mL ZnO respectively for 24 h. **E**, Viability of N417 cells treated for 48 h with or without 10 µg/mL ZnO and/or 10 µM Q-VD-OPh. Data are mean (SD), n = 4. ***, P < 0.0001; ns, not significant by one-way ANOVA and Dunnett’s test. **F**, Total glutathione in untreated cells. Data are mean (SD), n = 3. ***, P < 0.0001 by one-way ANOVA and Tukey’s multiple comparisons test. **G**, GSH-GSSG ratio of cells treated with or without 5 µg/mL ZnO for 1 h. Data are mean (SD), n = 3. *, P < 0.01; **, P < 0.001; ***, P < 0.0001 by one-way ANOVA and Tukey’s multiple comparisons test. **H** and **J**, Confocal fluorescent microscopy of N417 and OUS-11 cells maintained in PBS with 10 µM aminophenyl fluorescein and treated with 10 µg/mL ZnO for 90 min. **H**, Fluorescence due to highly reactive oxygen species in N417 cells. Scale bars, 10 µm. **I**, Viability of N417 cells treated for 48 h with 10 µg/mL ZnO and/or 0.64 mM Tiron. Data are mean (SD), n = 4. ***, P < 0.0001 by one-way ANOVA and Tukey’s test. **J**, Fluorescence due to highly reactive oxygen species in OUS-11 cells. Scale
bars, 30 µm. **K**, Confocal microscopy of DNA stained with propidium iodide in N417 cells treated with 10 µg/mL ZnO for 30 min. White arrows indicate chromatin leakage.

**Figure 4.**
ZnO inhibits proliferation of small-cell lung cancer cells resistant to CPT-11. **A**, Viability of CPT-11-susceptible N417 cells treated for 48 h with CPT-11 at indicated concentrations. Data are mean (SD), n = 3. ****, P < 0.0001 by one-way ANOVA and Dunnett’s test. **B**, Viability of CPT-11-resistant N417/IR cells exposed for 48 h to CPT-11 at indicated concentrations. Data are mean (SD), n = 3. Data were compared by one-way ANOVA and Dunnett’s test. **C**, Viability of CPT-11-resistant cells treated for 48 h with ZnO at indicated concentrations. Data are mean (SD), n = 4. Groups were compared by one-way ANOVA and Dunnett’s test. **D**, Fluorescence due to highly reactive oxygen species in N417/IR cells maintained in PBS with 10 µM aminophenyl fluorescein and treated with 10 µg/mL ZnO for 90 min. Scale bars, 30 µm.

**Figure 5.**
ZnO testing in an orthotopic mouse model of human small cell lung cancer. **A**, Experimental protocol. **B**, Body weight until day 34. Arrows mark the days of treatment. Data are mean (SD), n = 5. Groups were compared by two-way repeated measures ANOVA and Sidak’s test. **C**, Lung weights. Data are mean (SD), n = 5. Groups were compared by two-tailed Mann-Whitney test. **D**, Tumor volume. Data are mean (SD), n = 5. Groups were compared by two-tailed Mann-Whitney test. **E**, Tumor-inoculated lungs. Scale intervals, 1 mm. **F**, Hematoxylin, and eosin staining of tumor-burdened lungs. Scale bars, 1 mm. **G**, Hematoxylin, and eosin staining of normal adjacent tissues. Scale bars, 50 µm. **H**, Hematoxylin, and eosin staining of tumor tissues. Scale bars, 50 µm.

**Figure 6.**
Effects of ZnO in orthotopic lung tumors. **A** and **B**, DAPI staining. Scale bars, 30 µm (**A**), and 10 µm (**B**). **C** and **D**, TUNEL staining. Scale bars, 100 µm (**C**) and 10 µm (**D**). Arrows indicate TUNEL-stained leaking chromatin. **E**, Experimental protocol. **F**, Body weight until day 36. Arrows mark the days of ZnO treatment. MnTBAP or vehicle was intraperitoneally administered on 6 days per week until euthanasia. Data are mean (SD), n = 6 (Vehicle and ZnO), n = 5 (ZnO plus MnTBAP). Groups were compared by two-way repeated measures ANOVA and Tukey’s multiple comparisons test. **G**, Lung weights. Data are mean (SD), n = 6 (Vehicle and ZnO), n = 5 (ZnO plus MnTBAP). Groups were compared by one-way ANOVA and Tukey’s multiple comparisons test. **H**, Tumor volume. Data are mean (SD), n = 6 (Vehicle and ZnO), n = 5 (ZnO plus MnTBAP). Groups were compared by one-way ANOVA and Tukey’s multiple comparisons test. **I**, Tumor-inoculated lungs. Scale intervals, 1 mm.
Figure 1

A

Median: 17.0 nm
Average: 19.7 nm

B

Absorbance
362 nm

C

D

Intensity (a.u.)

E

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Figure 2

A

N417

0 m

15 m

2 h

12 h

24 h

48 h

B

N417

Cell viability (%)

0

50

100

150

ZnO (µg/mL)

C

OUS-11

0 h

48 h

D

OUS-11

Cell viability (%)

0

50

100

150

ZnO (µg/mL)

E

H82

H187

Cell viability (%)

0

50

100

150

ZnO (µg/mL)

F

BEAS-2B

Cell viability (%)

0

50

100

150

ZnO (µg/mL)

G

MCF-7

LS174T

Cell viability (%)

0

50

100

150

ZnO (µg/mL)

H

N417

PBS

ZnO

I

H82

PBS

ZnO

J

OUS-11

PBS

ZnO

K

BEAS-2B

PBS

ZnO
Figure 3

A) Flow cytometry analysis of cell viability over time with Annexin V and PI staining.

B) Graph showing the percentage of cell population in different states (Viable, Early apoptotic, Early necrotic, Dead) at 0, 3, 6, and 12 h.

C) Western blot analysis of BEAS-2B cells treated with PBS or ZnO, showing the levels of Cleaved PARP (D214) and Phospho-CHK2 (T68).

D) Western blot analysis of N417 and LS174T cells treated with PBS or ZnO, showing the levels of Cleaved PARP (D214) and Phospho-CHK2 (T68).

E) Bar graph showing cell viability percentages of untreated, ZnO, QVD, and ZnO + QVD treated cells.

F) Bar graph showing total glutathione levels in N417, H82, OUS-11, and BEAS-2B cells treated with PBS or ZnO.

G) Graph showing GSH-GSSG ratio in N417, H82, OUS-11, and BEAS-2B cells treated with PBS or ZnO.

H) Images of untreated and ZnO treated cells under microscope.

I) Bar graph showing cell viability percentages of untreated, ZnO, and ZnO + Tiron treated cells.

J) Images of untreated and ZnO treated cells under microscope.
Figure 4

A

Cell viability (%)

N417

CPT-11 (µM)

0 0.1 0.3 1 3 10 30

B

Cell viability (%)

N417/IR

CPT-11 (µM)

0 0.1 0.3 1 3 10 30

C

Cell viability (%)

N417/IR

ZnO (µg/mL)

0 0.3 1.5 3 6

D

Untreated

ZnO

Scale bars: 50 µm
Figure 5

A
Tumor inoculation to lung
Day 1

Treatment with vehicle or ZnO
Day 15, 22, 29

Lung collection
Day 36

B
Body weight (g)

Day

Vehicle
ZnO

C
Lung weight (mg)

D
Tumor volume (mm³)

E
Vehicle
ZnO

F
Vehicle 1
Vehicle 2
ZnO 1
ZnO 2

G
Vehicle 1
ZnO 1
Vehicle 2
ZnO 2

H
Vehicle 1
ZnO 1
Vehicle 2
ZnO 2

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Figure 6

A. Vehicle vs. ZnO

B. ZnO

C. Vehicle 1 vs. ZnO 1

D. Vehicle 2 vs. ZnO 2

E. Inoculation, Treatment, Euthanasia

Day 1: ZnO; day 8, 15, 22, 29 (i.v.)
MnTBAP: 6 days per week (i.p.)
Day 39

F. Body weight (g)

G. Lung weight (mg)

P = 0.0986

H. Tumor volume (mm³)

P = 0.0938

I. Tissue samples
# Molecular Cancer Therapeutics

## Anticancer Activity of ZnO Nanoparticles Against Human Small-Cell Lung Cancer in an Orthotopic Mouse Model

Ryosuke Tanino, Yoshihiro Amano, Xuexia Tong, et al.

*Mol Cancer Ther* Published OnlineFirst November 29, 2019.

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