Titania Nanosheets Generates Peroxynitrite for S-Nitrosylation and Enhanced p53 Function in Lung Cancer Cells

Rapeepun Soonnarong  
Chulalongkorn University

Sucharat Tungsukruthai  
Chulalongkorn University

Bodee Nutho  
Mahidol University Faculty of Science

Thanyada Rungrotmongkol  
Chulalongkorn University Faculty of Science

Chanida Vinayanuwattikun  
Chulalongkorn University Faculty of Medicine

Tosapol Maluangnont  
King Mongkut's Institute of Technology Ladkrabang

pithi chanvorachote (pithi.ch@gmail.com)  
Chulalongkorn University Faculty of Pharmaceutical Sciences  https://orcid.org/0000-0002-3103-3249

Research

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Titania Nanosheets Generates Peroxynitrite for S-Nitrosylation and Enhanced p53 Function in Lung Cancer Cells

Rapeepun Soonnarong¹,², Sucharat Tungsukruthai¹,², Bodee Nutho⁴, Thanyada Rungrotmongkol⁵,⁶, Chanida Vinayanuwattikun⁷, Tosapol Maluangnon⁸ and Pithi Chanvorachote²,³*

Abstract

Background: Metal oxide nanomaterials are increasingly being exploited in cancer therapy thanks to their unique properties, which can enhance the efficacy of current cancer therapies. However, the nanotoxicity and mechanism of Ti₈₀O₂ nanosheets for specific site-targeting strategies in NSCLC have not yet been investigated.

Methods: The effects of Ti₈₀O₂ nanosheets on cytotoxicity in NSCLC cells and normal cells were examined. The apoptosis characteristics, including condensed and fragmented nuclei, as assessed by positive staining with annexin V. The cellular uptake of the nanosheets and the induction of stress fiber were assessed via transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analyses, respectively. We also evaluated the expression of protein in death mechanism to identify the molecular mechanisms behind the toxicity of these cells. We investigated the relationship between S-nitrosylation and the increase in p53 stability by molecular dynamics.

Results: Ti₈₀O₂ nanosheets caused cytotoxicity in several lung cancer cells, but not in normal cells. The nanosheets could enter lung cancer cells and exert an apoptosis induction. Results for protein analysis further indicated the activation of p53, increased Bax, decreased Bcl-2 and Mcl-1, and activation of caspase-3. The nanosheets also exhibited a substantial apoptosis effect in drug-resistant metastatic primary lung cancer cells, and it was found that the potency of the nanosheets was dramatically higher than that of cisplatin and etoposide. In terms of their mechanism of action, we found that the mode of apoptosis induction was through the generation of cellular ONOO⁻ mediated the S-nitrosylation of p53 at C182. Molecular dynamics analysis further showed that the S-nitrosylation of one C182 stabilized the p53 dimer. Consequently, this nitrosylation of the protein led to an upregulation of p53 through its stabilization.

Conclusions: Taking all the evidence together, we provided information on the apoptosis induction effect of the nanosheets through a molecular mechanism involving reactive nitrogen species, which affects the protein stability; thus emphasizing the novel mechanism of action of nanomaterials for cancer therapy.

Keywords: Apoptosis, Nanosheets, Lung cancer, p53, S-nitrosylation, Peroxynitrite, Molecular dynamics
Background

Lung cancer is one of the most common cancers worldwide and has the highest mortality rate. Although a number of crucial components in the fight against lung cancer have been elucidated, including small molecule tyrosine kinase inhibitors, and immunotherapy developed, which have led to unprecedented survival benefits in selected patients, the overall cure and survival rates for non-small cell lung cancer (NSCLC) remain low [1]. Therefore, continued research into new drugs and combination therapies is required to expand the clinical benefit to a broader patient population and to improve the outcomes in NSCLC. The tumor suppressor p53 is an essential regulatory molecule that is implicated in cell cycle arrest and plays a mediator role in apoptosis in response to stress [2]. A key attribute of the p53 response is p53 stabilization, which results in a rapid increase in p53 steady-state levels. Considerable evidence has indicated that p53 stabilization largely depends on post-translational events that disengage p53 from its proteasomal degradation [3]. In all cases, this includes a series of post-translational modifications, some of which are known to impact the interaction between p53 and the mouse double minute 2 (MDM2) protein, representing the major mechanism for controlling p53 stability [4]. The activation of p53 results in an increase in BH3-only proteins promoting Bax/Bak oligomerization. The induction of pro-apoptotic signaling leads to the formation of mitochondrial pores, the release of cytochrome c into the cytosol, the activation of caspases, and finally cell apoptosis [5]. In contrast, the dysregulation of apoptosis in lung cancer can be caused by several mechanisms; however, upregulation of the expression of pro-survival proteins, including mammalian target of rapamycin (mTOR), and anti-apoptotic proteins of the Bcl-2 family, has been shown to be the predominant mechanism [6].

p53, an important tumor suppressor protein, has been intensively investigated as its functions are critical for cancers. The functions of the p53 protein are tightly associated with its protein conformation. The active conformation of this protein is the tetrameric form via an interaction of the tetramerization domain (TD) on the p53 protein. Studies have pointed out that the tetramerization of p53 is critical for DNA binding, and post-translational modification, as well as for p53 stability [7]. Cysteine thiol groups on the p53 molecule have been highlighted as sensitive sites for protein modification, and it was shown that the DNA-binding affinity could be altered by thiol blocking agents [8]. Certain cysteine residue amino acid replacements inhibit the binding of p53 to DNA [9]. In addition, several lines of evidence have demonstrated that the oxidative modification of cysteine residues within p53 can also influence the protein’s activity and stability [10-12].

Nitric oxide (NO) is a key intercellular messenger synthesized from l-arginine in a reaction catalyzed by NO synthases (NOS). Nitric oxide is recognized as an important signaling device for controlling practically all critical cellular functions, and it is also a strong mediator of cellular damage [13]. Nitric oxide plays an extensive role in regulating the expression of eukaryotic genes, and this effect is exerted in part through S-nitrosylation [14]. S-Nitrosylation of the controlling binding associates of the transcription factors can exert an extranuclear effect on transcription factor activation, stability, and/or nuclear targeting, as in the case of hypoxia-inducible factor-1α (HIF-1α) and p53 [15]. In addition, nitric oxide can react with superoxide (O2•−) to form the much more powerful oxidant peroxynitrite (ONOO−), which is a key component determining the contrasting roles of nitric oxide in physiology and pathology [16]. Many of the biological effects ascribed with nitric oxide are actually related to the intermediate peroxynitrite. Even though peroxynitrite is a powerful oxidant, it reacts at a moderately slow rate with most biological molecules and is able to reach cell membranes in part through anion channels [17]. This makes the biological and pathological insinuations of peroxynitrite much more interesting, because it can have more delicate and specific actions on cells.

Nanotechnology is a research field that has wide implications in the fields of chemistry, engineering, biology, and medicine. Nanotechnology has several applications in cancer biology, especially in the development of novel treatments [18].
Nanosheets are a developing class of nanomaterial that are highly anisotropic and flexible [19]. Moreover, Ti$_{0.8}$O$_2$ nanosheets have also been found to induce superoxide anions in cancer without harming normal dermal papilla cells [20]. However, to the best of our knowledge, the nanotoxicity and mechanism of Ti$_{0.8}$O$_2$ nanosheets for specific site-targeting strategies in NSCLC have not yet been investigated. Consequently, this study aimed to investigate the effects of Ti$_{0.8}$O$_2$ nanosheets on the cytotoxicity of human non-small cell lung cancer (NSCLC) cells and to identify the molecular mechanisms behind the toxicity of these cells, which we reveal to be related to ROS generation-mediated apoptosis via the mitochondrial pathway. This study could be valuable in the development of nanomaterials for anticancer approaches.

Results

Synthesis and characterization of the Ti$_{0.8}$O$_2$ nanosheets

The preparation of the Ti$_{0.8}$O$_2$ nanosheets from the lepidocrocite-type K$_{0.8}$Zn$_{0.4}$Ti$_{1.6}$O$_4$ is shown schematically in Figure 1A. Here, the K$^+$ ion (0.8 per the formula unit) alternately stacks with the negatively-charged sheets of edge-shared (Ti,Zn)O$_6$ octahedra (i.e., the Zn$_{0.4}$Ti$_{1.6}$O$_4$$^{4-}$ sheet with ∼1 nm thickness). Reacting the solid with 1 M HCl led to a quantitative replacement of K$^+$ with H$_2$O$^+$ and an almost complete Zn leaching. The subsequent reaction of the solid with the bulky TBA$^+$ ions resulted in the infinite separation of stacks of sheets into individual nanosheets.

As shown in Figure 1B, the obtained colloidal suspension was white and with a blue tint. It absorbed light at $\lambda$$_{max}$ = 261 nm, which is consistent with previous reports. A clear Tyndall effect could be observed where laser illumination was scattered throughout, suggesting the presence of nanosheets. The Dynamic Light Scattering provided the size estimation of the Ti$_{0.8}$O$_2$ nanosheets as ∼268 nm, see Figure 1C. The zeta potential of -30 mV agreed well with their negatively-charged nature. Figure 1D is a representative TEM image, showing several uniform-contrast, flat objects (i.e., nanosheets) with lateral dimensions of up to ∼200 nm. Altogether, the different characterization techniques confirmed the successful preparation of the negatively-charged Ti$_{0.8}$O$_2$ nanosheets (or simply Ti$_{0.8}$O$_2$ nanosheets) in contrast to typical TiO$_2$ with a charge-neutral. Detail characterizations can be found elsewhere (Mol Pharm 95 2019 418; ACS Appl Mater Interfaces 2 2019 3840).

Cytotoxicity of the Ti$_{0.8}$O$_2$ nanosheets on human lung cancer cells and normal cells

Cells were treated with various concentrations of Ti$_{0.8}$O$_2$ nanosheets (0–100 μg/mL) and analyzed by MTT assay. The results revealed the statistically significant cytotoxic effects of Ti$_{0.8}$O$_2$ nanosheets occurred at concentrations of 10–100 μg/mL in A549, H460, and H23 cells and at 20–100 μg/mL in H292 cells. In the primary dermal papilla cells from different sources (DP1 and DP2), cytotoxic effects of the Ti$_{0.8}$O$_2$ nanosheets were found at 50 μg/mL. Moreover, at the concentration of 30 μg/mL, the Ti$_{0.8}$O$_2$ nanosheets showed statistically significant cytotoxic effects on DP and HaCat cells (Fig. 2A–H). Characteristic apoptosis cells were identified using a nuclear staining assay. The results showed that the Ti$_{0.8}$O$_2$ nanosheets mediated apoptosis in lung cancer cells at concentrations of 1–10 μg/mL, with a small percentage of necrotic cells (Fig. 21-L). Flow cytometry analysis based on annexin V/PI detection also confirmed that 10 μg/mL of Ti$_{0.8}$O$_2$ nanosheets induced dramatic apoptosis in A549 and H460 cells when compared with untreated cells (Fig. 2M–N).

Uptake of the Ti$_{0.8}$O$_2$ nanosheets by cancer cells

Under SEM morphological analysis, it was seen that for H460 cells, the morphology of the cancer cells gradually changed, including the formation of stress fibers, when treated with Ti$_{0.8}$O$_2$ nanosheets at concentrations of 1–10 μg/mL (Fig. 3A). Moreover, TEM analysis showed that Ti$_{0.8}$O$_2$ nanosheets at 10 μg/mL could appropriately pass into the H460 cells (Fig. 3B).

Ti$_{0.8}$O$_2$ nanosheets modulate apoptosis-related proteins in H460 and A549 cells

In order to investigate the mechanism of Ti$_{0.8}$O$_2$ nanosheets-induced apoptosis, the apoptotic-related proteins were determined by Western blot analysis. A549 and H460 cells were treated with 0–10 μg/mL Ti$_{0.8}$O$_2$ nanosheets, and then the pro- and ant-
apoptotic proteins related to mitochondria-mediated apoptosis were evaluated. The results showed that the TiO$_2$ nanosheets increased the pro-apoptotic protein Bax, whereas the anti-apoptotic proteins Mcl-1 and Bcl-2 were downregulated in the cells treated with the Ti$_{0.8}$O$_2$ nanosheets. In addition, pro-caspase3 was decreased in a concentration-dependent manner. Moreover, p53 was found to be activated in response to the treatment with the Ti$_{0.8}$O$_2$ nanosheets (Fig. 3C and D). Taken together, it can be concluded that the Ti$_{0.8}$O$_2$ nanosheets mediated the apoptosis of lung cancer cells by increasing the pro-apoptotic proteins, which led to cell death by the mitochondria-dependent pathway.

**Cytotoxicity and apoptotic effects of Ti$_{0.8}$O$_2$ nanosheets on advanced lung cancer cells from patients**

To assess the potential pharmacological activities of synthetic Ti$_{0.8}$O$_2$ nanosheets compounds in advanced lung cancer cells, treatment with the current standard therapeutic agents was also performed for comparison. Two groups of cell lines were used for the investigations: panel A, an advanced non-small cell lung cancer cell line from patients with malignant pleural effusion who had never been treated by chemotherapy, targeted therapy, or immunotherapy; and panel B, an advanced non-small cell lung cancer cell line from patients with malignant pleural effusion who had been treated with standard platinum-doublet chemoRx with or without targeted therapy or a checkpoint inhibitor and second-line chemoRx. In total, six primary lung cancer cells were treated with the same concentrations of Ti$_{0.8}$O$_2$ nanosheets, cisplatin, and etoposide (0–100 μg/mL) for 24 hours and subjected to cell viability analysis by MTT assay. The Ti$_{0.8}$O$_2$ nanosheets could be considered nontoxic at doses lower than 1 μg/mL, while a concentration of more than 10 μg/mL caused a significant decrease in the cell viability of the cells (Fig. 4A–F); whereas, the standard drugs showed a slightly decreased cell viability from 0.5 μg/mL to 100 μg/mL, while doses of more than 20 μg/mL cisplatin and etoposide were considered toxic. Data analysis showed that the IC$_{50}$ of the Ti$_{0.8}$O$_2$ nanosheets was lower than 10 μg/mL at 24 h, which was significantly lower than for cisplatin and etoposide (Fig. 4M and N). The results showed that the Ti$_{0.8}$O$_2$ nanosheets reduced cell viability in a concentration-dependent manner compared with the untreated controls (Fig. 4A–F). To confirm the effect of the Ti$_{0.8}$O$_2$ nanosheets on advanced lung cancer cells from patients, a nuclear staining assay using Hoechst 33342 and propidium iodide was performed and the results analyzed. After treatment with the compounds at 10 μg/mL of Ti$_{0.8}$O$_2$ nanosheets, apoptotic cells were observed by the presence of a nuclear condensation morphology in the representative cell line (Fig. 4H–M). The percentage cell viability was determined using the MTT assay to determine the IC$_{50}$ values (Fig. 4G).

**Effect of Ti$_{0.8}$O$_2$ nanosheets on intracellular ROS induction in A549 and H460 cells**

Current developments in cancer research suggest that the generation of ROS through oxidative stress is a common mechanistic pathway of a number of apoptotic stimuli. ROS is considered an essential signaling molecule for the initiation and execution of apoptosis [32]. Consequently, we next investigated whether intracellular ROS generation was implicated in the anticancer effects of the Ti$_{0.8}$O$_2$ nanosheets. The intracellular ROS level was evaluated using the fluorescent probe DCFH-DA. The results showed that treatment with Ti$_{0.8}$O$_2$ nanosheets increased the intracellular ROS generation (Fig. 5A and B). In order to investigate the protective effect of N-acetylcysteine (NAC) or glutathione (GSH) as a potent antioxidant on Ti$_{0.8}$O$_2$ nanosheets -induced cytotoxicity mediated through ROS generation, H460 and A549 cell lines were pretreated with NAC or GSH for 1 h previous to treatment with the Ti$_{0.8}$O$_2$ nanosheets. We detected a decrease in the ROS level in all the cell lines treated with NAC and GSH (Fig. 5A and B), but the cell viability of the cancer cells could not be reversed by the pretreatment with NAC or GSH (Fig. 5C and D). These results suggested that the Ti$_{0.8}$O$_2$ nanosheets induce cytotoxicity in cancer cell lines but did not do this via the generation of ROS. Next, we investigated the specific ROS products using a DHE (dihydroethidium) fluorescent probe for the detection of ROS generation, and specifically for the detection of superoxide anions. The results showed that the Ti$_{0.8}$O$_2$ nanosheets had a significant effect on the superoxide anions in H460 cells.
when they were treated with TiO$_2$O$_2$ nanosheets in a concentration-dependent
manner (Fig. 5E); while the TiO$_2$O$_2$ nanosheets had only a slight effect on superoxide
anion generation in A549 cells (Fig. 5E). In addition, we also investigated the
generation of hydroxyl radicals using the HPF (hydroxyphenyl fluorescein)
fluorescent probe in both cell lines. The results showed that the TiO$_2$O$_2$ nanosheets
significantly generated hydroxyl radicals in both cell lines compared with the non-
treated cells (Fig. 5F). According to our obtained data, the pretreatment of cancer cell
lines with a potent antioxidant for 1 h could not inhibit H$_2$O$_2$ damage, while the TiO$_2$O$_2$
nanosheets generated superoxide anion hydroxyl radicals in both cell lines.

TiO$_2$O$_2$ nanosheets-mediated peroxynitrite induces apoptosis in A549 and H460 cells

Nitric oxide plays a role in apoptosis regulation through its ability to modulate ROS.
The cytotoxic capacity of nitric oxide has been confirmed in numerous systems using
diverse cell targets. In many circumstances, the cytotoxicity is the result of the
interaction of nitric oxide with superoxide to form peroxynitrite (ONOO$^-$), a potent
oxidant that is a key element in inducing cell death [33]. Consequently, we analyzed
the cellular nitric oxide levels in response to the TiO$_2$O$_2$ nanosheets using DAF-FM DA
as a fluorescent probe. The nitric oxide levels were found to be increased in a
concentration-dependent manner (Fig. 6A and C). Additionally, co-treatment with
PTIO (a nitric oxide scavenger) and/or MnTBAP (a superoxide anion inhibitor)
inhibited TiO$_2$O$_2$ nanosheets -induced cell death by increasing cell viability (Fig. 6B
and D). The results suggested that TiO$_2$O$_2$ nanosheets induce cytotoxicity in cancer cell
lines via the produced peroxynitrite. To confirm the previous results, we treated cells
with TiO$_2$O$_2$ nanosheets and/or pretreated them with PTIO and/or MnTBAP and then
determined the cellular nitric oxide level by staining with DAF-FM DA and then
visualizing under a fluorescence microscope. The results showed that the co-
treatment with these inhibitors decreased peroxynitrite levels in all the cancer cells
(Fig. 6F). Then, we observed whether increased peroxynitrite was required for cell
apoptosis induced by TiO$_2$O$_2$ nanosheets. The results showed that the co-treatment
with these inhibitors was able to inhibit apoptosis cell death, as shown in Figure 6E.
Collectively, these results indicated that peroxynitrite generation could play a role in
mediating TiO$_2$O$_2$ nanosheets -induced cell apoptosis.

TiO$_2$O$_2$ nanosheets-mediated peroxynitrite induces apoptosis in A549 and H460 cells via
p53 upregulation

Since then, several cell types have been shown to undergo apoptosis in response to
nitric oxide or peroxynitrite. A previous study reported that peroxynitrite was
associated with p53 regulation to induce cancer cell death [33]. Therefore, we
examined the effect of TiO$_2$O$_2$ nanosheets when combined with PTIO and/or MnTBAP.
Western blot analysis was performed to evaluate the p53 protein levels after 10
µg/ml TiO$_2$O$_2$ nanosheets treatment in all the cell lines. The results showed that the
p53 protein levels in all the cell lines were significantly increased with the TiO$_2$O$_2$
nanosheets alone treatment compared with those of the non-treatment control and
another condition treatment (Fig. 7A–C). Taking this together, it could be concluded
that the TiO$_2$O$_2$ nanosheets induced cancer cell death by the induction of peroxynitrite
generation, which activated p53, leading to cancer cell apoptosis. The
immunofluorescence staining results supported our finding that TiO$_2$O$_2$ nanosheets in
combination with PTIO and MnTBAP caused a dramatic decrease in the level of p53
and P-p53 in both cell lines (Fig. 7D and E). These results indicate that the pro-
apoptotic effect of TiO$_2$O$_2$ nanosheets for inducing nitric oxide was a result of the
formation of peroxynitrite, which then induced p53-dependent apoptosis in all the cell
lines.

TiO$_2$O$_2$ nanosheets increase p53 function but not through p53 proteasomal degradation

In healthy cells, p53 plays a pivotal role in responding to oncogenic stress signals and
helps to keep cells metabolically stable [34]. The importance of p53 is highlighted by
the fact that it is frequently altered in human cancers [35], [36]; indeed, even tumors
that retain wild-type p53 are often compromised in their ability to activate the p53
pathway. The acute activation of p53 leads to numerous responses that prevent
further cell division, including cell cycle arrest, senescence, and apoptosis. In this way, p53 can prevent the outgrowth of incipient cancer cells. A protein abundance reflects the balance of the rates of protein synthesis and protein degradation. Key to the regulation of p53 is control of the stability of the p53 protein, which is mainly arranged through a network of ubiquitination reactions. We further evaluated the effect of the TiO$_2$ nanosheets on the TiO$_2$ nanosheets stability in lung cancer cells. To compare the TiO$_2$ nanosheets stability between TiO$_2$ nanosheets -treated cells and the control cells, the cycloheximide (CHX) chasing assay was used followed by Western blot analysis. CHX, an inhibitor of protein biosynthesis, is widely used for determining the half-life of proteins of interest [37]. Therefore, H460 and A549 cells were treated with TiO$_2$ nanosheets (10 µg/ml) in the presence or absence of 50 µg/mL CHX, and the level of p53 over time was determined. Figures 8A, B show that in the condition where protein production was blocked, the TiO$_2$ nanosheets increased the stability of the p53 protein. A difference was first detected at 90 min after TiO$_2$ nanosheets treatment (Fig. 8A and B). We also determined the p53 protein half-life and found that the half-life of the p53 protein in the TiO$_2$ nanosheets -treated groups was about 60 min; whereas in the untreated control, the value was about 30–40 min (Fig. 8C).

Ubiquitin–proteasome degradation has been shown to influence protein turnover. Thus, MG132, a potent proteasome inhibitor, was used to prove that this increase in p53 stability was through proteasomal degradation of the protein by the TiO$_2$ nanosheets. We also checked the premise of ubiquitin-mediated p53 degradation using co-immunoprecipitation and evaluated the level of the p53-ubiquitin complex (poly Ub-p53) in H460 and A549 cells after treatment with 10 µg/ml of TiO$_2$ nanosheets and in non-treated control cells for 1 h. Figures 8D–E show that the polyubiquitination of p53 was noticeably diminished after TiO$_2$ nanosheets treatment when compared with the non-treated control; thus confirming that the TiO$_2$ nanosheets-mediated p53 stability did not occur through ubiquitin–proteasome degradation.

S-nitrosylation in the regulation of stability of the tetrameric p53 protein-protein complex

It has become increasingly evident that nitric oxide exerts its effects, in part, by the S-nitrosylation of cysteine (Cys) residues. The S-nitrosylation of a single Cys within HDM2 inhibits p53 binding and thereby stabilizes p53 and activates p53-dependent transcription [38]. We tested in vitro whether peroxynitrite may directly control p53 by S-nitrosylation and the activation of p53. Sulphydryl sensitivity and reversibility are consistent with nitrosylation. Finally, we identified a critical cysteine residue that nitric oxide modifies to disrupt p53 binding. To evaluate the structural stability of the p53 core domain tetramer (Fig. 9A), the number of intermolecular hydrogen bonds formed between each monomer at the protein–protein interface was monitored along with the simulation time. Note that a hydrogen bond was defined by the following geometric criteria: (i) the distance between the hydrogen bond donor (D) and acceptor (A) atoms is less than 3.5 Å, and (ii) the angle between D−H•••A is greater than 120°. The obtained results showed that there was an average of ~10 ± 2 hydrogen bonds steadily formed over the course of the simulation time (Fig. 9B, top).

This observation suggested that our simulation model was highly stable. Therefore, a hydrogen bond energy from each amino acid for the protein complex is shown in Figure 9C (top), where the positive and negative $\Delta A_{\text{H}}$ values are associated with protein destabilization and stabilization, respectively. It is noteworthy that only amino acids exhibiting a $\Delta A_{\text{H}}$ value of < −1.5 kcal/mol were marked as the key binding residues. The results showed that the crucial residues (L93, N167, M169, C176, P177, and E180 for monomer A; H178, E180, R181, N200, L201, and V225 for monomer B; C176, P177, H178, E180, R181, E198, L201, E224, V225, and H233 for monomer C; V97, N100,
N167, M169, C176, P177, E180, and R181 for monomer D) played a pivotal role in the tetrameric protein–protein stabilization. Based on this calculation and upon visual inspection, it can be assumed that the cysteine residue within the protein–protein interface, particularly C182, was most likely to be a critical residue, which would be expected to be related to the S-nitrosylation site and consequently lead to an increase in protein stability. To clarify such an hypothesis, the influence of S-nitrosylation at the C182 residue of p53 on its binding interaction with each monomeric p53 was investigated by means of MD simulation, as per the native p53 system. The simulation indicated that the total number of intermolecular hydrogen bonds between four monomeric proteins was slightly increased over the whole simulation, with an average value of \(~12 \pm 2\) hydrogen bonds, particularly in the last 10 ns (90–100 ns), in which the number of hydrogen bonds was found to be up to \(~20\) (Fig. 9B, bottom). This reflected that the S-nitrosylated C182 resulted in a higher stability of the tetrameric protein–protein complex compared to the native p53. In addition, the occurrence of the C182 S-nitrosylation appeared to induce the surrounding residues located in the interface region to bind more tightly to each other, especially the residues 176–186 (Fig. 9C, bottom and Fig. 9D). Among these amino acids, the lowest \(\Delta G_{\text{bind}}\) value (\(~−5\) to \(~−7\) kcal/mol) was observed for residue R181, most likely owing to the indirect stabilizing effect of the S-nitrosylation at C182. This was probably one of the reasons why the S-nitrosylation culminated in a higher stability of p53, as observed in the experimental data.

Discussion
Lung cancer remains the major cause of cancer death worldwide. Nowadays, nanomaterials are showing remarkable potential to aid the diagnosis and treatment of cancer by enabling the more effective targeting of tumors [39]. Previous studies have revealed that nanomaterials can selectively sink in solid tumors, whereby they increase the bioavailability and decrease the toxicity of the encapsulated cytotoxic agents [39,40]. Ti\(_{0.8}\)O\(_2\), an emerging 2D analog of TiO\(_2\), nanosheets can be derived from the potassium zinc titanate precursor K\(_{0.2}\)Zn\(_{0.8}\)Ti\(_{1.6}\)O\(_6\). The planar surface and functional motifs of such 2D inorganic nanosheets can be modified using a surface engineering process via chemical bonding or physical adsorption [22], which favors subsequent nanomedical applications in specific physiological environments, through e.g., their biostability improvement, site-specific targeting capability, and multiple theranostic functions to facilitate oncological applications [18,40,41].

Although nanoparticles and nanotubes of TiO\(_2\) have been extensively studied regarding biomedical applications [41,42], to the best of our knowledge, there are no studies on Ti\(_{0.8}\)O\(_2\) nanosheets. Consequently, here, we showed for the first time that Ti\(_{0.8}\)O\(_2\) nanosheets could distinctively induce anticancer activity in human non-small cell lung cancer cells and advanced lung cancer cells from patients. This effect was demonstrated in several lung cancer lines in comparison to common chemotherapeutic drugs used in lung cancer patients. The Ti\(_{0.8}\)O\(_2\) nanosheets significantly increased cancer cell death in a concentration-dependent manner (Fig. 2). Moreover, the Ti\(_{0.8}\)O\(_2\) nanosheets also mediated apoptosis in lung cancer cells in a concentration-dependent manner (Fig. 2I–N). Furthermore, SEM morphological analysis demonstrated that 10 \(\mu\)g/mL Ti\(_{0.8}\)O\(_2\) nanosheets initially changed the morphology, including the formation of stress fibers, while TEM analysis for characterization of the Ti\(_{0.8}\)O\(_2\) nanosheets in cells showed that the Ti\(_{0.8}\)O\(_2\) nanosheets could appropriately disperse into H460 cells more easily than in DP cells (Fig. 3B). A previous study showed that various types of nanoparticles (NPs) could be used to induce anticancer activity in cancer cells, such as copper oxide nanoparticles [43]. Next, we further examined whether the Ti\(_{0.8}\)O\(_2\) nanosheets could induce cell apoptosis in H460 and A549 cells. We found that the treatment of lung cancer cells with Ti\(_{0.8}\)O\(_2\) nanosheets resulted in a significant induction of p53, which may, at least in part, play a role in Ti\(_{0.8}\)O\(_2\) nanosheets-mediated apoptosis (Fig. 3C and D). Consistent with our findings, a previous study showed that FePt/GO nanosheets suppressed proliferation and induced apoptosis in H1975 cells and silver nanoparticles induced apoptosis in human colon cancer cells mediated by p53 [44,45].

The majority of human cancers appear to exhibit either abnormal p53 or...
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sites of Ser 15 and/or Ser 20, subsequently blocking p53–
cisplatin (CDDP) treatment, activates and stabilizes p53 via phosphorylation at the
resulted in a profound alteration of the protein
protein localization, and protein stability [56]. Cellular stress, such as through
the cysteine amino acid [54]. Until recently, more than 1000 proteins had been found
wherein nitric oxide is attached to a thiol moiety of the target protein forming S-NO at
interaction with the protein in
p53 occurs as a result of preventing its degradation process.
decrease in p53–upregulated (Fig. 6). Concomitantly, increased p53 was detected (Fig. 7A–
exposed to the nanosheets, the intracellular level of peroxynitrite was highly
the regulation of p53 tetramerization. Our results showed that when the cells were
upregulated the expression of p53-mediated apoptosis (Fig. 7). The data support the
that the generation of peroxynitrite after treatment with TiO₂ nanosheets
upregulated the expression of p53-mediated apoptosis (Fig. 7). The data support the
protein interaction, protein function,

p53 is an important tumor suppressor gene involved with the induction of
apoptosis and cell cycle regulation in cells that have sustained DNA damage. We found
that the generation of peroxynitrite after treatment with TiO₂ nanosheets
upregulated the expression of p53-mediated apoptosis (Fig. 7). The data support the
hypothesis that peroxynitrite contributes to the tumorigenic properties of p53
mutations. Peroxynitrite was found to induce mitochondrial permeability transition
changes and promote apoptosis in cell-free systems containing mitochondria [52].
Cancer predisposition, onset, and therapeutic response can be critically determined
by the integrity of the tumor suppressor p53. The degradation of p53 in normal cells
is regulated through ubiquitination by the E3 ubiquitin ligase Mdm2 [53]. In this
study, we found that under TiO₂ nanosheets treatment, the half-life of p53 was
dramatically increased. The cycloheximide-based assay showed that the half-life of
p53 in response to 10 µg/ml TiO₂ nanosheets was about 60 min in comparison to
30–40 min in the non-treated control cells (Fig. 8A–C). After applying the selective
proteasome inhibitor (MG132), we monitored the levels of the p53–ubiquitin complex
and found that the formation of the complex was dramatically decreased in the TiO₂
nanosheets -treated cancer cells (Fig. 8D and E). Here, we have revealed novel
information regarding the role of reactive nitrogen species, especially peroxynitrite, in
the regulation of p53 tetramerization. Our results showed that when the cells were
exposed to the nanosheets, the intracellular level of peroxynitrite was highly
upregulated (Fig. 6). Concomitantly, increased p53 was detected (Fig. 7A–C) with the
decrease in p53–ubiquitin complex (Fig. 8D and E), implying that the upregulation of
p53 occurs as a result of preventing its degradation process.
Peroxynitrite is considered an important biological inducer via its direct
interaction with the protein in S-nitrosylation. S-nitrosylation is a rapid interaction
wherein nitric oxide is attached to a thiol moiety of the target protein forming S-NO at
the cysteine amino acid [54]. Until recently, more than 1000 proteins had been found
as the targets of S-nitrosylation [55] and it was noted that such protein modification
resulted in a profound alteration of the protein–protein interaction, protein function,
protein localization, and protein stability [56]. Cellular stress, such as through
cisplatin (CDDP) treatment, activates and stabilizes p53 via phosphorylation at the
sites of Ser 15 and/or Ser 20, subsequently blocking p53–Mdm2 interaction and
suppressing p53 degradation [57]. Protein p53 is a key transcription factor that induces cell arrest when DNA is damaged and triggers the expression of DNA repair machinery, or apoptosis when the damage is irreversible [58]. The protein folds into several domains, the most relevant ones being the DNA-binding domain (p53DBD), at the core of the protein, and the tetramerization domain (p53TD), close to the C terminus [59]. However, the activity of the protein strongly depends on its tetrameric integrity [60]. Hence, molecules able to stabilize the tetrameric structure of mutated proteins with compromised tetramerization abilities could be valuable therapeutic tools. Furthermore, we report additional studies on the role of hydrogen bond interactions in protein stability and on the key binding residues of p53 to direct the effect of the S-nitrosylation (Fig. 9). In globular proteins, there are intermolecular hydrogen bonds between the protein and water molecules, and between water molecules that are bound with the proteins [61]. Here we used computational tools to predict the point of S-nitrosylation on the p53 protein, and found that peroxynitrite may directly control p53 by S-nitrosylation to stabilize the tetrameric structure of this protein. To estimate the contribution of these hydrogen bonds to the conformational stability of a protein compared with that of the native p53 and S-nitrosylation of p53 [62], we investigated the relationship between S-nitrosylation and the increase in p53 stability. We identified the H-bond intermolecular interactions between a monomer of native p53 compared to its S-nitrosylation form, and found a higher stability of the tetrameric protein–protein complex in comparison to the native p53, especially regarding the reactivity of the cysteine at residue 182 in p53. The high reactivity of specific cysteine thiol groups in p53 are likely important for the regulation of p53 and its degradation pathways [38]. Moreover, peroxynitrite has been shown to activate the opening of mitochondrial pores that release cytochrome c into the cytoplasm [63]. According to our results, we found that peroxynitrite induces p53 stability, and increases the activation of Bax and subsequently caspase 3. These changes are all hallmarks of cell death. Further, the nanosheets were shown to generate peroxynitrite in aggressively driven mechanisms, including the process for the S-nitrosylation of p53 for protein stabilization. This novel finding on the role of Ti0.8O2 nanosheets in p53-mediated apoptosis may have important implications in cancer treatment.

Conclusions
In conclusion, our present study, for the first time provides information on the effect of Ti0.8O2 nanosheets induce apoptosis through a molecular mechanism involving peroxynitrite generation. After treatment with Ti0.8O2 nanosheets, it may directly control p53 by S-nitrosylation to stabilize the tetrameric structure of this protein. This reflected that the S-nitrosylated at C182 of p53 resulted in a higher stability of the tetrameric protein–protein complex compared to the native p53. Therefore, the results of this study ingeminate the novel mechanism of action of nanomaterials for cancer therapy.

Methods

Ti0.8O2 nanosheets synthesis and characterization
The Ti0.8O2 nanosheets were prepared as reported previously. Briefly, the potassium zinc titanate K0.8Zn0.4Ti1.6O4 was first synthesized by heating the stoichiometric mixture of K2CO3, ZnO and TiO2 at 900 °C for 20 h. Then, the solid was soaked in 1 M HCl overnight (solid-to-solution ratio of 1 g-to-100 mL) for a total of 3 cycles, with the fresh acid replaced in between. The product is H1.6Ti1.6 O•0.8H2O, where 0.8H+ first exchanged for 0.8K+; another 0.8H+ for the leached [21] 0.4Zn2+; with water inclusion. Finally, 0.4 of the protonic form was mechanically shaken at 180 rpm for 14 days with diluted tetrabutylammonium hydroxide (TBAOH) solution (1 M, Sigma-Aldrich). The solid-to-solution was fixed at 0.4 g-to-100 mL, and the TBA+/H+ ratio at 1. The white colloid of Ti0.8O2 nanosheets was then obtained.

The absorption characteristics of the nanosheets colloid was measured using a T90+ UV/VIS spectrometer (PG Instruments). The “size” of the nanosheets (i.e., the
hydrodynamic radius as determined by Dynamic Light Scattering), and also the zeta potential, were measured using a Beckman Coulter Delsa Nano instrument. The nanosheets were also imaged using a JEOL JEM 2010 transmission electron microscope. Other results can be found in more detail elsewhere.

**Cell culture and reagents**

Non-small cell lung cancer cell lines including H460, H292, H23 and A549 were obtained from the American Type Culture Collection (Manassas, VA, USA). Human dermal papilla primary cell (primary DP1) was purchased from Celprogen (Benelux, Netherlands). The immortalized dermal papilla cells (DP) and human primary hair follicle dermal papilla cells (primary DP2) were purchased from Applied Biological Materials Inc, Richmond, BC. Furthermore, the human keratinocyte cell line (HaCaT) was purchased from Cell Lines Service (Heidelberg, Germany). H460, H292 and H23 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA), whereas A549, HaCaT, DP, primary DP1 and DP2 cells were cultivated DMEM medium (Gibco, Grand Island, NY, USA). The medium was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin and 2 mM L-glutamine (Gibco, MD, USA).

The cells were incubated in a 5% CO2 environment at 37˚C. Phosphate buffer saline (PBS) and trypsin-EDTA were purchased from GIBCO (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), Hoechst33342, propidium iodide (PI), bovine serum albumin (BSA) and dihydroethedium (DHE) were purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). Apoptosis Kit (FITC) was purchased from ImmunoTools (Germany). DCF, 2',7'-Dichlorofluorescein; and 3'-p-(hydroxyphenyl) fluorescein (HPF) were purchased from Invitrogen (USA). Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) was purchased from Merck (Germany). Antibody for mTOR, p53, Bcl-2, Mcl-1, Bax, caspase3 and β-actin as well as peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA).

**Patient-derived primary lung cancer cell line preparation from malignant pleural effusion**

The patient-derived malignant cancer cells were isolated from pleural effusions of recurrent or advanced stage non-small cell lung cancer patients who had been diagnosed at the King Chulalongkorn Memorial Hospital. The protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB 365/62) and was obtained informed consents from all contributors. Primary cancer cells were collected from pleural effusion (500-1,000 mL) through thoracentesis. The collected samples were centrifuged at 300 g for 10 min, at 4˚C and the cells were resuspended in RPMI medium with 10% FBS, 2 mM L-glutamine, and 100 units/ml of each of penicillin and streptomycin. After culturing for 10-15 passages, they were characterized as the patient-derived primary cancer cell lines (PM-4, ELC09, ELC12, ELC16, ELC17, and ELC20).

**Cytotoxicity assay**

Cells were seeded onto 96-well plates at the density of 1x10^4 cells/well and were allowed to incubate overnight. Then, cells were treated with various concentrations (0-100 μg/mL) of TiO_2 nanosheets for 24 h. at 37 °C and analyzed for cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT product was measured at 570 nm using a microplate reader. The cell viability was calculated by dividing the absorbance of the treated cells by that of the control cells and represented in percentage. All analyses were performed in 3 independent replicate cell cultures.

**Nuclear staining assay**

To determine apoptotic and necrotic cell death, H460 and A549 cells were seeded onto 96-well plates at the density of 1x10^4 cells/well, were incubated overnight and then were treated with TiO_2 nanosheets at various concentrations (0–10 μg/mL) for 24 h. at 37 °C. After that, the cells were incubated with Hoechst 33342 (10 μg/mL) and propidium iodide (PI) (5 μg/mL) for 30 min at 37 °C in the dark. These cells were
imaged under a fluorescence microscope (Nikon ECLIPSE Ts2).

**Cell apoptosis analysis**

Stage of apoptosis and necrosis cells were determined with Annexin V- FITC Apoptosis Kit (ImmunoTools, Germany). H460 and A549 cells were seeded in 24 well plates at a density of $1 \times 10^5$ cells/mL and were treated with Ti$_{0.8}$O$_2$ nanosheets at various concentrations (0–10 μg/mL) for 24 h. Then, cells were detached and suspended in 100 μL of 1X binding buffer and incubated in 5 μL of Annexin V-FITC and 1 μL of PI for 15 min at room temperature in the dark. Next, cells were analyzed by guava easyCyteTM flow cytometry systems.

**Scanning electron microscopy (SEM) morphological analysis**

H460 treated cells were fix in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1-2 h. Then, the cells were rinsed with phosphate buffer and distilled water. Next, cells were dehydrated with a graded series of ethanol (30%, 50%, 70%, and 95% for 5 minutes/each and 100% 3 times, 5 minutes/time), dried, mount and coat with gold (sputter coater, Balzers model SCD 040, Germany). Finally, cells were observed under a SEM (JEOL, model JSM6400, Japan).

**Transmission electron microscopy (TEM) for cellular uptake analysis**

To confirm that Ti$_{0.8}$O$_2$ nanosheets were uptake by cancer cell and/or normal cell, H460 and primary DP1 cells ($1 \times 10^6$ cells/mL) were seeded and treated with Ti$_{0.8}$O$_2$ nanosheets at 10 μg/mL for 24 h. Next, the treated cells were collected, washed with PBS and then fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in alcohol and embedding. Thin sections of resin embedded cells were cut and observed cellular uptake with a transmission electron microscope (TEM) JEM-1400 (Jeol Ltd., Tokyo, Japan).

**Western Blot Analysis**

Japan To determine proteins regulation in apoptosis pathway, the treated cells (H460 and A549) were lysed into cellular lysates as previous described [20] Equal amounts of protein from each sample were separated by SDS-PAGE and transferred to 0.45 μm nitrocellulose membranes (Bio-Rad). The blots were blocked for 1 h. with 5 % non-fat dry milk in TBST (Tris-buffer saline with 0.1 % Tween containing 25 mM Tris-HCl (pH 7.5), 125 mM NaCl and 0.1 % Tween 20) and incubated with specific primary antibodies against mTOR, p53, Bcl-2, Mcl-1, Bax, caspase3 and β-actin at 4 °C overnight. Then, the blots were washed in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 hours at room temperature. Finally, protein bands were detected using an enhancement chemiluminescence substrate (Supersignal West Pico; Pierce, Rockford, IL, USA) and exposed to film.

**ROS, superoxide anion and hydroxyl radical detection by flow cytometry**

A549 and H460 cells were seeded in 24-well plate and incubated overnight. Then, DCFHDA, DHE and HPF were added and then incubated for 30 min in dark. Next, cells were treated with Ti$_{0.8}$O$_2$ nanosheets for 3 h. Next, cells were washed and were resuspended in PBS. DCF fluorescence was quantified using guava easyCyteTM flow cytometry systems.

**Nitric oxide detection by DAF-FM DA assay**

After detachment, cells were collected and incubated with 10 μM DAF-FM DA for 30 min at 37°C. The cells were then washed, resuspended in phosphate buffered saline, and analyzed for fluorescence intensity using guava easyCyteTM flow cytometry systems. These cells were imaged under a fluorescence microscope (Nikon ECLIPSE Ts2).

**Immunofluorescence**

Cells were seeded onto 96-well plates at the density of $1 \times 10^5$ cells/well. After treatment 24 h, the cells were fixed with 4% (w/v) paraformaldehyde for 30 min and permeabilized with 0.1% (v/v) Triton-X for 20 min. Next, the cells were incubated
with 3% (w/v) BSA for 30 min, washed and incubated with an p53 or P-p53 antibody overnight at 4 °C, washed and incubated with Alexa Flour 488 (Invitrogen) conjugated goat anti-rabbit IgG (H+L) secondary antibody for 1 hour at room temperature in the dark. Therefore, the cells were washed with PBS, co-stained with 10 µg/mL Hoechst 33342 and visualized and imaged using fluorescence microscopy (Nikon ECLIPSE Ts2).

**Cycloheximide (CHX) chasing assay**

Cells were seeded and treated with 10 µg/mL of Ti$_{0.8}$O$_2$ nanosheets with or without 50 µg/mL CHX for 0, 15, 30, 45, 60 and 90 min. The treated cells were collected and lysed with RIPA lysis buffer containing the protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Western blot analysis was performed for detecting p53 protein levels. Protein bands were analyzed using the ImageJ software (version 1.52, National Institutes of Health, Bethesda, MD, USA), and the Mcl-1 protein half-life was calculated.

**Immunoprecipitation Assay**

Cells were seeded and treated with 0 and 10 µg/mL of Ti$_{0.8}$O$_2$ nanosheets for 60 min. The treated cells were collected and lysed with RIPA lysis buffer containing the protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). After that, immunoprecipitation was performed by using Dynabeads™ Protein G Immunoprecipitation Kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Magnetic beads were prepared and resuspended with the primary antibody of p53 in a binding buffer for 10 min. A suspension of the magnetic bead-Ab complex was mixed with lysed protein and incubated at 4 °C overnight to allow p53 antigen to bind with magnetic bead-Ab complex. After that, the magnetic bead-Ab-Ag complex was washed three times using 200 µL washing Buffer, separated on the magnet between each wash, and the supernatant was removed. Elution Buffer was added for releasing the Ab-Ag complex from magnetic beads. The supernatant contained the Ab-Ag complex was then used to perform Western blot analysis for detecting the ubiquitinated p53 protein.

**Computational method**

The X-ray structure of tetrameric p53 core domain was taken from the protein data bank (PDB ID: 3KMD) [22]. The H++ web server [23] was used to assign the protonation state of all ionizable groups of amino acids at pH 7.4. The modeled protein was then submitted to all-atom molecular dynamics (MD) simulations using AMBER16 software package according to standard procedures [24-26] as summarized below. In brief, the starting structure of p53 protein was firstly energy-minimized using steepest descent (500 steps) and conjugated gradient (1,500 steps) based on the ff14SB AMBER force field [27] to reduce the unfavorable contacts. After that, the 100-ns MD simulation with the NPT ensemble at 310 K and 1 atm was carried out by the PMEMD module of AMBER16. The SHAKE algorithm [28] was applied to restrain the covalent bond involved in hydrogen atoms, allowing a simulation time step of 2 fs. The particle mesh Ewald [29] summation method was used to treat the long-range electrostatic interactions, whereas a nonbonded cutoff distance was set to 10 Å. The MD trajectories in production phase were collected every 10 ps and analyzed in terms of intermolecular hydrogen bonding interaction using the CPPTRAJ module [30] of AMBER 16. To determine the essential residues associated with protein-protein binding at the interface of the four monomers and the effect of the S-nitrosylation at the cysteine (Cys) residue towards the protein stability, the per-residue decomposition free energy ($\Delta G_{\text{bind}}^{\text{residue}}$) based on the molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) method was performed using MMPBSA.py module [31] implemented in AMBER16.

**Statistical Analysis**

Data from three independent experiments are presented as mean ± standard error of mean (SEM). Multiple comparisons for statistically significant differences between multiple groups were performed using analysis of variance (ANOVA), followed by
Turkey’s post hoc test. P value ≤ 0.05 will be considered as statistically significant.

Abbreviations
NSCLC: Non-small cell lung cancer; 2D: Two-dimensional; TEM: Transmission electron microscopy; SEM: Scanning electron microscopy; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma 2; Mcl-1: Myeloid cell leukemia 1; C182: Cysteine residues at position 182; MDM2: Mouse double minute 2; mTOR: Mammalian target of rapamycin; TD: Tetramerization domain; NO: Nitric oxide; NOS: Nitric oxide synthases; HIF-1α: Hypoxia-inducible factor-1α; O2−: Superoxide anion; ONOO−: Peroxynitrite; DCF: 2′,7′-dichlorofluorescein; DHE: dihydroethidium; FBS: fetal bovine serum; HPP: 3′-p-(hydroxyphenyl) fluorescein; MnTBA: Mn(III)tetrakis(4-benzoic acid) porphyrin; MT3: 3,4,5-trimethylthiazolid-2-yl)-2,5-diphenyltetrazolium bromide; PBS: phosphate-buffered saline; PI: propidium iodide; DMSO: dimethyl sulfoxide; ROS: Reactive oxygen species; MD: Molecular dynamics; NAC: N-acetylcysteine; GSH: Glutathione; DAF-FM DA: Diaminofluorescein-FM diacetate; PTO: 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; CHX: Cycloheximide; NPs: Nanoparticles.

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Authors’ contributions
Conceptualization—P.C.; Methodology—T.R., C.V., T.M., and P.C.; Formal analysis—R.S., B.N., and P.C.; Investigation—R.S., S.T., B.N., T.M., and P.C.; Writing and original draft preparation—R.S., S.T., T.M.; Writing, review, and editing—P.C.; Funding acquisition—P.C.; Supervision, P.C. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All patient-derived malignant cancer cells experimental protocols were approved the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB 365/62) and was obtained informed consents from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Interdisciplinary Program of Pharmacology Graduate School, Chulalongkorn University, Bangkok, Thailand. 2Cell-based Drug and Health Products Development Research Unit, Chulalongkorn University, Bangkok, Thailand. 3Departments of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn, Thailand. 4Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok, Thailand. 5Bioscatalyst and Environmental Biotechnology Research Unit, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. 6Program in Bioinformatics and Computational Biology, Graduate School, Chulalongkorn University, Bangkok, Thailand. 7Division of Medical Oncology, Department of Medicine, Chulalongkorn University, Bangkok, Thailand. 8College of Materials Innovation and Technology, King Mongkut’s Institute of Technology Ladkrabang, Bangkok, Thailand.

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Figures

A

1) Proton exchange and zinc leaching with HCl
2) Exfoliation with TBAOH

K$_{0.2}$Zn$_{0.8}$Ti$_{0.8}$O$_4$

B

UV-VIS absorption spectrum and photograph of the nanosheets without (left) and with (right) the laser light shining through.

C

Size of the nanosheets.

D

Representative TEM image of the deposited nanosheets.

Fig. 1 Synthesis and characterization of the Ti$_{0.8}$O$_2$ nanosheets. A Schematic diagram of the Ti$_{0.8}$O$_2$ nanosheets preparation. B UV-VIS absorption spectrum and photograph of the nanosheets without (left) and with (right) the laser light shining through. C Size of the nanosheets. D Representative TEM image of the deposited nanosheets.
Fig. 2 Cytotoxicity of the Ti$_{0.8}$O$_2$ nanosheets on human lung cancer cells and normal cells. A–H Effect of the Ti$_{0.8}$O$_2$ nanosheets on the cell viability of lung cancer cells (A549, H460, H292, and H23) and normal cells (Dermal Papilla (DP) cell line, primary DP1, primary DP2, and HaCaT keratinocyte cells) over 24 h using the MTT assay. I–L Morphology of the apoptotic nuclei stained with Hoechst 33342 dye and propidium iodide in cells treated with Ti$_{0.8}$O$_2$ nanosheets, determined by visualization under a fluorescence microscope; the percentages of nuclear fragments and PI positive cells were calculated. M, N Apoptotic and necrotic cells were determined using annexin V-FITC/PI staining with flow cytometry. Data are shown as the mean ± SD (n = 3). * P < 0.05 versus non-treated control.
Fig. 3 Morphology and characterization of cells when treated with Ti₃O₂ nanosheets. A Morphology of H460 cells determined by scanning electron microscopy (SEM). B Cellular uptake of Ti₃O₂ nanosheets in H460 and primary DP cells at 24 h determined by transmission electron microscopy (TEM). C, D Effect of Ti₃O₂ nanosheets on apoptosis-related proteins measured by Western blot analysis. Blots were reprobed with β-actin to confirm the equal loading of samples. E, F Relative protein levels calculated by densitometry. Data are shown as the mean ± SD (n = 3). * P < 0.05 versus non-treated control.
Fig. 4 Cytotoxicity of the Ti$_{0.8}$O$_2$ nanosheets on malignant pleural effusion from advanced lung cancer patients. A-F Effect of the Ti$_{0.8}$O$_2$ nanosheets on the cell viability of malignant pleural effusion for 24 h using the MTT assay to determine the IC$_{50}$ values. G-H Percentages of cell viability were determined using the MTT assay. I-M Morphology of apoptotic nuclei stained with Hoechst 33342 dye and propidium iodide in cells treated with Ti$_{0.8}$O$_2$ nanosheets, cisplatin and etoposide were determined by visualization under a fluorescence microscope; the percentages of nuclear fragments and PI positive cells were calculated. Data are shown as the mean ± SD (n = 3). * P < 0.05 versus non-treated control.
Fig. 5. Ti$_{0.8}$O$_2$ nanosheets induced intracellular ROS in H460 and A549 cells. A, B. The effect of Ti$_{0.8}$O$_2$ nanosheets (0–10 µg/mL) on intracellular ROS induction at 24 h in H460 and A549 cells was determined by flow cytometry with the fluorescent probe DCF (10 µM). Cells were treated with Ti$_{0.8}$O$_2$ nanosheets (0–10 µg/mL) alone for 24 h or with the pretreatment of 2.5 mM NAC and 2.5 mM GSH. C, D. Effect of Ti$_{0.8}$O$_2$ nanosheets on cell viability in H460 and A549 cells at 24 h with the pretreatment of 2.5 mM NAC or 2.5 mM GSH was determined by the MTT assay. Data are shown as the mean ± SD (n = 3). E. The effect of Ti$_{0.8}$O$_2$ nanosheets (0–10 µg/mL) on superoxide anion induction at 24 h in H460 and A549 cells was determined by flow cytometry with the fluorescent probe DHE (10 µM). F. The effect of Ti$_{0.8}$O$_2$ nanosheets (0–10 µg/mL) on hydroxyl radical induction at 24 h in H460 and A549 cells was determined by flow cytometry with the fluorescent probe HPF (10 µM).
Fig. 6 Ti$_2$O$_2$ nanosheets induced NO in A549 and H460 cells and mediated peroxynitrite generation in these cells. A, C Effect of Ti$_2$O$_2$ nanosheets on NO levels in A549 and H460 cells. Cells were treated with DAF-FM DA and various concentrations of Ti$_2$O$_2$ nanosheets (0–10 µg/mL) for 30 min and the peroxynitrite levels were determined at 3 h by flow cytometry. B, D The cytotoxic effect of Ti$_2$O$_2$ nanosheets and NO scavenger (PTIO) on A549 and H460 cells. Effect of Ti$_2$O$_2$ nanosheets and NO scavenger (PTIO) on cell viability. Cells were treated with Ti$_2$O$_2$ nanosheets (10 µg/mL) in the presence or absence of pretreatment with a NO scavenger (PTIO) (100 µM) or pretreatment with a superoxide inhibitor (MnTBAP) (50 µM) for 24 h by MTT assay. E Apoptotic and necrotic cells were determined using annexin V-FITC/PI staining with flow cytometry. F Cellular NO level stained with DAF-FM DA in cells treated with Ti$_2$O$_2$ nanosheets (10 µg/mL) or pretreated with PTIO (100 µM) or pretreated with MnTBAP (50 µM) were determined by visualization under a fluorescence microscope.
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**Fig. 7** TiO₂ nanosheets associated with apoptosis in A549 and H460 cells via p53 upregulation. **A, B** Peroxynitrite-potentiated cell apoptosis through the p53 protein was measured by Western blot analysis. C Blots were reprobed with β-actin to confirm the equal loading of samples. The relative protein levels were calculated by densitometry. Data are shown as the mean ± SD (n = 3). * P < 0.05 versus non-treated control. **D, E** The expressions of p53 and P-p53 were analyzed by immunofluorescence staining in A549 and H460 cells.
Fig. 8 TiO\(_{2}\) nanosheets increased p53 stability but not through the p53 proteasomal degradation. A The half-life of p53 was confirmed using the cycloheximine (CHX) chasing assay. H460 and A549 cell lines were treated with 50 µg/ml of CHX with or without 10 µg/ml TiO\(_{2}\) nanosheets as indicated by the time in minutes. Western blot analysis was performed to evaluate the p53 protein level. B The relative p53 protein levels were calculated and compared with the non-treatment control at 0 min. C The half-lives of the p53 protein of H460 and A549 cells were calculated. D H460 and A549 cells were pretreated with 10 µM MG132 for 30 min followed by treatment with 10 µg/ml TiO\(_{2}\) nanosheets for 60 min. The protein lysates were collected and incubated with a mixture of beads and p53 primary antibodies to pull out the protein of interest. Then, the ubiquitinated protein levels were measured by Western blot analysis. E Ub–p53 levels were quantified by densitometry. The statistical calculation was compiled with repeated measured one-way ANOVA with Scheffe's post-hoc test for individual comparisons and the t-test for two group comparisons. The relative to control protein levels are reported. (n = 3) (*p < 0.05, **p < 0.01 compared with the untreated control at 0 min and #p < 0.05, ##p < 0.01 compared with the untreated control at the same time).
Fig. 9 S-nitrosylation in the regulation of p53 stability. 

A. Three-dimensional (3D) structure of the tetrameric p53 core domain without DNA bound (PDB ID: 3KMD). 
B. Time evolution of the total number of intermolecular hydrogen bonds formed between each monomer of the p53 core domain and its adjacent monomer. 
C. The plot of (kcal/mol) of the p53 tetramer for the native form (top) and the C182 S-nitrosylation (bottom) system. 
D. The representative 3D structure taken from the last MD snapshot of the S-nitrosylation system, with hydrogen bonds and electrostatic interactions represented by black dashed lines.
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