Carrier-free nanoprodrug for p53-mutated tumor therapy via concurrent delivery of zinc-manganese dual ions and ROS

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ABSTRACT

Human cancers typically express a high level of tumor-promoting mutant p53 protein (Mutp53) with a minimal level of tumor-suppressing wild-type p53 protein (WTp53). In this regard, inducing Mutp53 degradation while activating WTp53 is a viable strategy for precise anti-tumor therapy. Herein, a new carrier-free nanoprodrug (i.e., Mn-ZnO nanoparticles) was developed for concurrent delivery of dual Zn-Mn ions and reactive oxygen species (ROS) within tumor to regulate the p53 protein for high anti-tumor efficacy. In response to the mild tumor acidic environment, the released Mn$^{2+}$ and H$_2$O$_2$ from Mn-ZnO NPs could effectively elevate the intracellular dual Zn-Mn ions and ROS level and subsequently generate the cytotoxic hydroxyl radical ($\bullet$OH) through the Fenton-like reaction. With the integration of multiple functions (i.e., carrier-free ion and ROS delivery, tumor accumulation, p53 protein modulation, toxic $\bullet$OH generation, and pH-activated MRI contrast) in a single nanosystem, Mn-ZnO NPs demonstrate its superiority as a promising nanotherapeutics for p53-mutated tumor therapy.

1. Introduction

p53, a tumor suppressor protein, can regulate cancer cell death through transcriptional activation of multiple proapoptotic genes such as Bax [1–3]. However, in human cancers the level of wild-type p53 protein (WTp53) is rather low and in some cases even undetectable [4]. As a matter of fact, p53 mutation is oftentimes observed with more than 50% in most cancers, and up to 80% in those difficult-to-treat cancers such as high-grade serous ovarian cancers, triple-negative breast cancers, oesophageal cancers, small-cell lung cancers, and squamous cell lung cancers [5–7]. Typically, p53 mutation takes place in two distinct fashions [8]: 1) DNA contact mutation, e.g., p53$^{R248Q}$ and p53$^{R273H}$, caused by directly binding DNA to the protein domains, 2) conformational mutation, e.g., p53$^{R175H}$ and p53$^{H179R}$, caused by a full or partial distortion of the folding of DNA-binding domains. The mutant p53 proteins (Mutp53) not only completely lose the tumor-suppressive functions of the wild-type, but oftentimes acquire new inherent oncogenic functions, that is, a phenomenon termed as mutant p53 gain-of-function (GOF) [9,10]. Because of such a unique GOF, Mutp53 can actively promote tumor growth, invasiveness, and metastasis via regulation of the critical cellular processes from chromatin structure to metabolism [11–14]. To this end, elimination of Mutp53 while

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increasing WTp53 is of great benefit in tumor therapy.

Given the high mutation rate of p53, Mutp53 becomes an attractive target in p53-mutated tumor therapy [15]. Among various strategies to reduce the Mutp53 level, the most straightforward one is through the proteasome or autophagy-induced degradation. For example, efforts have been made to degrade Mutp53 by virtue of small molecules such as NSC59984, Hsp90 inhibitor 17-AAG, and statins (via the proteasomal pathway) [16-18], or MCB-613, gambogic acid, and SAHA (via the autophagic pathway) [19-21]. Despite their efficiency in Mutp53 degradation, the identified challenges such as chemical toxicity and non-target effects motivate continuous endeavors to seek for more robust yet safer mechanisms to regulate p53 protein. Recently, Chen and co-workers reported that zinc ions (Zn\(^{2+}\)) could inhibit the mitochondrial electron transport chain (mETC) and promote the production of reactive oxygen species (ROS) (such as O\(_2^−\) and H\(_2\)O\(_2\)) by increasing electron leakage to the oxygen at NADH-Q reductase (complex I) and ubiquinol-cytochrome-c reductase (complex III) of mETC [22]. More importantly, elevated intracellular Zn\(^{2+}\) level and endogenous ROS generation exhibit the capability of selectively degrading a panel of Mutp53 (both contact and conformational mutation), but not the WTp53, via the ubiquitination-mediated proteasomal (UPS) pathway [23]. Such observations partially prop our hypothesis that endogenous generation of ROS through Zn\(^{2+}\) in combination with exogenous supply of ROS would lead to more effective Mutp53 degradation.

Increasing evidence demonstrates that enhanced accumulation and stabilization of WTp53 could be achieved by activating the ataxia telangiectasia mutated (ATM)-p53 signaling pathway with a high concentration of manganese ions (Mn\(^{2+}\)) [24]. More specifically, Mn\(^{2+}\) exposure could cause the ATM activation via autophosphorylation and other established ATM phosphorylation targets (CHK2[T68] and H2AX [S139]), which subsequently induced the phosphorylation of p53 (serine 15). Thus, Mn\(^{2+}\) is actively involved in the ATM-regulated p53 phosphorylation [25] and the activated WTp53 then induce the transcription of proapoptotic Bax gene, responsible for tumor cell death or growth arrest [26]. Interestingly, ROS-induced ATM autophosphorylation on Ser1981 could also cause the phosphorylation of p53 on Ser15 [27]. Collectively, elevation of intracellular Mn\(^{2+}\), Zn\(^{2+}\), and ROS level could deliver an appealing strategy to activate WTp53 while simultaneously eliminating Mutp53 for cancer therapy. Furthermore, because of its Fenton catalytic activity, Mn\(^{2+}\) was also able to catalyze the formation of cytotoxic hydroxyl radical (•OH) from H\(_2\)O\(_2\) and subsequently cause the apoptosis of cancer cells for effective eradication [28]. However, it remains highly challenging to selectively transport and retain exogenous ROS and desirable metallic ions (e.g., Zn\(^{2+}\) and Mn\(^{2+}\)) within targeted cells through the membranous ion channels and intracellular trafficking against the intrinsic efflux/storage processes [29]. Taking advantage of the unique competence of certain exotic materials such as long circulating polyethylene glycol [30] and high ion-binding hyperbranched polyglycerol [31], metallic compounds or ions of interest can be formulated into nanocomposites with these materials for better endocytosis. However, the rising concerns on undesirable immunoresponses from such synthetic materials [32] divert the efforts toward reduction or elimination of the use of exotic materials. In this regard, the preferred and compelling delivery system for dual ions and exogenous ROS would be primarily composed of the stable formats of ions or ROS without the use of other materials. Ideally, such systems should maintain the structural integrity outside the cells and then exhibit the ability to mutually trigger the dual Zn\(^{2+}\)-Mn\(^{2+}\) and ROS sufficient to respectively induce Mutp53 degradation while improving WTp53 level upon cellular uptake. Very recent study has demonstrated the capability of simultaneously delivering exogenous H\(_2\)O\(_2\) and Zn\(^{2+}\) via pH-sensitive zinc peroxide nanoparticles (ZnO\(_2\) NPs) [22]. Meanwhile, the similar ionic radius between Mn and Zn (0.66 Å and 0.60 Å, respectively) would allow to substitute the Zn\(^{2+}\) of the crystal lattice of ZnO\(_2\) NPs with Mn\(^{2+}\) to yield the Mn-doped ZnO\(_2\) nanosystem [33]. To this end, it is highly feasible to develop a multifunctional nanosystem, enabling simultaneous delivery of Mn-Zn dual ions and ROS suitable for p53-mutated tumor therapy.

In this study, ZnO\(_2\) NPs doped with optimal Mn\(^{2+}\), i.e., Mn-ZnO\(_2\) NPs were accordingly synthesized and used as a carrier-free nanoprodruk in order to selectively elevate intracellular Zn\(^{2+}\) and Mn\(^{2+}\) concentration while enhancing ROS formation (H\(_2\)O\(_2\) and •OH) for Mutp53 degradation and WTp53 activation to achieve the synergistic cancer therapy (Scheme 1). The Mn-ZnO\(_2\) NPs were stable under a neutral pH environment but completely decomposed to Mn\(^{2+}\), Zn\(^{2+}\), and H\(_2\)O\(_2\) at the mild acidic circumstances. The physicochemical and biological properties of Mn-ZnO\(_2\) NPs were comprehensively evaluated in vitro and in vivo with particular focuses on the release performance of Zn-Mn dual ions and ROS, •OH generation capacity, cellular uptake, Mutp53 degradation efficiency, WTp53 activation, and tumor therapeutic effect along with their tissue distribution and biosafety. Several unique attributes could be identified with the multifunctional Mn-ZnO\(_2\) NPs including: 1) the rapid release of H\(_2\)O\(_2\) and Zn\(^{2+}\) in an acidic microenvironment after cellular internalization to elevate endogenous ROS formation and induce Mutp53 degradation, 2) an increase of WTP3p via the AT-Mp53-Bax pathway activated by the released Mn\(^{2+}\), 3) the high biosafety and selectivity for bio-specific transport of metallic ions and ROS without exotic materials, 4) the in vivo magnetic resonance imaging (MRI) capabilities of Mn\(^{2+}\) for tracking tissue distribution of Mn-ZnO\(_2\) NPs and guiding the therapeutic process, and 5) the generation of toxic •OH through the Fenton reaction of Mn\(^{2+}\) and H\(_2\)O\(_2\) for enhanced antitumor efficiency. Along with successful demonstration of such carrier-free Mn-ZnO\(_2\) NPs as a pH-sensitive prodrug to treat p53-mutated tumors, this study also provides blueprints to design other carrier-free nanosystems with varying ions and/or ROS delivery demands for cancer therapy.

2. Materials and methods

2.1. Materials

Zinc acetate (Zn(OAc)\(_2\)), polyvinylpyrrolidone (PVP, Mw = 360,000), hydrogen peroxide (H\(_2\)O\(_2\), 30 wt% in H\(_2\)O), zinc chloride (ZnCl\(_2\)), sodium acetate, acetic acid, 2,7′-dichlorofluorescin diacetate (DCFH-DA), ZnAF-2 DA, and calcein AM/propidium iodide (PI) staining kit were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Manganese (II) chloride (MnCl\(_2\)), methylene blue (MB), Pierce™ quantitative peroxide assay kit, and mitochondrial hydroxyl radical detection assay kit (MitoROS OHS80) were purchased from Fisher Scientific (Waltham, MA, USA). All chemicals were used as received without further treatment.

2.2. Synthesis of ZnO\(_2\) NPs

ZnO\(_2\) NPs were fabricated following a simple and green wet chemistry approach with modification [22]. Briefly, 0.1 g of Zn(OAc)\(_2\) and 0.1 g of PVP were dissolved in 5.0 mL of deionized (DI) water. Then, 0.5 mL of H\(_2\)O\(_2\) was quickly added under a vigorous stirring. After reaction for 24 h, the unreacted residue was removed by centrifugation at 15,000 rpm for 15 min and rinsing three times with DI water. The final PVP-modified ZnO\(_2\) NPs were re-dispersed in ethanol (95%) and used for the synthesis of Mn-ZnO\(_2\) NPs.

2.3. Synthesis of Mn–ZnO\(_2\) NPs

The Mn-doping was achieved using a cation-exchange approach. Specifically, 5 mL of the above-obtained ethanol solution of ZnO\(_2\) NPs was mixed with 5 mL of MnCl\(_2\) at different Mn concentrations and stirred at room temperature for 4 h. During the reaction, color of the solution gradually changed from milky white into yellowish-brown. Upon washing/centrifugation (15,000 rpm, 15 min), the resulting Mn-ZnO\(_2\) NPs were collected for further use.
2.4. Characterization

Transmission electron microscopic (TEM) imaging was taken with a Titan Themis 200 TEM (FEI, Hillsboro, USA) at an acceleration voltage of 200 kV. UV-vis-NIR absorption spectra were obtained with a multi-detection microplate reader (BioTek Instruments, Inc., Winooski, VT). The wide-angle powder X-ray diffraction (XRD) pattern was recorded using an X-ray diffractometer (Philips X'pert XRD system) with a Cu Kα (1.5406 Å) X-ray source at 40 kV and 40 mA and a scan rate of 5° (2θ)/min (scan range: 10-90°). The size of different nanoparticles was determined by dynamic light scattering (Zetasizer 3000HS; Malvern Instruments, Worcestershire, UK). The surface area and pore size of the nanoparticles were determined by using the Brunauer-Emmett-Teller (BET), nitrogen adsorption-desorption, and Barrett-Joyner-Halenda (BJH) methods (Micromeritics, ASAP 2020), respectively. The XPS measurements were performed using a PHI-5000 CESCA system (PerkinElmer) with the radiation from an Al Kα (1486.6 eV) X-ray source.

For in vitro MRI imaging, Mn-ZnO₂ NPs with different concentrations (0, 0.1, 0.2, 0.4, and 0.8 mg/mL) were added into 200 μL tubes for MRI signal detection using a microMRI instrument (Bruker BioSpec 94/30 9.4 T MRI).

2.5. pH-responsive Mn²⁺, Zn²⁺, and H₂O₂ release from Mn-ZnO₂ NPs

To detect the acid-induced release of Mn²⁺ and Zn²⁺, the Mn-ZnO₂ NPs were dialyzed against the buffer solutions at a specified pH (7.4 or 5.5). The dialytes were collected at predetermined time points and the released Mn²⁺ and Zn²⁺ were respectively detected by inductively coupled plasma mass spectrometry (ICP-MS).

The release of H₂O₂ from Mn-ZnO₂ NPs induced by acid was determined with a quantitative peroxide assay kit with a characteristic absorbance peak at 560 nm upon reaction with H₂O₂. Briefly, Mn-ZnO₂ NPs were dissolved in the buffer solutions at specified pH (7.4 or 5.5) and gently stirred for 1 h. Then, 20 μL of the above solution was mixed with the working reagent (200 μL) in the wells of 96-well plates. The UV-vis absorption spectra were measured using a multi-detection microplate reader.

2.6. •OH generation by Mn²⁺-driven Fenton-type reaction

MB degradation-based assay was used to evaluate the •OH generation. More specifically, 5 μg/mL MB was mixed with Mn-ZnO₂ or ZnO₂ suspension (200 μg/mL) and 25 mM NaHCO₃ under respective pH (7.4 or 5.5) conditions. After 3 h, the UV-vis absorption in the wavelength range from 300 to 800 nm for each sample was recorded using a multi-detection microplate reader. In addition, the UV-vis absorption spectra of Mn-ZnO₂ NPs at a series of concentrations (0-200 μg/mL) dissolved in the acetate buffer (pH = 5.5) containing 5 μg/mL MB were also obtained. For the kinetic analysis, the experiments were carried out in the acetate buffer (pH = 5.5) containing MB (5 μg/mL) and Mn-ZnO₂ NPs (200 μg/mL) and the corresponding absorption spectra were recorded at the designated time points (0-180 min).

Furthermore, electron spin resonance (ESR) spectra were also obtained to detect •OH production by ZnO₂ or Mn-ZnO₂ NPs (200 μg/mL) at specified pH conditions (pH 7.4 or 5.5). The samples were pipetted into the capillary tubes. The ESR signals were recorded using a Bruker EMX ESR spectroscope with the following settings: 9.872 GHz microwave frequency, 6.375 mW microwave power, 100.00 kHz modulation.
2.7. Intracellular Zn\(^{2+}\) and Mn\(^{2+}\) detection

ZnAF-2 DA as a cell-permeable fluorescent probe for Zn\(^{2+}\) was used to detect the intracellular Zn\(^{2+}\) level. After incubation with Zn\(^{2+}\) or Mn-ZnO\(_NPs\) for 4 h, the MDA-MB-231 cells were stained with Zn\(^{2+}\) dye (5 \(\mu\)M) and 4′,6-diamidino-2-phenylindole (DAPI) successively. Then, the fluorescence images were collected with the Nikon 80i epi-fluorescence microscope at Ex/Em = 488/530 nm for Zn\(^{2+}\) dye, and Ex/Em = 360/460 nm for DAPI.

To quantitatively detect the intracellular Mn\(^{2+}\) and Zn\(^{2+}\) level, MDA-MB-231 cells incubated with Mn-ZnO\(_{NPs}\) for 4 h were digested and treated with aqua regia. Quantitative analysis of Zn and Mn element was carried out by ICP-MS. The contents of Zn and Mn in the cells was calculated as nanogram per 1000 cells.

2.8. In vitro cellular ROS detection and anticancer performance

The MDA-MB-231 cells cultured in 6-well plates were incubated with Mn-ZnO\(_{NPs}\) for 4 h. Then, the cells were incubated for 20 min at 37 \(^\circ\)C with DCFH-DA (for total ROS detection, Ex/Em = 488/530 nm) or MitorOS OHS80 (for mitochondrial ROS detection, Ex/Em = 540/590 nm) in Opti-MEM without FBS and antibiotics. After gentle rinsing with sterile PBS for three times, fluorescence images of the cells were obtained with the Nikon 80i epi-fluorescence microscope.

The cell-killing efficiency of Mn-ZnO\(_{NPs}\) was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, MDA-MB-231 cells seeded and cultured in 96-well plates for 24 h at 37 \(^\circ\)C were respectively incubated with ZnO or Mn-ZnO at gradient concentrations. After incubation for 24 h, 20 \(\mu\)L of MTT (5 mg/mL in PBS) was added into each well. Upon removal of the media, 150 \(\mu\L\) of DMSO was added to extract the formazan under gently shaking for 5 min, and the optical density (OD) at 590 nm was recorded with a microplate reader. Similarly, the in vitro anticancer activity of H\(_2\)O\(_2\), Zn\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\) plus Mn\(^{2+}\), or Mn\(^{2+}\) plus H\(_2\)O\(_2\) (with the same concentration) was also examined.

Live/dead staining (i.e., calcein-AM/PI) was also performed to study the in vitro anticancer activity of Mn-ZnO\(_{NPs}\). The MDA-MB-231 cells cultured in 6-well plates were treated with Zn\(^{2+}\) plus Mn\(^{2+}\), ZnO or Mn-ZnO, respectively. After incubation for 6 h, the cells were stained with calcein-AM/PI and examined under the Nikon 80i epi-fluorescence microscope.

2.9. Ubiquitination analysis of mutant p53

Following previous studies [23,34], the lysates of MDA-MB-231 cells after the treatment with Mn-ZnO\(_{NPs}\) without or with the presence of MG132 (10 \(\mu\)M) for 6 h were subjected to immunoprecipitation with BeakerBeads\(^{TM}\) Protein A/G kit (BEAVER Biomedical, Suzhou, China) by using the antibody p53 (DO-1, 1 \(\mu\)g per sample). The pull-down complex was detected by western blotting with mutant p53 (ab32049, 1:1000 dilution), the Ubiquitin (ab134953, 1:1000 dilution) and K48-Ub (ab140601, 1:1000 dilution) antibodies.

2.10. Establishment of tumor xenograft model

Female Balb/c nude mice (6-8 weeks old, ~20 g) purchased from HuaFukang Biological Technology Co. Ltd (Beijing, China) were used to generate the MDA-MB-231 xenograft tumor models. Briefly, MDA-MB-231 cells (5 × 10\(^6\)) suspended in 100 \(\mu\L\) of PBS were subcutaneously injected to the back of each mouse. When the tumor volume reached ~80 mm\(^3\), the mice were used for in vivo experiments. All animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Hebei University of Technology.

2.11. In vivo biodistribution and MRI imaging

The biodistribution of Mn-ZnO\(_{NPs}\) in the tumor and major organs (heart, liver, spleen, lung, and kidney) was evaluated in the tumor-bearing mice (n = 3). Mn-ZnO\(_{NPs}\) at the dose of 5 mg/kg were intravenously (i.v.) injected into the MDA-MB-231 tumor-bearing mice. Then, the mice were sacrificed at different time intervals (0, 4, 12, and 24 h), and the dissected tissues were weighed, homogenized, and treated with aqua regia. Quantitative analysis of Zn and Mn element was carried out with ICP-MS. The biodistribution of Zn and Mn in different tissues was calculated as the percentage of injected dose per gram of tissue (% ID/g).

For MRI imaging, MDA-MB-231 tumor-bearing mice were intravenously injected with Mn-ZnO\(_{NPs}\) at a dose of 5 mg/kg. The coronal and transverse plane MRI images at 0, 4, 12, and 24 h were obtained by using an microMRI instrument (Bruker Biospec 94/30 9.4 T MRI).

2.12. In vivo therapeutic effect of Mn-ZnO\(_{NPs}\)

In order to evaluate the in vivo therapeutic effect of Mn-ZnO\(_{NPs}\), MDA-MB-231 tumor-bearing mice were stochastically divided into four groups (n = 5); (1) saline, (2) Zn\(^{2+}\) plus Mn\(^{2+}\) (with 1.21 mg/mL ZnCl\(_2\) and 0.25 mg/mL MnCl\(_2\), 100 \(\mu\L\)), (3) Zn\(^{2+}\) (1 mg/mL, 100 \(\mu\L\)), and (4) Mn-ZnO\(_{NPs}\) (1 mg/mL, 100 \(\mu\L\)). During the therapeutic treatment, the mice received various treatments via i.v. injection every 2 days (a total of 8 injections received for the entire treatment period). The body weight and tumor volume were also recorded every 2 days up to 16 days. The tumor volume was calculated according to the following formula: tumor volume = length × width\(^2\)/2. By the time of sacrificing, the tumors and all major organs were collected and processed for histological analysis upon hematoxylin & eosin (H&E) staining of their cross-sections. The ROS level in tumor tissues was assessed by staining the tissue cross-sections with dihydroethidium (DHE) (20 mM, Invitrogen) and then examining with the Nikon 80i epi-fluorescence microscope. The obtained fluorescence images of randomly selected fields (n = 5) were analyzed for fluorescence intensity using the NIH ImageJ software (1.46r).

2.13. TUNEL assay

At 48 h after the last administration, tumors with various treatments were performed for TUNEL assay (One Step TUNEL Apoptosis Assay Kit, C1088, Beyotime). Briefly, 4-\(\mu\)m thick paraffin sections of the resected tumors were dewaxed in xylene and rehydrated with graded ethanol solutions prior to the treatment with proteinase K for 30 min at 37 \(^\circ\)C. Then, the sections were washed with PBS for three times and then incubated with the TUNEL reaction mixture for 60 min at 37 \(^\circ\)C in a humidified chamber. After rinsing with PBS, the sections were stained with DAPI for nuclei, and then examined under the Nikon 80i epi-fluorescence microscope.

2.14. Western blotting

Proteins of MDA-MB-231 cells (at 12 h after treatment) or tumor tissues (at 48 h after last injection) from tumor-bearing mice were extracted and separated using 8-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to the polyvinylidene fluoride membranes and then incubated with 5% fat-free milk in PBS-Tween (0.2%) for 1 h to block the nonspecific binding. After incubation with appropriate primary antibodies, the membranes were washed three times with PBS-Tween, blotted with secondary antibodies conjugated with horseradish peroxidase for 1 h, and then imaged with the gel imaging system (Tanon-5200Multi). The primary antibodies used in this study were anti-mutant p53 (ab32049, 1:1000 dilution), anti-wildtype p53 (bs-00338, 1:500 dilution), anti-phospho p53 (S15, ab278683, 1:10000 dilution), anti-
ATM (BA0655-2, 1:2000 dilution), anti-phospho ATM (S1981, BM4008, 1:1000 dilution), anti-Bax (ab32503, 1:1000 dilution), and anti-GADPH (ab9485, 1:2500 dilution).

2.15. Quantitative real-time PCR (qPCR)

RNA was extracted from the cells or tumor tissues and the expression of selected genes was then detected with a real-time quantitative PCR instrument (QuantStudio 1, ThermoFisher Scientific) according to the manufacturer’s instructions. The gene expression level was normalized with β-actin (housekeeping gene) and presented as average ± standard deviation from duplicates or triplicates of the repeated experiments. The primers used in the qPCR analysis were listed in Table S1.

2.16. Statistical analysis

All experiments were repeated at least 3 times and presented as the mean ± SD. Significant differences among groups were determined by one-way ANOVA with Tukey multiple comparison tests. Statistical significance was set at *p < 0.05, **p < 0.01.

3. Results and discussion

3.1. Synthesis and characterization of Mn-ZnO$_2$ nanoprodrug

ZnO$_2$ NPs were synthesized from zinc acetate (Zn(OAc)$_2$) by reacting with H$_2$O$_2$ at room temperature in the presence of PVP, and a relatively good yield (12.3%) was achieved (Scheme 1). During the synthesis, the presence of PVP, which complexed with zinc ions, was able to provide the protection to ZnO$_2$ to form stable nanostructures, thereby affecting the growth and control the uniformity of the spherical aggregates. In the absence of PVP, however, the ZnO$_2$ nanocrystals tended to aggregate in a noncontrollable manner, resulting in irregular round shapes (Fig. S1a) with poor storage stability (Fig. S1b). With the aid of PVP, on the other hand, the assembly of ZnO$_2$ nanocrystals occurred in a controlled fashion and the formed aggregates exhibited as spherical particles with relatively uniform size and good stability. Such a distinct difference most likely resulted from PVP that could lower the surface energy of ZnO$_2$ nanocrystals and provide stabilization to the final nanoaggregate via its entangled polymeric chains [35]. Fourier transform infrared (FTIR) spectra revealed the presence of signature absorptions at 1670 cm$^{-1}$.
cm⁻¹ (C=O stretching of PVP) and 1285 cm⁻¹ (C–N stretching of PVP) [36], thereby confirming successful incorporation of PVP within the as-prepared ZnO NPs (Fig. S2). To endow ZnO NPs with the capability of •OH generation, WtP53 modulation, and MRI contrast, a facile cation-exchange step was therefore adopted to construct the Mn-doped ZnO NPs. As a result of the similar ionic radius between Mn and Zn (0.66 Å and 0.60 Å, respectively) [35], Mn²⁺ would be readily doped into the crystal lattice of ZnO via a gradient-driven substitution of Zn²⁺ (Fig. 1a). To determine the desirable Mn-ZnO formulation for further use, ZnO NPs at a constant concentration were substituted with different Mn²⁺ weight fractions. As shown in Fig. S3 and Fig. S4f, increasing Mn²⁺ in the reaction led to gradual darkening of the obtained Mn-ZnO solutions, displaying the dark brownish at 80%. With the weight fraction of Mn at 50% or below, the Mn-ZnO NPs retained their spherical morphology with a good uniformity (Figs. S4a–e). To correlate the Mn²⁺ weight fraction with •OH formation from H₂O₂, methylene blue (MB) was used as the indicator, which could be decomposed by •OH to lose its blue color. Interestingly, Mn-ZnO NPs of 30% Mn²⁺ weight fraction exhibited the strongest •OH generation capacity (Fig. S4g). To determine the optimal Mn/Zn ratio for maximal tumor inhibitory effect, MDA-MB-231 cells were treated with Mn-ZnO NPs containing different Mn²⁺ amounts and evaluated for their viability using MTT assay. To our surprise, the Mn-ZnO NPs with 30% Mn²⁺ weight fraction also showed the maximal tumor cell killing efficiency (Fig. S5). Thus, Mn-ZnO NPs with 30% Mn²⁺ weight fraction were particularly chosen for further experimental use.

As confirmed by transmission electron microscopy (TEM), the as-prepared ZnO NPs existed as monodispersity with the spherical morphology and rather uniform size (Fig. 1b). After 30% Mn doping, the color of the obtained Mn-ZnO NPs solution changed from milk white (ZnO NPs suspension) into yellow-brownish (Fig. S6 inset) and the absorbance at 350 nm was also significantly increased (Fig. S6). The microstructure of obtained Mn-ZnO NPs was further characterized by TEM and they remained the uniform spherical shape (Fig. 1c). As measured, the mean diameter of Mn-ZnO NPs was 42.1 ± 2.5 nm, which was very comparable if not identical to that of the ZnO NPs (44.2 ± 3.8 nm) (Fig. S7), and such sizes allow for prolonged circulation in the blood for enhanced extravasation into tumors [37]. Energy-dispersive X-ray spectroscopy (EDS) elemental mapping of the prepared Mn-ZnO NPs confirmed the presence of Zn, Mn, and O (Fig. 1d and Fig. S8). X-ray diffraction (XRD) characterization further validated the remaining crystal structure of ZnO in the Mn-ZnO NPs (Fig. S9) with completely matched diffraction peaks to the typical cubic structure of nano ZnO as described in the literature [38,39]. To further verify the composition of Mn-ZnO NPs especially the valence state of elements, X-ray photoelectron spectroscopy (XPS) analysis was performed. The total spectrum (Fig. 1f) confirmed the presence of Zn, Mn, O elements and the bonding energy of Zn 2p, Zn 3s, Zn 3p, Mn 2p and O 1s. As shown in Fig. 1g, the two splitting peaks at 1044.8 and 1021.7 eV corresponded to the Zn 2p1/2 and Zn 3p3/2 orbits of Zn²⁺, respectively. And the two peaks at 655.3 and 640.3 eV with a peak spacing of 15 eV in Fig. 1h were in a good agreement with the Zn 2p3/2 and Mn 2p3/2 peaks of Mn²⁺. The O 1s peak at 532.6 eV was assigned to O−O, suggesting the existence of peroxide groups (Fig. 1i). Once again, XPS results confirmed the sole presence of Zn²⁺ and Mn²⁺ in Mn-ZnO NPs as the peroxide compound.

Interestingly, mesopores were identified in the as-prepared Mn-ZnO NPs via the specific surface area determined from the nitrogen adsorption isotherms (Fig. 1e) was as high as 1746.4 m²/g, clearly offering more active sites for decomposition reactions. Mapping the pore-size distribution (inset of Fig. 1e) revealed the peak pore diameter at 3.75 nm, large enough for free transport of the released ions and H₂O₂ in and out. To assure the stability of Mn-ZnO NPs for future in vivo and in vivo use, additional tests were performed by extending the storage period up to 30 days. As summarized in Table S2, even incubation in the media containing 10% FBS (close to physiological conditions) for 30 days, the Mn-ZnO NPs remained well dispersed without noticeable aggregation/precipitation and the size also kept similar despite a slight increase over the time (Table S2). Apparently, the demonstrated stability of Mn-ZnO NPs mainly results from the presence of PVP in the nanostructures [40].

3.2. Acid-induced dual Zn-Mn ions/H₂O₂ release and •OH generation from Mn-ZnO NPs

As the carrier-free nanoprdug, we expect these Mn-ZnO NPs would decompose and release the dual ions (Mn²⁺ and Zn²⁺) and ROS in an acidic tumor microenvironment while being stable under the physiological pH of other tissues (Fig. 2a). To demonstrate such a pH sensitivity of Mn-ZnO NPs, we accordingly measured the release of H₂O₂, Zn²⁺, and Mn²⁺ from Mn-ZnO NPs under the neutral (pH 7.4, physiological) or acidic (pH 5.5, tumoral) conditions. We analyzed the release of H₂O₂ from Mn-ZnO NPs by using a ferrous (Fe) ion oxidation xylene orange (XO)-based assay with the Pierce™ Quantitative Peroxide (PQX) Assay Kit [41,42]. In this assay, the peroxide, i.e., released H₂O₂, can directly convert the Fe²⁺ to Fe³⁺, which react with the XO dye to yield a purple product with a maximum absorbance at 560 nm. As shown in Fig. 2b, a significant absorption peak appeared at 560 nm upon the incubation of PQX solution with Mn-ZnO NPs at pH 5.5, while only a marginal absorbance at 560 nm was seen at pH 7.4, confirming a mild acidic environment would effectively cause the generation of H₂O₂. Time-dependent release of Zn²⁺ and Mn²⁺ from Mn-ZnO NPs under different pH conditions (i.e., 7.4 versus 5.5) was measured by inducibly coupled plasma mass spectrometry (ICP-MS), showing a rapid release of Zn²⁺ and Mn²⁺ from the Mn-ZnO NPs at pH 5.5 and reaching their releasing plateau around 6 h (Fig. 2c and d). In contrast, the release of Zn²⁺ and Mn²⁺ in pH 7.4 buffer solution was rather slow with very low release rates. Interestingly, upon switching the pH from 7.4 to 5.5, the color of Mn-ZnO solution (yellow-brownish) turned to colorless rapidly and the strong UV absorbance also disappeared immediately (Fig. S10a). TEM examination of the Mn-ZnO NPs incubated in different pH buffers for 1 h showed that Mn–ZnO NPs remained unchanged at pH 7.4 while almost completely dissociated and decomposed at pH 5.5 (Figs. S10b and c). The accelerated release of Zn²⁺ and Mn²⁺ in a mild acidic environment is most likely due to the facilitated decomposition of Mn-ZnO NPs. Apparently, such a pH-sensitive decomposition of Mn-ZnO NPs displays noticeable advantages for in vivo tumor treatment especially considering that the acidic endo/lysosomes (pH 5.0-5.6) of cancer cells [43] would similarly trigger the release of H₂O₂ and ions in an on-demand manner.

Given the paramagnetic property of Mn²⁺ [44] and the pH-dependent release of Mn²⁺, we also compared the MR imaging signal and longitudinal (T₁) relaxivity of Mn-ZnO NPs solutions with different concentrations at pH 7.4 or 5.5. As shown in the T₁-weighted MR imaging (Fig. 2e), the signal intensity detected from the Mn-ZnO solutions was proportional to their concentration in a linear function at pH 5.5, while the signal intensity at pH 7.4 remained hypointense up to 0.8 mM. Additionally, the T₁ relaxation rate (r₁) also increased drastically from 0.81 (pH 7.4) to 5.53 mM⁻¹ s⁻¹ (pH 5.5) (Fig. 2e), revealing the unique pH-activatable MRI contrast of Mn-ZnO NPs, mainly due to enhanced chemical exchange between protons and Mn²⁺ released from Mn-ZnO NPs at pH 5.5. The off-to-on high MR contrast enabled by Mn-ZnO NPs in response to the mild acidic conditions delivers a promising MRI modality to selectively visualize the targeted tumor with a minimum interference from surrounding healthy tissues.

Theoretically, the concurrently released Mn²⁺ and H₂O₂ from Mn-ZnO NPs in an acidic environment would lead to the generation of cytotoxic •OH via the Fenton catalytic reaction [45-48]. To demonstrate this, electron spin resonance (ESR) spectroscopy was used to detect •OH formation through the Mn-based Fenton-like reaction upon incubation of Mn-ZnO NPs in the simulated acidic tumor environment. In contrast to the extremely weak ESR signals of •OH at pH 7.4, incubation of
Mn-ZnO NPs within the pH 5.5 buffer solution did cause a much stronger signal (Fig. 2f), confirming the capability of Mn-ZnO NPs in efficiently generating •OH under an acidic condition. On the other hand, no detectable ESR signals were seen with ZnO NPs solutions either at pH 7.4 or pH 5.5, further illustrating the importance of Mn$^{2+}$ for •OH formation. In addition, methylene blue (MB), a dye probe that is specifically decomposed by •OH, was used to further quantify •OH formation. Consistent with ESR measurements, Mn-ZnO NPs (pH 5.5) yielded the most decomposition of MB while only a marginal decomposition with Mn-ZnO NPs (pH 7.4) or no change with other circumstances (Fig. 2g). Furthermore, decomposition of MB by acidified Mn-ZnO NPs was both concentration- (Fig. 2h) and time-dependent (Fig. 2i). All these results affirmed the capacity and efficiency of self-generating •OH by Mn-ZnO NPs under an acidic tumor-like environment.

3.3. Intracellular Zn$^{2+}$, Mn$^{2+}$, and ROS elevation by Mn-ZnO NPs

As a result of the acidic (pH 5.0-6.0) endo/lysosomal environment of cancer cells, pH-sensitive Mn-ZnO NPs after endocytosis should be decomposed into Zn$^{2+}$, Mn$^{2+}$, and H$_2$O$_2$ and then followed with intracellular •OH production via the Fenton-like reaction between Mn$^{2+}$ and H$_2$O$_2$ (Fig. 3a). To verify the onset of such expected events, breast cancer cells (MDA-MB-231) exhibiting high p53 mutation [49] was particularly selected for the in vitro cellular study. To visualize the elevated intracellular Zn$^{2+}$ level, a Zn$^{2+}$ specific fluorescent indicator (ZnAF-2 DA) was used to stain the cells upon incubation with Mn-ZnO NPs. As shown in Fig. 3b and Fig. S11, significantly higher green fluorescence was seen with the cells incubated with Mn-ZnO NPs than those untreated control or treated with ZnCl$_2$. To better quantify the increases of intracellular Mn$^{2+}$ and Zn$^{2+}$ by Mn-ZnO NPs, ICP-MS measurement was performed. Clearly, the Mn-ZnO NP treatment elevated both Mn$^{2+}$ and Zn$^{2+}$ levels
dramatically, about 189.7 and 690.5 times of the nontreated controls, respectively (Fig. 3c), implying the efficiency in releasing ions from Mn-ZnO\(_2\) NPs within a tumor cell environment.

Next, the boosted level of intracellular ROS caused by released H\(_2\)O\(_2\) was detected using 2',7'-dichlorofluorescin diacetate (DCFH-DA) as the indicator. Upon cellular uptake, DCFH-DA undergoes deacetylation by intracellular esterase to yield nonfluorescent DCFH, which can be oxidized by ROS to emit green fluorescence [50–52]. As demonstrated in Fig. 3d, MDA-MB-231 cells incubated with ZnCl\(_2\) displayed relatively stronger green fluorescence compared to those nontreated cells. The enhanced intracellular ROS upon Zn\(^{2+}\) exposure might be mainly attributed to Zn\(^{2+}\)-induced mitochondrial ROS production [53–56]. As expected, the cells incubated with either ZnO\(_2\) NPs or Mn-ZnO\(_2\) NPs yielded comparable green fluorescence but much higher intensity than that of nontreated controls and even Zn\(^{2+}\)-challenged ones. Such results suggest intracellular ROS elevation is the combined effect of H\(_2\)O\(_2\) and Zn\(^{2+}\). Semi-quantification of the fluorescence intensity using ImageJ revealed that Mn-ZnO\(_2\) group was approximately 14.4 times of the control group. Above results confirmed the ROS generation capability of Mn-ZnO\(_2\) NPs in cancer cells via both endogenous (by Zn\(^{2+}\)) and exogenous (by H\(_2\)O\(_2\)) avenues. Considering that DCFH-DA reacts with all ROS including H\(_2\)O\(_2\) and \(\cdot\)OH [57], a novel live-cell permeant hydroxyl radical probe (MitoROS™ OH580) with a good selectivity toward \(\cdot\)OH was used to evaluate the intracellular \(\cdot\)OH level, which reacts with MitoROS™ OH580 to rapidly generate the red fluorescence [58]. Compared to negligible fluorescence in other groups (no treatment or ZnO\(_2\) NPs), cells treated with MnCl\(_2\) and H\(_2\)O\(_2\) did show red fluorescence, but still relatively weak (Fig. 3g). In contrast, the cells with Mn-ZnO\(_2\) NPs had much stronger red fluorescence, about 10.9 times of the nontreated controls (Fig. 3f), suggesting effective generation of \(\cdot\)OH within the tumor cells through the Fenton-like reaction from the released Mn\(^{2+}\) and H\(_2\)O\(_2\). The noted difference in \(\cdot\)OH generation between MnCl\(_2\)/H\(_2\)O\(_2\) and Mn-ZnO\(_2\) NPs might mainly come from the limited and varying transport efficiency of Mn\(^{2+}\) and H\(_2\)O\(_2\) from exogenous MnCl\(_2\)/H\(_2\)O\(_2\) through the cell membrane, and on the other hand also highlighted the advantages of Mn-ZnO\(_2\) NPs in terms of effective intracellular delivery and on-demand release of Mn\(^{2+}\)/Zn\(^{2+}\) and H\(_2\)O\(_2\) for desired biological functions while minimizing the unwanted side effects.
3.4. In vitro antitumor effect of Mn-ZnO₂ NPs via modulation of p53 protein and enhancement of •OH formation

Encouraged by the above results, we further hypothesized that the released Zn²⁺, Mn²⁺, and ROS might be able to elicit Mutp53 degradation and activate the ATM-WTp53-Bax signal pathway, synergistically inducing the death of p53 mutant tumor cells (Fig. 4a). To verify this, we firstly investigated whether Mn-ZnO₂ NPs could cause Mutp53 degradation in the highly p53 mutant MDA-MB-231 cells. In comparison to negligible Mutp53-degrading capacity in other groups (no treatment or Mn²⁺), both ZnO₂ and Mn-ZnO₂ NPs markedly decreased the level of Mutp53 (Fig. 4b and c). However, ZnCl₂ at an equivalent zinc-concentration was much less effective in causing Mutp53 degradation, most likely due to its poor ability to diffuse through the cell membrane via ion channels [29]. Meanwhile, H₂O₂ alone was also able to reduce the Mutp53 level to some extent. These results confirmed the dependence of Mutp53 degradation on Zn²⁺ and H₂O₂ released from Mn-ZnO₂ NPs but independent of Mn²⁺. To better interrogate the underlying mechanism, especially the involvement of intracellular oxidative level, we examined the Mutp53 protein level in MDA-MB-231 cells treated with Mn-ZnO₂ NPs with or without the presence of a global antioxidant N-acetyl cysteine (NAC), which was expected to abolish the ROS.

Fig. 4. Mn-ZnO₂ NP-mediated modulation of p53 proteins and cell-based killing efficiency. (a) The proposed mechanism on tumor cell death induced by Mn-ZnO₂ NPs via the regulation of p53 proteins. (b) Western blotting of Mutp53 in MDA-MB-231 cells after various treatments (non-treated control, MnCl₂, ZnCl₂, H₂O₂, ZnO₂ NPs, or Mn-ZnO₂ NPs). (c) Semi-quantification of the Mutp53 level shown in b. *p < 0.05, **p < 0.01. (d) Fluorescence images of ROS production in MDA-MB-231 cells after treated with Mn-ZnO₂ NPs or Mn-ZnO₂ NPs + NAC. (e) Western blotting of Mutp53 in MDA-MB-231 cells after the Mn-ZnO₂ NPs treatment without or with NAC. (f) Western blotting of ATM, WTp53, and Bax in MDA-MB-231 cells after various treatments (non-treated control, ZnCl₂, MnCl₂, ZnO₂ NPs, or Mn-ZnO₂ NPs). (g) Western blotting (WB) of total ubiquitination (Ub) and K48-polyubiquitination (K48-Ub) of the immunoprecipitated (IP) p53 in MDA-MB-231 cells after the Mn-ZnO₂ NPs treatment without or with MG132. (h) Viability of MDA-MB-231 cells after various treatments. Data are expressed as mean ± SD (n = 6). *p < 0.05, **p < 0.01. (i) Viability of MDA-MB-231 cells treated with ZnO₂ NPs or Mn–ZnO₂ NPs, respectively. Data are expressed as mean ± SD (n = 6). *p < 0.05, **p < 0.01. (j) Fluorescence images of MDA-MB-231 cells after different treatments (non-treated control, Zn²⁺ + Mn²⁺, ZnO₂ NPs, or Mn-ZnO₂ NPs). Cells were stained live (green) with calcein-AM and dead (red) with PI.
elevated by Mn-ZnO NPs. Notably, the ROS level was indeed dramatically reduced by NAC (Fig. 4d). As such, the degradation of Mutp53 by Mn-ZnO NPs was fully rescued (Fig. 4e and Fig. S12). Thus, intracellular ROS elevation is crucial toward Mutp53 proteasomal degradation induced by Mn-ZnO NPs. To further determine whether such a decreased Mutp53 level was from a reduced transcription, we conducted qPCR analysis to the cells with different treatments. Interestingly, no difference in Mutp53 mRNA expression was seen with ZnO2 or Mn-ZnO2 NPs from that of non-treated controls (Fig. S13), indicating that neither Zn\(^{2+}\) nor ROS released from Mn-ZnO2 NPs altered the transcription of Mutp53 and the corresponding Mutp53 reduction most likely occurred through the post-translational degradation. Thus, the involvement of ubiquitination-mediated proteasome-degradation of Mutp53 [34] was verified using the MG132, a potent and cell-permeable proteasome inhibitor [59]. As shown in Fig. S14, MG132 could completely abolish the ability of Mn-ZnO NPs to degrade Mutp53. As confirmed, Mn-ZnO2 NPs enhanced ubiquitination and K48 polyubiquitination of total cellular proteins (Fig. S15) and of Mutp53 (Fig. 4g) in MDA-MB-231 cells. MG132 treatment further increased the level of Mutp53 ubiquitination and particularly K48 polyubiquitination associated with proteasomal degradation [60]. Above results proved that Mn-ZnO2 NPs degraded Mutp53 protein through the post-translational degradation via the ubiquitination-dependent proteasomal pathway.

The possible activation of ATM-WTP53-Bax pathway in MDA-MB-231 cells by Mn-ZnO2 NPs was also investigated by examining the essential protein levels. In comparison to the low improvement of WTP53 accumulation by Zn\(^{2+}\) stimulation alone, noted accumulation of WTP53 was observed with Mn\(^{2+}\), ZnO2 NPs, and Mn-ZnO2 NPs treatment, particularly Mn-ZnO2 NPs, which showed about 4-fold increase (Fig. 4f and Fig. S16). Meanwhile, Mn-ZnO2 NPs also led to marked activation of the ATM-WTP53-Bax pathway by elevating the marker proteins of phosphorylated ATM (Ser1981, p-ATM), total WT p53, phosphorylated WTP53 (Ser15, p-WTP53), and Bax (Fig. 4f and Fig. S16). To our surprise, p-ATM, total WTP53, p-WTP53, and Bax were also augmented in the ZnO2 NPs-treated group while the total ATM levels remained unaltered, which might be attributed to the activation of ATM autophosphorylation by intracellular ROS [61]. More importantly, the highest WTP53 accumulation detected in MDA-MB-231 cells with Mn-ZnO2 NPs implied the potential for enhanced cellular apoptosis. Analyses of several key mRNAs by qPCR (Fig. S13) revealed that the ATM mRNA expression level was not affected by Mn-ZnO2 NPs, consistent with previous report on the involvement of ATM kinase in p53 phosphorylation through a post-translational modification [62,63]. Whereas the increase of WTP53 and Bax mRNA level may come from the ROS-mediated p53 gene upregulation and the subsequent transcriptional regulation of Bax expression [64]. The obtained data affirmed that the release of Mn\(^{2+}\) and ROS from Mn-ZnO2 NPs within tumor cells was able to activate the ATM-WTP53-Bax pathway by triggering the ATM phosphorylation, following with stabilization and accumulation of WTP53, and eventually inducing the Bax anticancer effect.

Increasing evidence has shown that the highly stabilized Mutp53 favors the growth and survival of cancer cells, whereas WTP53 exhibits the opposite effect [5]. To this end, the Mn-ZnO2 NP-induced dual events, i.e., degrading Mutp53 and activating WTP53, were expected to cause more destruction of p53 mutant cancer cells. (i.e., low cell viability). To validate this assumption, several selected cell lines with Mutp53, WTP53 or p53 absence were respectively treated with Mn-ZnO2 NPs (Fig. 5). Mn-ZnO2 NPs (64.7% and 76.1%, respectively) most likely comes from the ready uptake of stable nanoparticles (ZnO2 and Mn-ZnO2 NPs) by MDA-MB-231 cells and then intracellular rapid release of ions and ROS upon decomposition. The noted further enhancement of cell killing by Mn-ZnO2 NPs was a combinatorial anticancer effect from p53 protein and •OH, which were regulated by the pH-responsive release of H2O2 and Zn-Mn dual ions in the acidic endo/lysosomes [43]. Next, the concentration-dependent anticancer effect of ZnO2 or Mn-ZnO2 NPs was also evaluated. Apparently, the cell killing capacity closely correlated with the concentration of ZnO2 or Mn-ZnO2 NPs and reached as high as 94% cell killing with 50 μg/ml Mn-ZnO2 NPs (Fig. 4f). To further confirm the anticancer effect of Mn-ZnO2 NPs via causation of cell death, the cells were fluorescently stained with calcine-AM (live, green) and PI (dead, red). Mn-ZnO2 NPs did lead to significant cell death compared to either Zn\(^{2+}\) plus Mn\(^{2+}\) or ZnO2 NPs (Fig. 4j). In addition, Mn-ZnO2 NP-induced degradation of mutp53 and wild-type p53 accumulation in MDA-MB-231 cells also caused the cell cycle arrest at the G2M phase (Fig. S18), consistent with previous evidence [23]. Taken together, the obtained results demonstrated that Mn-ZnO2 NPs could induce Mutp53 degradation via the released Zn\(^{2+}\) and ROS, activate WTP53 mainly via the released Mn\(^{2+}\), and promote cytotoxic •OH generation via the Fenton reaction, which synergistically eradicated the p53-mutated tumor cells.

### 3.5. In vivo therapeutic effect of Mn-ZnO2 NPs in p53-mutated tumor

In view of the anticancer effects of Mn-ZnO2 NPs with cell culture, efforts were made to further understand their efficacy in in vivo therapy of p53-mutated cancer on MDA-MB-231-tumor-bearing mice. Following the cellular study results (Fig. 3), it is reasonable to anticipate that Mn-ZnO2 NPs could accumulate in the tumors via the enhanced permeability and retention (EPR) effect [65]. Thus, tumor-specific accumulation and biodistribution of Zn and Mn were first analyzed to the organs/tissues (heart, liver, spleen, lung, kidney, and tumor) harvested from the MDA-MB-231-tumor-bearing mice. Following 12 h post-injection, both Zn and Mn NPs could accumulate in the tumors (Fig. 5a and b), Mn-ZnO2 NPs could efficiently accumulate in the tumors and release dual Zn\(^{2+}\) and Mn\(^{2+}\) ions with the respective enrichment rate of 32.1%, 47.1%, and 40.6% for Zn and Mn, respectively (Fig. 5c). The results were further suggested by the significant cell death compared to either Zn\(^{2+}\) plus Mn\(^{2+}\) or ZnO2 NPs (Fig. 4j). In addition, Mn-ZnO2 NP-induced degradation of mutp53 and wild-type p53 accumulation in MDA-MB-231 cells also caused the cell cycle arrest at the G2M phase (Fig. S18), consistent with previous evidence [23]. Taken together, the obtained results demonstrated that Mn-ZnO2 NPs could induce Mutp53 degradation via the released Zn\(^{2+}\) and ROS, activate WTP53 mainly via the released Mn\(^{2+}\), and promote cytotoxic •OH generation via the Fenton reaction, which synergistically eradicated the p53-mutated tumor cells.

With the demonstrated advantages of Mn-ZnO2 NPs, i.e., high tumorous accumulation, on-demand (low pH triggered) release of dual ions (Mn\(^{2+}\), Zn\(^{2+}\)) and ROS, effective modulation of p53 protein, efficient generation of highly toxic •OH, as well as pH-activated MR imaging modality, the therapeutic effect on p53-mutated MDA-MB-231 tumors was accordingly performed (Fig. 5e). Tumor bearing mice were randomly divided into four groups (n = 5) and respectively treated with: 1) saline, 2) Zn\(^{2+}\) + Mn\(^{2+}\), 3) ZnO2 NPs, and 4) Mn-ZnO2 NPs. Time-resolved tumor progression was monitored by recording the tumor images (Fig. 5f) and measuring the tumor volume. As shown in Fig. 5g, Zn\(^{2+}\) plus Mn\(^{2+}\) exhibited negligible therapeutic effect on tumor inhibition with no statistical difference from controls (saline only). In contrast to a partial tumor suppression by ZnO2 NPs, significantly reduced tumor growth was observed with the Mn-ZnO2 NPs treatment,
showing no change of tumor volume throughout the experimental period. The terminal weight of excised tumors agreed well with the tumor volume measurements (Fig. 5h and i), and again Mn-ZnO$_2$ NPs yielded the highest antitumor capacity.

To evaluate the cell phenotype within Mn-ZnO$_2$ NP-treated tumors, the cross-sections of tumors were stained with hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL). H&E staining revealed that Mn-ZnO$_2$ NP-treatment led to significant necrosis and anucleate of cells within the treated tumors (Fig. 6a and inset). Similarly, the TUNEL assay also showed the highest cell apoptosis (82.1%) occurred to the Mn-ZnO$_2$ NP-treated tumors (Fig. 6b and c). To determine whether Mn-ZnO$_2$ NPs could elevate the tumoral ROS level, cryosections of tumors were stained with dihydroethidium (DHE) probe, which can be oxidized into ethidium by intracellular ROS and then intercalated into DNA to produce red fluorescence [66]. As shown in Fig. 6e, strong red fluorescence was seen in the tumors treated with ZnO$_2$ or Mn-ZnO$_2$ NPs, reaching as high as 12.0 times and 11.7 times of the saline controls, respectively (Fig. 6d). Negligible fluorescence was detected in the tumors treated with dual ions (Zn$^{2+}$ + Mn$^{2+}$). These in vivo results suggested that Mn-ZnO$_2$ NPs were able to accumulate in the tumors, locally release dual Zn$^{2+}$/Mn$^{2+}$ ions and ROS and subsequently cause tumor cell death.

To assure that Mn-ZnO$_2$ NPs could elicit Mutp53 degradation and activate the ATM-WTp53-Bax signaling under an in vivo circumstance, we also assessed the key proteins in the excised tumors by western blotting. Notably, efficient degradation of Mutp53 protein did happen in the tumors treated with ZnO$_2$ or Mn-ZnO$_2$ NPs (Fig. 6f and Fig. S19), but no change in Mutp53 mRNA (Fig. S20), implying the post-translational event of Mutp53 degradation by Mn-ZnO$_2$ NPs. Activation of the ATM-WTp53-Bax signaling by Mn-ZnO$_2$ NPs took place through the phosphorylation of ATM instead of elevating the overall ATM protein level, i.e., no change in total ATM among all the experimental groups (Fig. 6f and Fig. S19). Compared to the saline controls, Zn$^{2+}$ + Mn$^{2+}$ treatment did not induce any noticeable difference in terms of the phosphorylation...
of ATM and WTp53, and the total WTp53 and Bax. The treatment with ZnO NPs on the other hand was able to partially boost the phosphorylation of ATM and WTp53 while enhancing the total WTp53 and Bax level. Interestingly, Mn-ZnO NPs yielded the highest protein level of p-ATM, p-WTp53, WTp53, and Bax (Fig. 6f) and upregulation of both WTp53 and Bax gene (Fig. S20). Collectively, the above results demonstrated the in vivo superiority of Mn-ZnO nanoprodrug for enhanced therapy of p53 mutant cancer.

To maximize the use of animals, biosafety of Mn-ZnO NPs was also evaluated along with the therapeutic assessment. First, no significant body weight change was observed under various therapeutic treatments (Fig. S21a). Biochemical analyses of blood collected from the mice showed comparable liver and kidney functions between Mn-ZnO NP-treated and nontreated mice at day 8 and 16 (Figs. S21b–e). Routine blood analysis of Mn-ZnO NP-treated mice for 16 days also confirmed the maintenance of normal range of eleven blood indexes including white blood cell (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV), platelets (PLT), red cell distribution width (RDW), and plateletcrit (PCT), suggesting negligible blood toxicity of Mn-ZnO NPs (Fig. S21f–p). Histological analyses of major organs harvested after 16-day treatment with Mn-ZnO NPs showed no signs of tissue damage or inflammatory injury (Fig. S22), confirming negligible toxicity to major organs as well. All above analyses demonstrate that Mn-ZnO nanosystems have good in vivo biocompatibility as a promising therapeutic agent for future p53-mutated cancer therapy.

4. Conclusion

In summary, we developed a new concept of carrier-free nanoprodrug (Mn-ZnO2) to simultaneously deliver dual ions (Mn2+ and Zn2+) and ROS to the tumor site without other materials, which would reduce unwanted inflammation and eliminate the concerns on incomplete clearance. Meanwhile, the demonstrated Mn-ZnO2 nanoprodrug is a T1-weighted MRI-ready NP with the capabilities of degrading Mutp53, activating WTp53, and generating toxic •OH for selective therapy of p53-mutant tumors. More specifically, due to their pH sensitivity, such Mn-ZnO2 NPs can completely decompose to Mn2+, Zn2+, and H2O2 at a mild acidic microenvironment upon cellular ingestion. Elevated intracellular Zn2+ level in conjunction of ROS generation induces Mutp53 degradation via the ubiquitination-mediated proteasomal pathway. In parallel, the released Mn2+ activates the ATM-WTp53-Bax pathway for the prominent anticancer effect. Consistent with the in vitro evidence that Mn-ZnO2 NPs can elevate the intracellular Zn2+/Mn2+ and ROS level, generate cytotoxic •OH through the Fenton-like reaction and...
rebalance the Mutp53/WTp53 level, in vivo studies further demonstrate that such a nanoprodrg can effectively modulate the p53 level to achieve a high therapeutic efficiency for p53-mutant tumor. Overall, the reported findings provide a simple yet efficient route to synthesize carrier-free nanoprodrg for potential utility in p53-mutant tumor therapy. The synthesis method can be readily extended to other ions-doped metal peroxide nanoparticles for additional ion and ROS delivery. Besides, the strategy of using engineered nanomaterials to address the challenges imposed by Mutp53 degradation and WTp53 delivery. Besides, the strategy of using engineered nanomaterials to balance the Mutp53/WTp53 level, -

Multiscale Imaging at Stevens Institute of Technology for his help in -

Data curation.

Ethics approval

Declaration of competing interest

The authors declare no known competing financial interest.

Ethics approval

All animal experiments were carried out in accordance with the guidelines evaluated and approved by the ethics committee of Hebei University of Technology.

CRediT authorship contribution statement

Jinping Wang: Investigation, Methodology, Data curation, Funding acquisition, Writing – original draft. Chang Qu: Methodology, Data curation. Xinyue Shao: Investigation, Data curation. Guoqiang Song: Data curation. Jingyu Sun: Data curation. Donghong Shi: Methodology. Ran Jia: Methodology. Hailong An: Supervision, Funding acquisition, Writing – review & editing. Hengjun Wang: Conceptualization, Supervision, Funding acquisition, Writing -review & editing.

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Appendix A. Supplementary data

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