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miRNA-driven sensitization of breast cancer cells to Doxorubicin treatment following exposure to low dose of Zinc Oxide nanoparticles

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ABSTRACT

The impact of Engineered nanomaterials (ENMs) (i.e., Zinc Oxide nanoparticles (ZnO NPs)) on human health has been investigated at high and unrealistic exposure levels, overlooking the potential indirect harm of subtoxic and long exposures. Therefore, this study aimed to investigate the impacts of subtoxic concentrations of zinc oxide (ZnO NPs) on breast cancer cells' response to Doxorubicin. Zinc oxide nanoparticles caused a concentration-dependent reduction of cell viability in multiple breast cancer cell lines. A subtoxic concentration of 1.56 μg/mL (i.e., no observed adverse effect level) was used in subsequent mechanistic studies. Molecularly, miRNA profiling revealed significant downregulation of 13 oncogenic miRNAs (OncomiRs) in cells exposed to the subtoxic dose of ZnO NPs followed by doxorubicin treatment. Our comprehensive bioinformatic analysis has identified 617 target genes enriched in ten pathways, mainly regulating gene expression and transcription, cell cycle, and apoptotic cell death. Several tumor suppressor genes emerged as validated direct targets of the 13 OncomiRs, including TFDP2, YWHAG, SMAD2, SMAD4, CDKN1A, CDKN1B, BCL2L11, and TGIF2. This study insinuates the importance of miRNAs in regulating the responsiveness of cancer cells to chemotherapy. Our findings further indicate that being exposed to environmental ENMs, even at levels below toxicity, might still modulate cancer cells' response to chemotherapy, which highlights the need to reestablish endpoints of ENM exposure and toxicity in cancer patients receiving chemotherapeutics.

1. Introduction

Cancer remains the leading cause of mortality before the age of 70 years in more than 60 % of countries worldwide. Recent studies have placed breast cancer at the top of the most commonly diagnosed cancers globally, with a staggering 2.3 million new cases and nearly 685 thousand deaths in 2020 (Sung et al. 2021). Histologically, breast cancer is divided into four major subtypes based on the expression of hormone receptors: estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) to luminal A, luminal B, HER2-positive, and triple-negative (Ensenyat-Mendez et al. 2021). Even though the 5-year relative survival is about 90 % for all races, subtypes, and stages, it significantly drops in distant diseases (Siegel et al. 2022). The choice of the treatment strategy depends solely on the molecular subtype, grade, and stage of the disease to ensure maximum efficiency and safety. The approved strategies and approaches include surgery, radiotherapy, endocrine therapy, anti-HER2 therapy, PARP Inhibitors, and CDK4/6 Inhibitors.

Nevertheless, neoadjuvant and adjuvant chemotherapies remain the cornerstone of almost every treatment regimen despite the aggressiveness and progressiveness of breast cancer (Burguin, Diorio, and Durocher 2021). Doxorubicin (DOX) is a member of a large family of cytotoxic anthracyclines that mediate their cytotoxicity through multiple frontiers, including DNA intercalation and chromatin damage, DNA adduct formation, Topoisomerase II poisoning, redox state disruption, mitochondrial dysfunction, calcium homeostasis and immune

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modulations, and induction of apoptosis (Mattioli et al. 2023). Countless factors could interfere with the treatment effectiveness and result in DOX resistance (Holohan et al. 2013; Housman et al. 2014), the mechanisms of which have yet to be fully understood (Gatti and Zunino 2005). In the last two decades, advances in genetics revealed that the mammalian genome transcribes thousands of short, non-coding RNAs (miRNAs) that regulate various biological processes and diseases, including gynecological malignancies (Alshamrani 2020).

MicroRNAs are highly conserved, single-stranded, short, non-coding RNA molecules (19–25) that bind their target protein-coding genes post-transcriptionally, resulting in the cessation of translating those genes or facilitating their degradation (He and Hannon 2004). The human genome transcribes more than 1900 miRNA precursor sequences, generating nearly 2600 mature miRNA sequences (Kozomara, Birgaoanu, and Griffiths-Jones 2019), estimated to regulate two-thirds of all human genes (Friedländer et al. 2014; Plotnikova, Baranova, and Skoblov 2019). Dysregulation of the miRNAome has been strongly linked with chemosensitivity and resistance in various human malignancies, including breast cancer (Si et al. 2019). To date, approximately 60 miRNAs have been documented to play a pivotal role in anthracycline resistance in various cancers by regulating several pathways, including cell cycle and proliferation, apoptosis and autophagy, drug efflux, epithelial-to-mesenchymal transition, DNA repair, epigenetics, among others (Mattioli et al. 2023). However, none of these studies have accounted for pre-treatment exposure to other contaminants that might have interfered with the therapeutic efficacy. Several lines of evidence suggest that chronic exposure of cancer patients to environmental pollutants (e.g., nanoparticles) could indeed impact the effectiveness of chemotherapeutic drugs (Lagunas-Rangel, Liu, and Schiöth 2022).

Over the last few years, the development and use of engineered nanomaterials (ENMs), sized at 1–100 nm, has grown exponentially (Liu and Xia 2020; Vance et al. 2015; Albalawi et al. 2021). The unique physiochemical properties of ENMs have opened avenues for new and innovative applications in various fields, including electronics, energy, healthcare, and biotechnology (Albalawi et al., 2021; Vance et al., 2015; Oksel Karakus et al., 2021). Nevertheless, the environmental and human safety and the risks ENMs might pose are valid concerns that continue to attract the scientific community's attention to date (Johnston et al., 2020; Liu and Xia, 2020; Singh et al., 2019; Vance et al., 2015; Deng et al., 2021; Oksel Karakus et al., 2021). Most of these studies have focused on the toxicities associated with high and unrealistic ENM concentrations exposure (Liu and Xia 2020; Johnston et al. 2020; Nel et al. 2006; Nel et al. 2009). Despite the importance of such approaches, the undetectable impacts of low and subtoxic ENM exposure levels could be neglected. Humans are constantly exposed to very low doses of ENMs (e.g., in pharmaceutical and household products) for extended periods (Liu and Xia, 2020; Singh et al., 2019; Vance et al., 2015; Oksel Karakus et al., 2021). Therefore, assessing adverse responses following exposure to subtoxic levels of ENMs is crucial to avoid potential risks that could be developed unintentionally, jeopardizing human health safety.

Zinc oxide nanoparticles (ZnO NPs) belong to a metal oxide nanomaterial family that have been exploited in cancer diagnosis, drug delivery, and treatment (Saha et al. 2023). An increasing number of studies have demonstrated that ZnO-based nanoparticles possess unique physicochemical properties that can be easily controlled, facilitating their use in a variety of applications such as nano-electronic/nano-optical devices, nano-sensors, energy storage, and cosmetic products (Smijs and Pavel 2011; Ruszkiewicz et al. 2017; Vidor et al. 2017; Vasantharaj et al. 2021; Zhang et al. 2013). Studies have reported that ZnO NPs could be an effective anticancer agent, whether alone or in combination with other medications (Jha et al., 2023). In this study, we sought to investigate adverse cellular responses associated with exposing breast cancer cells to subtoxic concentrations of ZnO NPs prior to DOX treatment. Specifically, we assessed the global changes in the miRNA expression profiles, their potential target genes, and their affected cellular pathways. To our knowledge, this study is the first to investigate the role of miRNAs in driving the impact of subtoxic concentrations of environmental metal oxide nanoparticles on the response of breast cancer to chemotherapy.

2. Materials and Methods

2.1. Nanoparticle preparation and characterization

Fresh ZnO NPs and NiO NPs powder stocks were generously provided to us by our collaborators, King Abdullah Institute for Nanotechnology, King Saud University, Riyadh, Saudi Arabia. NiO NPs were prepared by solid-state reaction using nickel acetate and sodium hydroxide, while the ZnO NPs were synthesized using a wet chemical method from zinc nitrate. The detailed processes of NP synthesis and preparing the final working stock were previously reported (Ahamed et al., 2013; Alsaleh et al., 2023; Sridar et al., 2018). Briefly we used a water-bath sonicator to ensure total dispersion of the original ZnO NP stock at a concentration of 1 mg/mL. Before cell treatment, serial working concentrations of the original stock were made in cell culture media with thorough vortexing of vials to ensure a homogenous NP solution. ZnO NPs characteristics, including the hydrodynamic size (nm), zeta potential (mV), and polydispersity index (PDI), were measured using Zetasizer (Malvern Panalytical, Westborough, MA, USA). For qualitative characterization of the ZnO NPs, samples were re-dispersed by sonication for 10 min, dropped onto the copper mesh (Copper, Ted Pella, 300 mesh), and left to dry overnight. ZnO NPs were visualized the next day, and images were captured using an 80 kV JEOL JEM1010 transmission electron microscope (TEM) (Jeol, Tokyo, Japan).

2.2. Cell culture

The ER-positive luminal A cell line MCF-7 and the triple-negative human breast cancer cells MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were then frozen in liquid nitrogen in a freezing medium consisting of 10 % Dimethylsulfoxide (DMSO) (Sigma Chemical, St. Louis, MO) with Fetal Bovine Serum (FBS) (GIBCO Life Technologies, Gaithersburg, MD) until the day of use. Cells were then thawed and cultured in T-75 tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % FBS and 100 U penicillin/mL: 100 g streptomycin/mL (GIBCO Life Technologies, Gaithersburg, MD) at 37° C under a 5 % CO_2 humidified environment. Cells were passaged at least three times before the first experiment to restore their physiochemical properties and then used below 13 passages. The number of cultured cells, treatment conditions, and incubation times were identical throughout this study, and cells were incubated overnight to allow for adherence before treatment.

2.3. Measuring cell viability

The MCF-7 and MDA-MB-231 cellular metabolic activities were determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric staining (Tokyo Chemical Industry, Tokyo, Japan). Briefly, 10,000 cells per well were cultured in 96-well plates in 10 % DMEM and incubated overnight until they reached ~ 80 % confluency. The supplemented medium was then replaced with serum-free media, and cells were treated at doses of Dox, ZnO NPs, or NiO NPs (1.56–100 μg/mL) for 24hr. In the case of combination therapy, cells were treated with a subtoxic dose of the ZnO NPs or NiO NPs (1.56 μg/mL) for 24hr, then changed the media to a serum-free medium containing the IC50 dose of DOX (0.171 μM) for another 24hr. The cell culture medium was aspirated carefully, then 50 μL MTT in PBS (500 μg/mL) was added to each well and incubated for three hours at 37° C under a 5 % CO_2 humidified environment and protected from light. After carefully removing the media, 150 μL of Isopropyl alcohol (IPA) was added to each well and placed on a microplate shaker for 10 min to

solubilize the formed formazan crystals. A microplate reader was used to measure the absorbance of each well at a wavelength of 570 nm (Synergy HT system, BioTek, Winooski, VT, USA). Cell viability percentages were calculated using the formula: Cell viability (%) = 100 × (absorbance of the compound) / (absorbance of the control), and the IC50 values were calculated using GraphPad 9.0.

2.4. Nanoparticle cellular uptake

We utilized the inductively coupled plasma mass spectrometry (ICP-MS) to quantify the cellular ZnO NPs uptake. Briefly, MCF-7 cells were cultured in 35 mm dishes in 10 % DMEM medium at a density of 2.5 × 10^5 cells per dish for 24hr to reach a confluency of ~ 80 %. Cells were then treated with 1.56 μM ZnO in a serum-free medium for another 24hr. Excess ZnO NPs were carefully rinsed three times with ice-cold PBS, and cells were collected in 1.5 mL Eppendorf tubes for processing. Cell pellets were then digested overnight in 70 % HNO_3 at room temperature. Afterward, samples were diluted with purified water to reach a final HNO_3 concentration of 1 % and filtrated to remove any remaining solid cell components. Cellular ZnO NP content was quantified using the Perkin Elmer "Elan" 9000 ICP-MS system (Waltham, MA, USA). An internal standard containing lithium (Li), yttrium (Y), and indium (In) was used. ZnO NP was detected at a level of 0.05 *ppb* resolution.

2.5. Small RNA extraction and purification

Total RNA, including miRNAs, was isolated using GeneAll® Hybrid-R™miRNA extraction kit (GeneAll, South Korea) following the manufacturer's protocol. The concentration and quality of RNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Only samples with an A260/A280 ratio ≥ 1.8 were further processed. For long-term storage, RNA samples were aliquoted and maintained at −80 °C.

2.6. NanoString nCounter profiling analysis

Samples were generously processed by our collaborators at the NanoString's European Customer Experience Laboratory (CX Lab) in Amsterdam, the Netherlands. A 100 ng of total RNA per sample was used for miRNA profiling utilizing the nCounter Human v3 miRNA Expression Assay Kit (Nanostring Technologies, Seattle, WA), that covers up to 827 miRNAs, following the manufacturer's instructions. Data were exported and analyzed by our King Saud University team using the Nanostring nSolver software v4.0 (Nanostring Technologies, Seattle, WA).

2.7. miRNAs targets prediction and validation

Putative binding sites for the dysregulated miRNAs at the 3-UTR of all known human mRNAs were computationally screened using the miRWalk 3.0 database (https://mirwalk.umm.uni-heidelberg.de/) (Sticht et al. 2018). A score ≥ 0.95 was considered a critical criterion for the miRWalk predictive analysis. Validated gene targets were identified using the miRTarBase database to reduce false-positive results, which miRWalk suffers from (https://mirtarbase.cuhk.edu.cn/php/index.php) (Huang et al. 2019).

2.8. Pathway enrichment and network analyses

Target genes data were used to perform functional enrichment analyses using DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/). The Reactome pathway database was utilized to present the systematic analysis, annotation, and visualization of gene functions. A Fisher exact *p*-value of 0.001 was used as a cut-off to identify significantly enriched pathways (Huang et al. 2007).

2.9. Statistical analysis

Data is presented as mean ± standard error of the mean (SEM). Student's *t*-test, One-way analysis of variance (ANOVA), mixed effect model (REML) followed by Tukey's honest significance test, used as Appropriate. The statistical difference is considered significant when the *p*-value is less than 0.05. We utilized GraphPad Prism 9 software for statistical analysis and graph generation (GraphPad Inc., San Diego, CA, USA).

3. Results

3.1. ZnO nanoparticles characterization

We first characterized the shape, size, and surface charge of ZnO NPs. Fig. 1 shows a representative transmission electron microscopy (TEM) image of ZnO NPs with an approximate diameter of 20 nm. The dynamic light scattering (DLS) analysis shows that ZnO NPs have a hydrodynamic size of 480.9 ± 48.7 nm in ddH_2O and a slightly larger size at 485.8 ± 40.9 nm in the cell culture media (DMEM) (Table 1). The tendency of these NPs to aggregate in different liquid media could be solved by sonicating the stock solution for a few minutes right before conducting any treatment experiments. Interestingly, ZnO NPs displayed a significantly positive surface charge in cell culture media compared to ddH2O (−1.3 ± 1.25 mV vs. −19.1 ± 1.1 mV) (Table 1). The data suggest that the positively charged surface could have impacted the interaction between the NPs and cell surface, further facilitating cellular internalization of ZnO NPs (Bannunah et al. 2014).

3.2. Cell viability following exposure to subtoxic concentrations of the ZnO nanoparticles

The mitochondrial metabolic activity of MCF-7 and MDA-MB-231 cells was assessed using an MTT assay to evaluate cellular viability. The higher intensity of the MTT signal corresponds to increased metabolic activity and, thus, cell viability, and vice versa. The growth inhibitory effect and IC50 values of ZnO NPs were measured against another inorganic ENM, NiO NPs. We treated both cell lines with increasing ZnO NPs and NiO NPs concentrations for 24hr. Our results showed that both inorganic nanoparticles caused a dose-dependent reduction in cell viability starting at 6.25 μg/mL and higher (Fig. 2 A, B). Unlike the gradual reduction in cell viability of MCF-7, we noticed a sudden drop in the viability of MDA-MB-231 with almost diminished

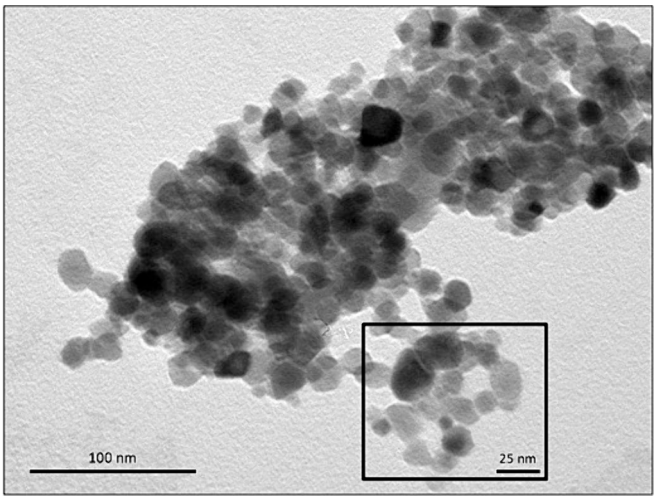

Fig. 1. Transmission electron microscopy images of ZnO nanoparticles. Representative transmission electron microscopy (TEM) image of ZnO NPs for qualitative assessment of the size and shape.

Table 1
Characterization of ZnO nanoparticles in dd water (ddH2O) and cell culture media (DMEM).

ZnO NPs	Hydrodynamic size (nm)	Charge(ζ) (mV)
In ddH_2O	480.9 ± 48.7	−19.1 ± 1.1
In DMEM (serum-free)	485.8 ± 40.9	−1.3* ± 1.25

* Statistically different $p < 0.05$.

viability at higher concentrations. A significant difference in the cytotoxic effects between the two inorganic nanoparticles was noticed at 12.5 μg/mL and higher ($p < 0.05$) in both cell lines. ZnO NPs were five times more potent against MCF-7 cells and fifteen times against MDA-MB-231 when compared with NiO NPs (IC50 = 21.17 μg/mL vs. 109.4 μg/mL; IC50 = 15.64 μg/mL vs. 235.4 μg/mL), respectively. Accordingly, we chose a concentration of 1.56 μg/mL as our subtoxic ZnO NPs pre-exposure dose, which resulted in no observable effects on cellular morphology or viability, to be used in all subsequent experiments.

Next, we assessed whether pre-exposing breast cancer cells with subtoxic concentrations of inorganic nanoparticles would alter cellular response to the chemotherapeutic agent Dox. To achieve that, we treated cells with 1.56 μg/mL of either ZnO NPs or NiO NPs for 24hr, then treated cells with increasing concentrations of Dox (0.03 – 30 μM) for another 24hr. Pre-exposing MDA-MB-231 to the sub-toxic doses of both nanomaterials did not significantly change cellular sensitivity to Dox (Fig. 2C). Interestingly, unlike pre-exposure with NiO NPs, pre-exposing MCF-7 cells with ZnO NPs resulted in a significant reduction in Dox IC50 (0.082 μM vs. 0.177 μM), respectively (Fig. 2D). Therefore, we fixed the treatment conditions with 1.56 μg/mL for ZnO NPs and 0.171 μM for Dox in all subsequent functional experiments in MCF-7 cells.

3.3. Cellular internalization following exposure to a subtoxic concentration of the ZnO nanoparticles

To understand how the subtoxic concentration of ZnO NPs would interact with breast cancer cells, we assessed the nature of ZnO NPs' physical interaction with the MCF-7 cell surface. We measured the MCF-7 intracellular concentrations of ZnO NPs using inductively coupled plasma mass spectrometry (ICP-MS) to achieve that. Even though untreated breast cancer cells displayed a high level of internal ZnO (6.41 ppb), our results showed that a 24-hour exposure to a subtoxic concentration of ZnO NPs resulted in a significantly higher cellular internalization compared with untreated cells (7.22 ppb) (Fig. 3). These data suggest that cell surface interaction, and internalization are prerequisites for the consequent observed effects of subtoxic ZnO NPs concentrations on cancer cells.

3.4. Identification of downregulated OncomiRs following pre-exposure to subtoxic concentrations of ZnO nanoparticles in Dox-treated cells

To assess the involvement of miRNAs in regulating the cell death of Dox-treated cells pre-exposed to subtoxic ZnO dose, we utilized the NanoString nCounter system, which directly counts the actual number of miRNA transcripts in samples rather than relative expression, which requires the amplification step, thus preventing PCR-related errors (Kulkarni 2011). Of the 800 plus screened miRNAs, 44 miRNAs were significantly downregulated in cells pre-exposed to subtoxic ZnO dose prior to Dox treatment compared to untreated and Dox-treated cells, respectively (Table 2).

Further analysis revealed that 13 of those miRNAs have been previously reported by others as OncomiRs associated with worsened progression in breast cancer (Xu et al. 2015; Huang et al. 2023; Shao et al. 2023; Cao et al. 2014; Yang et al. 2017; Stiff et al. 2024; Fu et al. 2016; Liu and Yang 2023; Nair et al. 2020; Zhao et al. 2017; Cabello et al. 2023; Guan et al. 2023; Parashar et al. 2019; Wang et al. 2019) (Table 3). Supervised hierarchical clustering based on the 13 differentially expressed miRNAs in different treatment groups is shown in Fig. 4. All 13 OncomiRs were significantly downregulated in the Dox-treated group compared with the untreated one. Similarly, cells pre-exposed to ZnO followed by Dox treatment had significantly lower expression of the 13 OncomiRs than cells treated with Dox alone. The data suggest

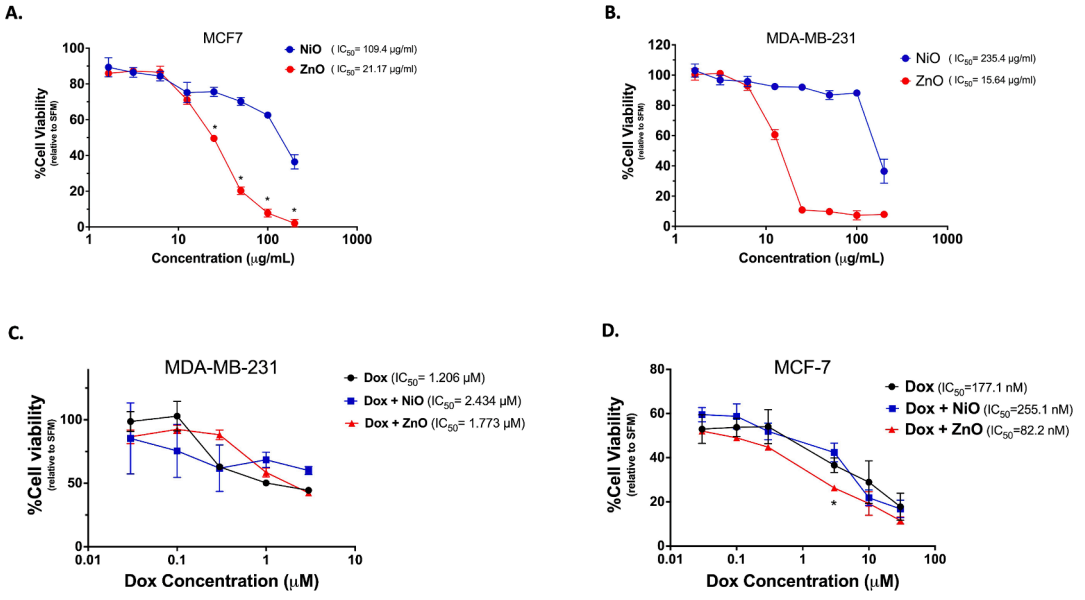

Fig. 2. Pre-exposure to subtoxic concentrations of ZnO NPs increases susceptibility to Dox-mediated cytotoxicity. To evaluate the potential cytotoxic effects of inorganic nanoparticles on different breast cancer cell lines, A) MCF-7 and B) MDA-MB-231 cells were treated with increasing concentrations (1.56–100 μg/mL) of ZnO and NiO nanoparticles for 24hr. IC50 was computed using nonlinear regression, best fit model. To assess the potential impact of pre-exposure to subtoxic concentrations of inorganic nanoparticles on the IC50 of Dox, MDA-MB-231 (C) and MCF-7 (D) cells were pre-treated with subtoxic concentration of ZnO NPs or NiO NPs (1.56 μg/mL) for 24hr, then changed the media to a serum-free medium containing the IC50 dose of DOX (0.171 μM) for another 24hr. The data are expressed as the percentages of serum-free medium controls from three independent experiments as mean ± SEM, (n = 3) per treatment group. *$p < 0.05$ indicates significant differences between similar doses across the treatment groups analyzed by mixed effect model (REML) followed by Tukey's honest significance test.

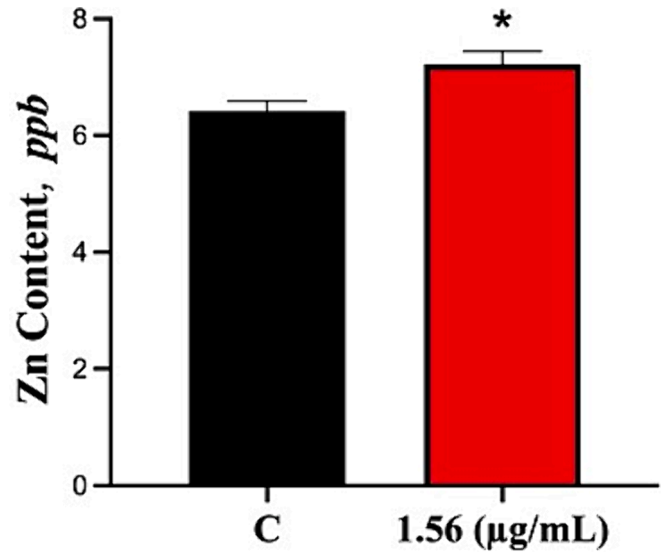

Fig. 3. Cellular internalization of ZnO nanoparticles. Cellular uptake of ZnO NPs was evaluated by inductively coupled plasma mass spectrometry (ICP-MS). The red bar represents the internal cellular content of ZnO after exposing cells to 1.56 µg/mL of ZnO NPs for 24hr. The data were analyzed by unpaired *t*-test, with bars displaying mean ± SEM of at least three independent experiments (n > 3). * $p < 0.05$ indicates a statistical difference compared to the untreated control group.

that suppression of multiple oncogenic miRNAs following pre-exposure to a subtoxic ZnO dose could play a pivotal regulatory role in sensitizing the breast cancer cells to Dox treatment.

3.5. Identification of predicted and validated gene targets of the downregulated OncomiRs and pathway enrichment analyses

To gain an insight into the potential role of the downregulated OncomiRs in the significant death of Dox-treated MCF-7 cells following pre-exposure to subtoxic ZnO dose, we screened for putative binding sites in all known human mRNAs using the miRWalk database (v3) and then filtered the predicted gene targets based on literature validation using miRTarBase database. Of the 10,425 predicted target genes, we identified 617 validated targets for the 13 downregulated miRNAs, representing 5.6 % of the total number of predicted target genes (Fig. 5) (Supplementary File S1). hsa-miR-665 had 364 validated target genes, representing ~ 59 % of total targets, the highest among the down-regulated OncomiRs, followed by hsa-miR-1260b and hsa-miR-18a-5p (Table 4).

We performed pathway enrichment analyses to determine which pathways those validated target genes of the differentially expressed miRNAs are involved in. We identified 54 significantly enriched pathways ($p < 0.05$) (Supplementary File S2). However, ten pathways emerged as the top significantly enriched pathways in which gene expression and transcription, cellular apoptosis, and cell cycle pathways were among the most enriched by those validated target genes (Table 5).

Twenty validated target genes were predicted to regulate ≥50 % of the ten enriched pathways (Table 6). The TFDP2 and YWHAG genes were enriched in seven pathways, whereas the other 6 and 12 genes were enriched in six and five pathways, respectively. Ninety percent of these target genes (18/20) are targets of only one miRNA. SMAD2 was found to be a target of five OncomiRs, followed by CDKN1A, which was targeted by two OncomiRs. These data support the hypothesis that miRNAs regulate essential cellular genes involved in crucial pathways, resulting in breast cancer cell sensitization to Dox treatment following the pre-exposure to subtoxic ZnO NPs dose.

Table 2
Significantly downregulated miRNAs in MCF-7 cells pre-exposed to subtoxic ZnO followed by Dox compared with untreated controls and Dox-treated cells, respectively.

miRNA	Fold Change Relative to Untreated Cells	*p*-value *	Fold Change Relative to Dox-treated Cells	*p*-value *
hsa-miR-106a-5p + hsa-miR-17-5p	−1.56	0.0293	−2.74	0.0162
hsa-miR-10b-5p	−3.06	0.0331	−1.78	0.0443
hsa-miR-1258	−2.11	0.0207	−3.74	0.0066
hsa-miR-1260b	−1.98	0.0066	−4.42	0.0101
hsa-miR-1305	−1.49	0.0069	−2.48	0.0123
hsa-miR-133a-3p	−2.22	0.0442	−3.53	0.0076
hsa-miR-146a-5p	−2.18	0.0198	−1.97	0.0264
hsa-miR-153-3p	−1.55	0.0194	−2.15	0.0238
hsa-miR-181c-5p	−2.96	0.0311	−3.21	0.0028
hsa-miR-188-5p	−3.5	0.0016	−1.66	0.0411
hsa-miR-18a-5p	−2.31	0.0067	−2.3	0.0313
hsa-miR-192-5p	−2.62	0.0047	−2.26	0.0201
hsa-miR-2053	−2.36	0.0086	−1.84	0.0154
hsa-miR-2116-5p	−2.13	0.0331	−2.3	0.0496
hsa-miR-299-5p	−2.74	0.0114	−2.52	0.048
hsa-miR-300	−2.6	0.0016	−5.45	0.0064
hsa-miR-345-5p	−2.08	0.0389	−2.26	0.0361
hsa-miR-346	−2.18	0.0451	−2.45	0.0428
hsa-miR-3605-3p	−2.27	0.0213	−2.1	0.0203
hsa-miR-363-3p	−2.4	0.0058	−2.88	0.0316
hsa-miR-367-3p	−2.27	0.0392	−2.24	0.0412
hsa-miR-371a-5p	−1.83	0.0108	−3.27	0.0156
hsa-miR-377-3p	−1.56	0.0186	−2.88	0.0008
hsa-miR-382-3p	−1.77	0.0051	−2.32	0.0434
hsa-miR-3934-5p	−1.99	0.0327	−2.15	0.0208
hsa-miR-4787-5p	−1.69	0.0009	−2.33	0.0039
hsa-miR-487b-5p	−1.83	0.0188	−2.5	0.0256
hsa-miR-495-5p	−2.17	0.0426	−2.86	0.0282
hsa-miR-499b-5p	−2.4	0.046	−2.21	0.0126
hsa-miR-503-3p	−2.05	0.0406	−2.29	0.04
hsa-miR-516b-5p	−1.62	0.042	−3.06	0.0108
hsa-miR-518f-3p	−2.19	0.0035	−2.91	0.0459
hsa-miR-543	−2.63	0.0175	−2.62	0.0091
hsa-miR-548 *m*	−1.73	0.0186	−2.72	0.0273
hsa-miR-551b-3p	−2.37	0.0228	−1.85	0.005
hsa-miR-564	−3.79	0.0124	−3.31	0.011
hsa-miR-597-5p	−1.83	0.0247	−2.05	0.0033
hsa-miR-628-3p	−2.1	0.0238	−3.09	0.0432
hsa-miR-665	−3.5	0.0016	−1.93	0.0181
hsa-miR-758-3p + hsa-miR-411-3p	−3.18	0.0499	−1.47	0.0368
hsa-miR-764	−1.79	0.023	−2.61	0.0234
hsa-miR-766-5p	−2.07	0.0116	−3.67	0.0125
hsa-miR-891b	−1.78	0.0047	−3.36	0.0138
hsa-miR-922	−2.53	0.0275	−1.47	0.0344

* *p*-value based on *t*-Test.

4. Discussion

The response of breast cancer cells to chemotherapeutics could fluctuate due to different internal and external factors, including exposure to environmental insults (Alfarouk et al. 2015; Koual et al. 2020). In cancer research, the overwhelming number of studies tend to assess the impact of high and impractical doses of different environmental

Table 3
Significantly downregulated OncomiRs in MCF-7 cells pre-exposed to subtoxic ZnO followed by Dox compared with Dox-treated cells.

OncomiRs	Fold Change Relative to Dox-treated Cells	*p*-value*
hsa-miR-300	−5.45	0.0064
hsa-miR-1260b	−4.42	0.0101
hsa-miR-377-3p	−2.88	0.0008
hsa-miR-495-5p	−2.86	0.0282
hsa-miR-346	−2.45	0.0428
hsa-miR-4787-5p	−2.33	0.0039
hsa-miR-382-3p	−2.32	0.0434
hsa-miR-18a-5p	−2.3	0.0313
hsa-miR-503-3p	−2.29	0.04
hsa-miR-146a-5p	−1.97	0.0264
hsa-miR-665	−1.93	0.0181
hsa-miR-551b-3p	−1.85	0.005
hsa-miR-10b-5p	−1.78	0.0443

* *p*-value based on *t*-Test.

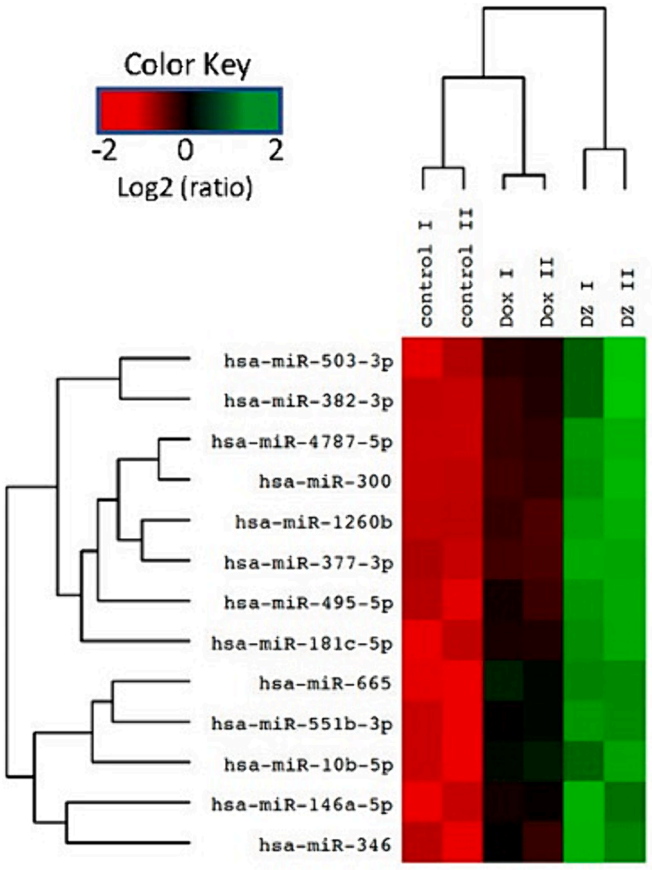

Fig. 4. Supervised hierarchical clustering analysis based on 13 differentially expressed OncomiRs. Heatmap colors represent relative miRNA expression in the untreated control cells, cells treated with only Dox, and cells pre-exposed to a single subtoxic dose of ZnO followed by Dox. Dark boxes represent the downregulated OncomiRs in the Dox-treated cells compared with untreated control cells, whereas the green boxes represent the downregulated OncomiRs in cells pre-exposed to subtoxic ZnO dose followed by Dox treatment compared with the other two groups.

pollutants on the response of cancer cells or tumors to different therapeutics (Koual et al. 2020). An example of such materials are the ENMs, which have gained much attention given their physiochemical properties and usefulness in several biomedical applications (Jamkhande et al., 2019; Jha et al., 2023). However, the cellular impact of low doses or subtoxic concentrations of these nanomaterials remains an open question. In our study, we sought to determine the impact of subtoxic

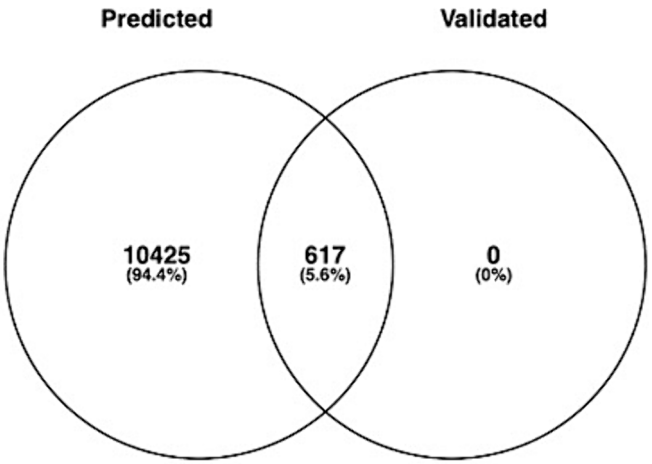

Fig. 5. Bioinformatic identification of OncomiRs-target genes. A Venn diagram drafted from https://bioinfogp.cnb.csic.es/tools/venny/, showing the total number of predicted (by miRWalk database) and validated (by miRTarBase database) target genes of the 13 differentially expressed OncomiRs.

Table 4
Number of validated target genes identified by miRTarBase database for each of the 13 OncomiRs.

#	OncomiRs	# of Validated Target Genes
1	hsa-miR-665	364
2	hsa-miR-1260b	67
3	hsa-miR-18a-5p	59
4	hsa-miR-10b-5p	34
5	hsa-miR-346	25
6	hsa-miR-146a-5p	24
7	hsa-miR-300	23
8	hsa-miR-495-5p	11
9	hsa-miR-503-3p	11
10	hsa-miR-377-3p	9
11	hsa-miR-4787-5p	5
12	hsa-miR-382-3p	4
13	hsa-miR-551b-3p	2

Table 5
Top 10 significantly enriched pathways of the downregulated OncomiRs target genes.

#	Pathway	# Target Genes	% Target Genes	Fold Enrichment	*p*-value *
1	RNA Polymerase II Transcription	86	13.96	1.79	5.07E-08
2	Generic Transcription Pathway	80	12.99	1.83	6.96E-08
3	Gene Expression (Transcription)	92	14.94	1.71	1.37E-07
4	Intrinsic Pathway for Apoptosis	10	1.62	5.22	1.04E-04
5	FOXO-mediated Transcription	10	1.62	4.41	3.86E-04
6	Nuclear Receptor Transcription pathway	9	1.46	4.96	3.91E-04
7	Downregulation of SMAD2/3: SMAD4 Transcriptional Activity	7	1.14	6.69	4.97E-04
8	Apoptosis	16	2.6	2.55	0.0015
9	Transcriptional Activity of SMAD2/SMAD3: SMAD4 Heterotrimer	8	1.3	4.5	0.0017
10	Cell Cycle	40	6.49	1.66	0.0018

* *p*-value based on Fisher's exact test.

Table 6
Most regulated validated target genes of the top ten enriched pathways identified by DAVID database and their targeting OncomiRs.

Most Regulated Genes	# of Pathways	# of miRNAs Targeting that Gene
TFDP2	7	hsa-miR-665
YWHAG	7	hsa-miR-377-3p
BBC3	6	hsa-miR-665
BCL2L11	6	hsa-miR-18a-5p
CDKN2A	6	hsa-miR-10b-5p
SMAD2	6	hsa-miR-146a-5p, hsa-miR-18a-5p, hsa-miR-300, hsa-miR-495-5p, hsa-miR-503-3p
SMAD4	6	hsa-miR-1260b
TP53	6	hsa-miR-18a-5p
APAF1	5	hsa-miR-665
CDKN1A	5	hsa-miR-10b-5p, hsa-miR-665
CDKN1B	5	hsa-miR-1260b
NEDD4L	5	hsa-miR-665
NR3C1	5	hsa-miR-18a-5p
PMAIP1	5	hsa-miR-146a-5p
PSME3	5	hsa-miR-503-3p
PSMF1	5	hsa-miR-665
SKIL	5	hsa-miR-665
TGIF1	5	hsa-miR-665
TGIF2	5	hsa-miR-1260b
WWTR1	5	hsa-miR-18a-5p

concentrations of the ENMs, particularly ZnO NPs, on the response of breast cancer cells to Dox treatment. To our knowledge, this study is the first to assess, at a sub-genetic level, the cellular effects of ENMs on breast cancer response to chemotherapy. We found that exposure to a subtoxic dose of ZnO NPs sensitized the MCF-7 breast cancer cells to the Dox treatment. Furthermore, molecular analysis using the state-of-art Nanostring nCounter technology identified 13 oncogenic miRNAs that regulate more than 600 target genes, most of which were involved in gene expression, transcription, and apoptosis pathways.

The unique properties of ENMs make them of great importance in many industrial and biomedical applications (Ijaz et al. 2020). ZnO NPs have become one of the most commonly investigated metal oxide nanomaterials due to their reported potential applications as an antibiotic, antioxidant, anti-diabetic, and anti-cancer agents, in addition to the ability to efficiently absorb UV radiation, which makes it an essential component of many pharmaceutical formulations including sunscreen and cosmetics (Ijaz et al. 2020). Several studies have investigated the breast cancer cell response to high doses and prolonged exposures to ZnO NPs. For instance, treating MCF-7 cells with concentrations as low as 6.25 μg/mL resulted in a significant, dose-dependent reduction in cell growth, clonogenicity, and proliferative capabilities (Kadhem et al. 2019). Stepankova H. et al. reported similar antiproliferative effects of different ZnO NP formulations at high concentrations on Triple-negative breast cancer cells with 24hr IC50 values ranging from 65 − 110 μg/mL for MDA-MB-231, 150 − 210 μg/mL for MDA-MB-468, and 340 − 350 μg/mL for HBL-100 (Stepankova et al. 2021).

Similarly, our results demonstrated a concentration-dependent toxicity to MCF-7 cells following exposure to ZnO NPs. The cytotoxic effects of ZnO NPs were at least five times more potent when compared with another metal oxide (NiO) (Kouhbanani et al. 2021), with 24hr IC50 values of 21.17 μg/mL and 109.4 μg/mL, respectively. Even though our reported IC50 value was less than those of other breast cancer cell lines, it lies within with what others have demonstrated, where treating MCF-7 with ZnO NPs resulted in IC50 values around 15.88 μg/mL (Kadhem et al. 2019). In addition, the aggressiveness and the subtype of the breast cancer cell lines, their drug resistance and efflux mechanisms, and the nanoparticles' internalization mechanisms may have also counted for differences in the IC50 values, along with other nanoparticles-related factors, including the method of ZnO NPs preparation, final particle size, and the NP surface charge (Bannunah et al. 2014).

Based on the viability study, a sub-toxic dose of ZnO NPs 1.56 μg/mL, which resulted in no observed adverse effect, was used in subsequent experiments to assess any associated adverse response following a 24-hour exposure to the nanoparticles. Despite using such a low dose, our ICP-MS results showed that exposure to ZnO NPs was associated with a significant cellular internalization compared with untreated cells. The detected Zinc particles in the untreated cell were attributed to the Zinc deposits normally found in Eukaryotic cells to regulate different biological processes inside the cells (Chen et al., 2024a). Based on these results, we speculate that any ZnO NP-induced biological outcomes would have been mediated via cellular internalization and accumulation rather than a cell membrane-driven mechanism (Nel et al. 2006). Indeed, our results showed enhanced cell sensitivity to Dox treatment following pre-exposing MCF-7 cells to a sub-toxic dose of ZnO NPs. In agreement with other studies (Shawki et al. 2022), neither observable mitochondrial dysfunction nor reactive oxygen species increase were behind the increased cytotoxicity (Data not shown). Collectively, these data suggest that other underlying molecular mechanisms could have driven such modulation of cellular sensitivity to chemotherapy following exposure to subtoxic levels of ZnO NPs.

Over the last two decades, the role of miRNAs in regulating the response of different cancers, including gynecological malignancies, has been well characterized and documented (Alshamrani 2020; Si et al. 2019). Nearly all hallmarks of cancer cell tumorigenesis (Hanahan 2022) have been strongly linked with either upregulation of oncogenic miRNAs (OncomiRs), which act by repressing the expression of tumor suppressor genes, or downregulation of tumor suppressor miRNAs, resulting in upregulation of their downstream target oncogenes (Hamam et al. 2017; Kurozumi et al. 2017; He et al. 2016; Singh et al. 2023). Indeed, we report here in 13 OncomiRs to be significantly downregulated in sensitized Dox-treated MCF-7 cells following prior exposure to a sub-toxic dose of ZnO NPs. Several previous studies have demonstrated the oncogenic role of these miRNAs in breast cancer. For instance, miR-665 overexpression has been reported by several lines of evidence to be associated with accelerated cancer growth, invasion, metastasis, and poor prognosis (Guan et al. 2023). Our data confirms previous reports that miR-665 was significantly downregulated in response to ZnO NP-mediated sensitization of MCF-7 cells to Dox treatment. Such an effect was achieved by regulating about 364 validated target genes, among which eight tumor suppressor genes that were involved in regulating multiple pathways, including cell cycle, transcription, and apoptosis. The oncogenic nature of miR-1260b was also reported by (Huang et al. 2023) and (Park et al. 2022), where they demonstrated that human breast cancer tissues, plasma, and different human breast cancer cell lines had significantly elevated levels of miR-1260b. Our data rimes with others that miR-1260b could have served as an OncomiR and that its downregulation consequently upregulated several repressed tumor suppressor genes, including SMAD4, CDKN1B, and TGIF2 that might have driven sensitization of MCF-7 by a sub-toxic dose of ZnO NPs.

miR-18a-5p is another overexpressed oncogenic miRNA that has been shown to enhance the cell proliferation ability of several human ER-positive (ER+) breast cancer cell lines, including MCF-7, and is associated with poorer prognosis (Nair et al. 2020). Furthermore, research by (Filho et al. 2014) revealed similar significant upregulation of miR-18a-5p in the triple-negative breast invasive ductal carcinoma compared with the luminal A breast invasive ductal carcinoma and the normal breast parenchyma controls. Some miRNAs might have dual functions as an oncogene or a tumor suppressor. miR-18a-5p, for instance, has been reported by other studies to have tumor suppressive capabilities when overexpressed in certain breast cancer cell lines, including HER2-positive cells (Liu and Yang 2023) and ER-negative cells (Guo et al. 2013). Our results suggest that the downregulation of miR-18a-5p might have contributed to the cytotoxicity in Dox-treated breast cancer cells following the pre-exposure to the sub-toxic dose of ZnO NPs by releasing the inhibitory effect on several tumor suppressor

genes, including BCL2L11, SMAD2, TP53, NR3C1.

Cellular sensitization to chemotherapy can be achieved by downregulating one or more OncomiRs and consequently upregulating one or more tumor suppressor target genes. In other words, the downregulation of all 13 identified OncomiRs and the upregulation of all 600 plus genes are not required to achieve this level of cell sensitivity to Dox treatment; some could have a higher impact than others. For instance, our comprehensive bioinformatic analysis revealed that SMAD2 emerged as the highest tumor suppressor gene to be targeted by five different OncomiRs. The role of SMAD2 as a tumor suppressor is well-documented in several cancers, including breast cancer (Chen et al., 2024b; Petersen et al., 2010). The involvement of SMAD2 in 6 different pathways suggests that it might have had a higher impact on cellular response to Dox treatment than other genes. However, such a conclusion cannot be withdrawn without conducting gain-of-function and lose-of-function experiments. The focus of this study was not to identify the synergistic effects of toxic concentrations of ENMs with chemotherapy but to gain some insight into whether exposure to ENMs at subtoxic concentrations may influence breast cancer cell response to chemotherapy. To the best of our knowledge, this is the first study to assess such an impact on cancer cells at such low concentrations of ENMs. Our findings demonstrated that exposure to a sub-toxic dose of ENMs (ZnO NPs in particular) sensitized the breast cancer cells to the cytotoxic effects of Dox. Mechanistically, the downregulation of multiple oncogenic miRNAs and the corresponding upregulations of their tumor suppressor target genes might have mainly driven the cellular response to chemotherapy. The current study did not experimentally validate the expression of the identified target genes. However, despite the technical limitations in this study, the findings suggest that it would be key to future studies to validate these miRNAs and their targets, and specifically identify essential genes involved in breast cancer cellular sensitization of Dox following a pre-exposure to sub-toxic concentrations of ENMs.

5. Conclusions

Engineered nanomaterials (ENMs), including ZnO nanoparticles, have always been investigated for their potential cytotoxic effects on normal and cancer cells at high and unrealistic concentrations. We provide here a first-of-a-kind study to demonstrate how pre-exposure to a sub-toxic dose of ZnO NPs manipulates breast cancer cell response to chemotherapy. Our findings reveal global changes in the miRNome of these cells that might account for the enhanced cellular response to Dox-mediated cytotoxicity. The data reported in the study emphasizes the importance of assessing the safety of ENMs at low and subtoxic exposure levels, especially in patients using chemotherapeutic drugs to avoid consequential side effects.

Supplementary Materials: SuppFile S1: All Predicted and Validated miRNA-target Genes; Supplementary File S2: Pathway Enrichment Analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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