Zinc oxide nanoparticles provide anti-cholera activity by disrupting the interaction of cholera toxin with the human GM1 receptor

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ABSTRACT

\textit{Vibrio cholerae} causes cholera and is the leading cause of diarrhea in the developing countries, highlighting the need for the development of new treatment strategies to combat this disease agent. While exploring the possibility of using zinc oxide (ZnO) nanoparticles (NPs) in cholera treatment, we previously found that ZnO NPs reduce fluid accumulation in mouse ileum induced by the cholera toxin (CT) protein. To uncover the mechanism of action of ZnO NPs on CT activity, here we used classical (O\textsuperscript{395}) and El Tor (C6706) \textit{V. cholerae} biotypes in growth and biochemical assays. We found that a ZnO NP concentration of 10 μg/ml did not affect the growth rates of these two strains, nor did we observe that ZnO NPs reduce the expression levels of CT mRNA and protein. It was observed that ZnO NPs form a complex with CT, appear to disrupt the CT secondary structure, and block its interaction with the GM1 ganglioside receptor in the outer leaflet of the plasma membrane in intestinal (HT-29) cells and thereby reduce CT uptake into the cells. In the range of 2.5–10 μg/ml, ZnO NPs exhibited no cytotoxicity on kidney (HEK293) and HT-29 cells. We conclude that ZnO NPs prevent the first step in the translocation of cholera toxin into intestinal epithelial cells without exerting measurable toxic effects on HEK293 and HT-29 cells.

INTRODUCTION

In 2015, WHO reported 172,454 cases and 1340 deaths in 42 countries due to cholera (1); the impact of this is enormous on the society (2). Cholera, an acute diarrheal disease, lethal if left untreated, is caused by the intestinal infection of \textit{Vibrio cholerae} (3). Of more than 200 “O” serogroups, only O1 and O139 are responsible for cholera epidemics. Serogroup O1 has been classified into two biotypes, classical (Cl) and El Tor (ET), the latter is associated with the ongoing 7th pandemic first reported in 1961 (4, 5). It affects mostly the places with poor sanitation resources (6), highly populated areas (7), war affected regions (8), and famine-struck territory (9). When contaminated foods or water are consumed, \textit{V. cholerae} that has survived the acidic pH of the stomach colonizes the lining of the intestinal wall secreting cholera toxin (CT), an AB5 type of toxin (10, 11). Rapid onset of vomiting with profuse rice water stools leading to the dehydration and the hypovolemic shock mostly contributes the severity of cholera (12).

Presently, the key management of cholera is the oral rehydration solution (ORS) (13, 14). It reduces the rate of mortality but it cannot reduce the duration of illness (15). So,
antibiotics are recommended for severely ill patients (16). But WHO does not recommend the general use of antibiotics (17, 18) because (i) *V. cholerae* possesses a unique property of genetic plasticity (i.e. exchange of mobile genetic elements and genomic islands) through which several variants of *V. cholerae* have emerged. (ii) Multiple drug resistant strains are emerging at an alarming rate. Multidrug efflux pumps, spontaneous mutation in the chromosome, horizontal gene transfer are the mechanisms employed by *V. cholerae* for developing genetic resistance. (iii) *V. cholerae* has the potential to acquire antibiotic resistance gene from resistant bacteria and can also share with other bacteria through mobile genetic elements. In the human gut, *V. cholerae* may share this gene with other enteric pathogens and in the process may complicate the treatment for an array of infections (19). These conditions necessitate the finding of a new therapeutic strategy for the proper management of the disease.

Recently, NPs are gaining popularity as an alternative to antibiotics (20–22). Unlike antibiotics that act on specific target, NPs destroy pathogens via direct contact with bacteria cell wall and thus bacteria are less likely to develop resistance to NPs (23, 24). Out of several NPs, we had chosen ZnO NP for studying its anti-cholera activity. The World Health Organisation recommends oral zinc combined with ORS as an effective therapy to decrease the morbidity and mortality associated with cholera (25, 26). However, there are reports saying adding zinc to ORS did not reduce total stool output or recovery time or disease duration (27, 28). Hence we employed ZnO NP in our study. Nano-sized particles can easily penetrate into cells and can interact with biological molecules within or on the cell surface. Furthermore, NP possess larger surface-to-volume ratio that enables better interaction with biomolecules (29). Apart from that, ZnO NP has excellent stability and long shelf life (30). Recent studies have shown that ZnO NP exhibits minimal effects on human cells and have selective toxicity to bacteria (31). Our previous work demonstrated the antibacterial activity of ZnO NP against *V. cholerae* (32). In this work, we had induced the fluid accumulation in mice ileal loop using CT, which got alleviated using the NPs. This raises the question how ZnO NP achieves this. Here, we have examined the action of ZnO NP on CT production by *V. cholerae* and the mechanism by which it inhibits its activity.

**RESULTS**

**Characterization of ZnO NP**

ZnO NP was characterized using dynamic light scattering (DLS) and X-ray diffraction (XRD). The diffraction pattern obtained from XRD (Figure S1) is identical to that obtained from the hexagonal wurtzite phase of ZnO. Figure S2 represents the particle size distribution of ZnO NP as obtained from DLS, which shows that the hydrodynamic diameter of ZnO NP is 83.29 nm (Z-Average diameter = 68.24 nm).

**Deterioration of CT binding to GM1 after ZnO NP treatment**

In order to observe the effect of ZnO NP on CT production by two biotypes of *V. cholerae*, we selected O395 (Cl) and C6706 (ET) strains. We explored the classical GM1 ganglioside based ELISA for detecting secreted CT level in the supernatant of the culture grown alone and in presence of ZnO NP (0–50 µg/ml) under CT-induced condition. It was observed that the level of detectable CT gets reduced by ZnO NP in a dose dependent manner. In both the strains, the percentage of detectable CT reduced to 50–57% at concentration of 20 µg/ml of ZnO NP (Figure 1). This could result from either (i) the growth inhibition of *V. cholerae* or suppression of CT production by ZnO NP, or (ii) ZnO NP interaction either with GM1 or CT and prevention of CT-GM1 binding.

**Effect of ZnO NP on bacterial growth**

To test the above hypotheses, we first looked upon the growth kinetics of *V. cholerae* without and with various concentrations of ZnO NP. Surprisingly up to 10 µg/ml of ZnO NP, neither strain showed any significant reduction in growth (Figure 2AB); although we had observed a prominent reduction of ~40% in the level of detectable CT (Figure 1). We also checked the impact of ZnO NP (10 µg/ml) on the bacterial membrane by monitoring change in membrane fluidity and membrane potential. No significant change in the membrane structure...
was observed (Figure S3) suggesting healthy condition of *Vibrio* under the concentration of NP used. Viability of the cell was also assessed by counting CFU and by MTT assay at the end of 18h, and we confirmed that the reduction of CT level up to ZnO NP concentration of 10 µg/ml does not correspond to the stressed condition of cells induced by ZnO NP (Figure 2CD). It thus appears that ZnO NP is directly responsible for the low detectable CT in culture supernatant.

**ZnO NP does not affect CT expression at mRNA and protein level**

CT production is under the control of master virulence regulator ToxT which in turn is activated by a protein complex comprised of two pairs of inner membrane proteins, ToxRS and TcpPH. AphA and AphB proteins sense cell density, anaerobiosis, and other environmental cues and subsequently activate TcpPH (33).

The finding of decrease in CT level in both the strains while cells are in healthy condition made us speculate that either CT mRNA expression or CT protein production or both were altered. To validate our elucidation, we isolated total RNA from the untreated and NP-treated cells, made the cDNA to determine the amount of CT-specific cDNA using semi-quantitative and Quantitative RT PCR. These analyses revealed that CT mRNA expression in NP-treated cells was same as that of the control, signifying no hindrance due to ZnO NP in the upstream regulatory cascade controlling the expression of CT (Figure 3A).

The next possibility of reduced CT protein level was examined by Immunoblot analysis of untreated and NP-treated culture supernatant using anti-CT antibody. Importantly, no change in the concentration of CT protein was seen with varying concentration of ZnO NP (Figure 3B).

These results annulled our first assumption that GM1 ELISA detected low CT in presence of ZnO NP due to growth inhibition of *V.cholerae* or suppression of CT production.

**ZnO NP does not interact with GM1**

Since ZnO NP exhibits no effect on CT production and secretion, next we studied if ZnO NP binds to GM1 receptor and inhibits its activity to attach with CT. We treated the GM1 coated wells with ZnO NP for 2h, washed thrice with PBS (1X) prior its incubation with CT, followed with the standard GM1 ganglioside based ELISA protocols. No change in CT binding to the NP-treated GM1 was observed (Figure 4A), suggesting no role of GM1. However, when ELISA was performed with NP-treated CT (2h), there was a continuous decrease in CT binding to GM1 with increasing concentration of ZnO NP, indicating the importance of the interaction of ZnO NP with CT (Figure 4B).

**ZnO NP disturbs the secondary structure of CT via complex formation**

The interaction of ZnO NP with CT protein was studied using biophysical techniques. Fluorescence quenching is one of the widely used processes to study the interactions between biomolecules and ligand. For quenching to occur, the fluorophore and the quencher need to be in contact with each other. The quenching may be collisional (dynamic) or complex formation (static) (34). The intrinsic fluorescence emission of Trp of CT in presence of different concentrations of ZnO NP is shown in Figure 5A. The fluorescence intensity decreased gradually with increasing concentration of ZnO NP with a slight red shift in the emission maxima signifying the exposure of Trp residue to polar environment. The linear Stern-Volmer plot of $F_o/F$ versus the concentration of ZnO NP indicates the presence of only one type of quenching of CT in presence of ZnO NP. To elucidate the molecular mechanism of CT–ZnO NP interaction, Stern-Volmer plots of $F_o/F_c$ against the concentration of ZnO NP were plotted for two different temperatures (18°C and 45°C). The linear plot of $F_o/F_c$ against the concentration of ZnO NP and decreasing slope with increase in temperature suggest the homogeneous static quenching (Figure 5B).

To determine the effect of ZnO NP on the secondary and tertiary structure of CT, we performed far-UV and near-UV circular dichroism study (35). As evident from the far-UV CD spectra given in Figure 5C, there was a slight decrease in the negative ellipticity in the far-UV region of CD without any significant shift of the peak, thereby suggesting subtle change in the $\alpha$-helical content of the CT in presence of ZnO NP. Likewise, near-UV
circular dichroism also did not indicate any major change in tertiary structure of CT (Figure S4).

To further confirm the interaction between CT protein and ZnO NP, we incubated ZnO NP with the CT-induced culture supernatant of both the strains for 1 h at room temperature followed by washing with PBS. The pellet was dissolved in Laemmli buffer and was examined for CT protein that could adsorb on ZnO NP via Immunoblot assay using anti-CT antibody. As revealed in Figure 5D with increasing concentration of ZnO NP, there is an enhancement of CT adsorption. Furthermore, we also used anti-HAP antibody to check if another protein in the supernatant co-pellet with ZnO NP. HAP is an extracellular metalloprotease secreted by V. cholerae during nutrient limitation, while entering into stationary phase and at high cell population density (36). The absence of HAP specific bands in the ZnO NP treated samples indicates no interaction of HAP with ZnO NP (Figure S5A). We further compared the interaction of ZnO NP with the purified proteins, such as heat shock protein (HSP90α), tubulin and CT. As shown in Figure S5B, ZnO NP adsorbs CT in concentration dependent manner but no significant interaction was found with either HSP90α or tubulin.

To examine if ZnO NP affects the integrity of CT (AB5), the holotoxin was incubated with ZnO NP (10 µg/ml) and in 3 M urea, pH 3.5 (positive control) separately for 1 h. After incubation, gel filtration chromatography was performed. Figure S6B represents the elution patterns of CT alone, in presence of ZnO NP and with urea, pH 3.5. The profiles demonstrate no dissociation of CT subunits in presence of ZnO NP, whereas urea treated sample showed significant dissociation (positive control). Albumin (67 kDa), chymotrypsinogen A (25 kDa) and lysozyme (14.3 kDa) were used as molecular weight markers (Figure S6A). We further confirmed the results by running 7.5% native polyacrylamide gel. For positive control, CT was heat denatured (Figure S7). Again, the oligomeric structure of CT seemed to have remained unchanged.

Structure and activity of some standard proteins in presence of ZnO NP

To understand if the inhibitory activity of ZnO NP is specific to CT, we studied the specificity of four standard proteins (lysozyme, human serum albumin (HSA), HSP90α and tubulin), the first three of which were also studied for their activity. Intrinsic Trp fluorescence quenching experiment indicates ZnO NP does not interact with tubulin and HSP90α, whereas it binds with lysozyme and HSA at the working concentration (Figure S8). Though interaction takes place between HSA, lysozyme and ZnO NP, the activities of these proteins are not compromised (Figure S9).

**HT-29 cell bioassay**

HT-29 is a human colorectal adenocarcinoma cell line, established in 1964 from the primary tumor of a 44-year-old Caucasian female with colorectal adenocarcinoma. This cell line is used as an invitro model to study cholera toxin binding by intestinal cells. In presence of CT, large pleomorphic vacuoles are formed in HT-29 cells (37). We utilized this bioassay to check the effectiveness of ZnO NP against biological activity of CT. HT-29 cells were treated with CT alone and NP-treated CT. The CT induced vacuoles formation was inhibited by ZnO NP in a concentration dependent manner emphasizing the inhibition of biological activity of CT as shown in Figure 6A.

**Effect of ZnO NP on CT uptake by HT-29 cells**

We further investigated the effects of ZnO NP on CT uptake by cells. For this study, Immunoblot assay was done with the lysates of HT-29 cells incubated with CT and NP-treated CT. Blot analysis clearly demonstrated the fact that ZnO NP interacts with CT and does not allow its binding with GM1 present on the cell surface hence reducing its uptake by the cells (Figure 6BC).

**Cytotoxicity in HEK293 cells**

The study of cytotoxicity of ZnO NP on the human embryonic kidney cells (HEK293) and HT-29 cells would be helpful to determine whether the concentrations of ZnO NP used in this study are non-cytotoxic. For this, HEK293 and HT-29 were treated with various concentrations (2.5, 5, 10µg/ml) of ZnO NP for 48h and the percentage of viable cells were measured by MTT assay. As evident from
Figure 6D, ZnO NP was found to be non-toxic in the effective range of concentration.

**DISCUSSION**

In our previous paper (32), we reported the detailed antibacterial activity of ZnO NP (~7-10 nm) against two biotypes of O1 serogroup of *V. cholerae* – Classical (O395) and El Tor (N16961). We observed that ZnO NP exerts its anti-*Vibrio* activity by producing reactive oxygen species and disrupting bacterial membrane that leads to depolarization of membrane, increased permeabilization, protein leakage, and DNA damage. We had also shown that ZnO NP effectively blocked intestinal fluid secretion induced by purified CT (32). These results prompted us to examine the mechanistic study of the anti-cholera activity of ZnO NP at a concentration (10 µg/ml) at which the bacterial cells remain unaffected.

CT initiates its pathogenic effect by binding to GM1 ganglioside receptor present on the intestinal epithelial barrier. This complex is endocytosed and transported to endoplasmic reticulum for the activation of cholera toxin A (CTA) subunit. The activated CTA, in turn, activates the G protein by adenosine diphosphate (ADP)-ribosylation of its G\(_{\alpha}\) subunit thereby increasing adenylate cyclase activity to produce cyclic adenosine monophosphate (cAMP). The high level of intracellular cAMP stimulates protein kinase A (PKA) to turn on the cystic fibrosis transmembrane conductance regulator (CFTR), leading to the efflux of Cl\(^-\) ions followed with Na\(^+\) ions and water, resulting in rice water stools (38).

The present management of cholera aims at recovery rather than cure. The usage of antibiotics in severely ill patients may kill bacteria but cannot inhibit the action of cholera toxin once produced. Hence, the finding of a drug that can kill pathogen as well as block the activity of cholera toxin already secreted by colonized bacteria may boost the present therapeutic strategies. In literature, various compounds are reported to inhibit cholera toxin activity via different mechanisms. For example, Virstatin inhibits the transcriptional regulator ToxT, thereby preventing the expression of toxin-coregulated pilus and cholera toxin (39, 40). Plant-derived dihydroisosteviol reversibly inhibits CFTR chloride channels (41). Capsaicin, one of the active compounds of red chili, acts as a repressor of CT production by enhancing the transcription of the hns gene coding for the histone-like nucleoid structure protein (42). In a related study gold NPs have been found to destabilize the structure of another *V. cholerae* toxin Ace, and thereby prevent fluid accumulation in mouse ileal loop (43).

In this paper, we report the anti-cholera toxin activity of ZnO NP. We showed ZnO NP binds with CT and prevent its attachment with receptor GM1 thus inhibiting the very first crucial step of CT-induced diarrhea. To study the anti-cholera toxin activity, we selected the optimal dose at which ZnO NP is neither detrimental to *V. cholerae* nor to HEK293 and HT-29 cells. Our findings suggest that ZnO NP when added to O395 and C6706 culture supernatant brought about decrease in CT level as detected by ELISA (Figure 1), though no change in CT concentration was observed by Western blot analysis (Figure 3B). This was also corroborated with no alteration in the transcription of CT mRNA (Figure 3A). These outcomes point towards the effect of ZnO NP on CT-GM1 binding. We further extended our study to investigate the exact mechanism of action of ZnO NP. We found no change in CT level when GM1 was treated with different concentration of ZnO NP whereas CT-GM1 interactions decreased in a dose dependent manner when CT was treated with ZnO NP (Figure 4). We explored the CT-ZnO NP interaction by performing biophysical studies. Fluorescence study revealed decrease in fluorescence intensities with a slight spectral red shift of 2 nm indicating unfolding of CT secondary structure upon binding to ZnO NP (Figure 5AB). Far-UV CD analysis indicated that the \(\alpha\)-helical content of CT was reduced with the concomitant increase in random coil, when CT was treated with ZnO NP (Figure 5C).

Finally, we checked the effectiveness of ZnO NP in reducing CT induced pathological effects and CT binding on HT-29 cells. According to literature, large vacuoles are formed when HT-29 cells are incubated with CT. Our results demonstrated that NP-treated CT failed to induce vacuoles and also uptake of
CT by HT-29 cells decrease in a dose-dependent manner (Figure 6).

ZnO NP has been shown to have antibacterial effect [32]. It may be mentioned that the antibacterial effect of any NP is influenced by its size. ZnO NP used here is about 10 times larger than the previous work (32). While 25 µg /ml was found to disrupt the membrane structure (32), a concentration of 10 µg /ml did not affect the membrane integrity to any significant extent (Figure S3). But even at this concentration, which is non-cytotoxic (Figure 6D), and which does not inhibit the growth of V. cholerae (Figure 2), it can inhibit cholera toxin activity. All these results suggest that ZnO NP has the potential for clinical application in preventing cholera infection, though further studies need to be done to translate it as a cholera treatment for human.

**EXPERIMENTAL PROCEDURES**

*V. cholerae* classical biotype strain O395 and El Tor strain C6706 were used in these studies. These strains were gift from Dr. R. K. Nandy (NICED, Kolkata) and Dr. Rukhsana Chowdhury (IICB, Kolkata), respectively, and maintained in our laboratory. Cholera toxin (AB5) (CT), anti-rabbit IgG (whole molecule)– peroxidase antibody produced in goat, anti-cholera toxin antibody produced in rabbit, monosialoganglioside GM1 from bovine brain, streptomycin, and ZnO NP were purchased from Sigma-Aldrich. Anti-HAP antibody produced in rabbit was a gift from Dr. Amit Pal (NICED, Kolkata).

**Characterization of ZnO nanoparticles**

Zinc oxide, dispersion nanoparticles, < 100 nm particle size, was purchased from Sigma-Aldrich. Prior to use, ZnO NP was characterized. Particle size was determined by dynamic light scattering (DLS) technique using Zetasizer Nano ZS Malvern Instrument (Worcestershire, UK). X-ray diffraction pattern for the ZnO NPs was recorded using an X-Ray Powder Diffractometer, Rigaku MiniFlex (The Woodlands, Texas, USA), using Cu Kα radiation of wavelength λ=0.1541 nm, maintaining applied voltage of 30kV and current at 15 mA in the scan range 2θ=10-80°.

**GM1 ganglioside based ELISA**

Cultures of both the strain (O395 and C6706) were grown under toxin inducing condition in absence and presence of varying concentration of ZnO NP. After 18h cells were pelleted down and supernatant was collected. To estimate the CT content supernatants were added to GM1 coated microtiter well and ELISA was performed (44) using anti-cholera toxin antibody and anti-rabbit IgG (whole molecule)– peroxidase antibody. The color intensity was measured at 450 nm in an ELISA plate- reader.

**Bacterial susceptibility test**

From the overnight grown cultures of the respective strains, 1% was inoculated into fresh Luria–Bertani broth and grown up to the turbidity of 0.5 McFarland standards. Growth inhibition study was performed under toxin inducing condition, 200 µl of culture (equivalent to ~5X10⁵ CFU) was incubated at 30°C in the presence and absence of different concentration of ZnO NP in a 96-well flat bottom microtiter plate (Tarsons), and the absorbance was recorded at 595 nm at every 15 mins up to 18h in a microplate reader. Cell viability was checked by plating appropriate dilution of culture onto LB plates at 18h.

**RNA isolation, Semi-Quantitative and Quantitative RT-PCR**

For RNA isolation cells were treated as before for 18h. Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies). Furthermore, the pooled RNA (600 ng) was converted to one-strand cDNA using Verso cDNA synthesis kit (Thermo Scientific) as per manufacturer’s instruction. Then semi-quantitative RT-PCR was performed using CT- and 16S rRNA- specific primers. Amplification was performed for 37 cycles (96°C for 30 s, 55°C for 45 s, and 72°C for 45 s). 10 µl of each PCR product was electrophoresed on a 2% agarose gel and visualized using ethidium bromide. PCR products were normalized according to the amount of 16S rRNA detected in the same cDNA sample. The data for the mRNA transcript levels were further validated by performing quantitative-PCR (qPCR) (7500 Fast Real-time PCR system, Applied Biosystems) using 2X SYBR green (hot jumpstart sybr green, Sigma) and CT- specific primer. Data analysis was done according to
Livak method using 16S rRNA as an internal control.

**Western blotting**

Electrophoresis was carried out using the treated culture supernatants of the respective strains and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). Immunoblotting was done using anti-cholera toxin antibody (1:2000) followed by incubation with anti-rabbit IgG (whole molecule)-peroxidase antibody (1:1000). The horseradish peroxidase-positive bands were visualized using Lumi-Light Western Blotting Substrate (Roche) in dark.

**Fluorescence spectroscopy**

The fluorescence spectrum of CT (3 µM) was studied on a Perkin Elmer fluorescence spectrophotometer using quartz cuvette with a 1 cm path length at specified temperatures. The excitation and emission spectra were set at 282 nm and 310 to 400 nm respectively. For fluorescence quenching measurements, ZnO NP was added successively to CT and the final fluorescence values were corrected for the inner filter effect using the following equation (45):

\[
F_{corr} = F_{obs} \times 10^{-\frac{A_{ex} + A_{em}}{2}}
\]

Where \( F_{corr} \) and \( F_{obs} \) are the corrected and the observed fluorescence intensities respectively, \( A_{ex} \) and \( A_{em} \) are the absorbances of the sample at the excitation and the emission wavelength respectively.

The fluorescence intensities were determined at \( \lambda_{max} \), and data were analyzed using the Stern-Volmer equation (45):

\[
F_0 \times F_c = 1 + K_{SV} \times [NP]
\]

**Circular dichroism spectroscopy**

The far- and near-UV CD spectra ranging from 200-260 nm and 240-340 nm, respectively were obtained using a JASCO-810 spectropolarimeter equipped with a thermostatically controlled cell holder. The concentration of CT used for far- and near-UV CD was 3 µM and 30 µM, respectively and that of ZnO NP varied from 0 to 15 µg/ml. The final data represents the average of three scans and a bandwidth of 1 nm. From each sample spectra, contribution of the buffer was subtracted.

**Pull down assay**

The CT-induced culture supernatant of O395 and C6706 strains were incubated with ZnO NP for 1h at room temperature. Cells were centrifuged at 6000rpm for 10 mins at 4°C followed by washing three times with PBS. The NP-protein pellet was dissolved in Laemml buffer and boiled for 10 mins. Finally, Immunoblotting was done using anti-cholera toxin antibody as explained before.

**Activity assay**

For HSA, esterase activity was checked in presence of ZnO NP (0-25 µg/ml). 1 ml of 1 mg/ml HSA in 10 mM Tris-Cl, pH 8.0 was incubated with different concentration of ZnO NP for 1h. Then 5 µl of 0.1M p-nitrophenyl acetate was added and kept at 22 °C. Finally, absorbance was measured at 400 nm at different time points (46). For the respective ZnO NP concentration, a control sample without albumin was followed in a similar manner to subtract the effect of ZnO NP on p-nitrophenyl acetate.

For lysozyme, lysis of *Micrococcus lysodeikticus* cells was monitored to determine its activity (47). 300 units/ml of lysozyme in phosphate buffer, pH 6.2 was incubated with ZnO NP (0-25 µg/ml) for 1h. These samples were used to monitor the lyses of cells at 450 nm for 5 mins.

Lastly, HSP90α activity was determined by measuring the release of inorganic phosphate due to hydrolysis of ATP using a malachite green assay (48).

**Gel filtration chromatography**

Gel filtration chromatography was used to analyze the effect of ZnO NP on the quaternary structure of CT. Size-exclusion high pressure liquid chromatography of CT, CT + ZnO NP (10 µg/ml) was performed with a Polysep-GFC-P 4000 column (300x7.8 mm; Phenomenex) equilibrated with 20 mM HEPES, 100 mM NaCl, pH 7.5. For positive control, CT was incubated in 0.1 M glycine-HCl buffer (pH 3.3) containing 3 M urea for 1h (49).

**Cell culture**

Human embryonic kidney cell line (HEK293), human colon adenocarcinoma cell line (HT-29) were purchased from American Type Culture Collection (ATCC #HTB-38, Manassas, VA). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 1 mM L-glutamine, 10%
fetal bovine serum, 50 µg/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B and non-essential amino acids at 37°C under 5% CO₂. Cells were grown not more than 80-90% confluency.

**HT-29 cell bioassay**

1 × 10⁶ HT-29 cells cultured in DMEM were incubated with 20 µg/ml of CT alone or with various concentrations of ZnO NP for 2 h at 37°C. The morphology of the cells was evaluated by phase-contrast microscopy (Leica DFC450 C, Leica Microsystems India Pvt. Ltd.).

**MTT assay**

The growth inhibition and lack of metabolic activity of *V. cholerae* strains and HEK 293 and HT-29 cell lines were determined using MTT assay. *V. cholerae* and HEK 293 cells were grown alone in presence of different concentration of ZnO NP for 18h and 48h respectively, washed with PBS and incubated with MTT solution for 2h at 37°C. To dissolve formazan crystals, 100 µl of DMSO was added to each well and optical density was monitored at 595 nm.

The percentage of viability was calculated as cytotoxicity (32):

$$\left(1 - \frac{A_{test}}{A_{control}} \right) \times 100$$

**Statistical analysis**

Data represented as the mean ± SD. All statistical analyses were performed with GraphPad Prism 5 software, using 1way ANOVA followed with Dunnett’s Multiple Comparison Test. P<0.05 was considered statistically significant.

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**Conflicts of interest:** None.

**Author contributions:** SS designed and performed the experiments, analyzed the data, and wrote the manuscript. AA and MP performed cell line experiments and analyzed the data. AA also studied the interaction and activity of HSP90α. PC designed the experiments, wrote and edited the manuscript, supervised and directed overall research. All authors reviewed the results and approved the final version of the manuscript.

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FIGURE LEGENDS:

FIGURE 1. Determination of secreted CT level in the CT-induced culture supernatant of (A) O395 and (B) C6706 strains, grown alone and in presence of ZnO NP (0-35 µg/ml) by GM1 ganglioside based ELISA. A value of 1 indicates no change relative to control. Data were analyzed using 1-way ANOVA with Dunnett’s Multiple Comparison Test. **P<0.01, ***P<0.001, n=3.

FIGURE 2. Effect of ZnO NP on V. cholerae growth. Growth curves of V. cholerae (A) O395 and (B) C6706 in presence of ZnO NP (0-50 µg/ml). Viability of the cells after exposure to ZnO NP (0-10 µg/ml) for 18h, as measured by (C) CFU analysis and (D) MTT assay. ns defines no statistically significant differences. All data represent the value of three independent experiments.

FIGURE 3. Effect of ZnO NP on production of CT at RNA and protein level. (Ai,ii) Ethidium bromide stained agarose gels, demonstrating semi-quantitative RT-PCR data of CT mRNA and 16S rRNA (loading control) expression in V. cholerae treated with ZnO NP (0-10 µg/ml). (Aiii,iv) Scatter plot demonstrating fold change of CT at mRNA level by quantitative RT-PCR, normalized against 16S rRNA. (Bi) Immunoblot of CT protein present in the supernatant of the bacterial culture cultivated in absence and presence of ZnO NP (0-10 µg/ml), normalized against cell density. (Bii) Scatter plot, demonstrating the quantitation of CT protein of figure (Bi) by ImageJ software. All data represent the value of three independent experiments.

FIGURE 4. Effect of ZnO NP on the activity of CT by GM1 ELISA. Determination of CT level by GM1 ganglioside based ELISA, (A) GM1 coated wells incubated with ZnO NP (0-50 µg/ml) followed by 3 µM of purified CT. (B) GM1 coated wells incubated with 3 µM of purified CT treated with ZnO NP (0-35 µg/ml). Data were analyzed using 1-way ANOVA with Dunnett’s Multiple Comparison Test. ***P<0.001, n=3.

FIGURE 5. Evaluation of ZnO NP interaction with CT using spectroscopic methods. (A) Fluorescence emission spectra of CT (3 µM) with increasing concentration of ZnO NP (0-36 µg/ml). (B) Stern–Volmer plots of fluorescence quenching for CT with ZnO NP at two different temperatures (18° and 45°C). Data shown are corrected for inner filter effect. (C) Secondary structure of native CT and CT bound to ZnO NP (0-15 µg/ml) monitored by far-UV CD spectra. (D) Estimation of CT protein adsorbed on ZnO NP by Immunoblot assay.

FIGURE 6. Uptake of CT by HT-29 cells. (A) Phase-contrast microscopic images showing that ZnO NP ameliorates vacuole formation by CT in a concentration dependent manner (magnification: 20X; scale bar: 10 µm); dashed box indicates an enlarged view of that section. Cells with no ZnO NP or CT treatment constitute the control. (B, C) Level of internalized CT as determined by Immunoblot from whole cell lysate of HT-29 cells; β-actin is used as the loading control. (D) Percentage of viable HT-29 and HEK293 cells after exposure to ZnO NP (0-10 µg/ml) for 48h, as measured by MTT assay. Data were analyzed using 1-way ANOVA with Dunnett’s Multiple Comparison Test. ***P<0.001, n=3.
FIGURE 1.

(A) O395

(B) C6706

Relative change in CT level vs. ZnO NP, µg/ml
FIGURE 2.
FIGURE 3.

(A) (i) O395 (ii) C6706

ZnO NP (µg/ml) 0 2.5 5 10

CT mRNA

16S rRNA

(iii) (iv) (ii)

CT mRNA (Relative expression)

0.0 0.5 1.0

ZnO NP, µg/ml 0 2.5 5 10

(B) (i)

ZnO NP (µg/ml) 0 2.5 5 10

kDa 25 35

35 35

C6706 C395

CT protein

(ii)

CT protein (Relative expression)

0.0 0.5 1.0

ZnO NP, µg/ml 0 2.5 5 10

C6706 O395
FIGURE 6.
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