Zinc at Cytotoxic Concentrations Affects Posttranscriptional Events of Gene Expression in Cancer Cells

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Key Words
Clioquinol • Zinc • microRNA • p-body • Dicer

Abstract
Zinc at cytotoxic concentrations has been shown to regulate gene transcription in cancer cells, though zinc’s involvement in posttranscriptional regulation is less characterized. In this study, we investigated the involvement of cytotoxic zinc in the posttranscriptional steps of gene expression. Clioquinol, a well-established zinc ionophore, was used to raise intracellular zinc to reported cytotoxic levels. The MCF-7 human cancer cell line was applied as a cell model system. Several parameters were used as indictors of posttranscriptional regulation, including p-body formation, microRNA profiling, expression level of proteins known to regulate mRNA degradation, microRNA processing, and protein translation. p-body formation was observed in MCF-7 cells using several molecules known as p-body components. Clioquinol plus zinc enhanced p-body assembly in MCF-7 cells. This enhancement was zinc-specific and could be blocked by a high affinity zinc chelator. The enhancement does not seem to be due to a stress response, as paclitaxel, a commonly used chemotherapeutic, did not cause enhanced p-body formation at a highly cytotoxic concentration. microRNA profiling indicated that clioquinol plus zinc globally down-regulates microRNA expression in this model system, which is associated with the reduced expression of Dicer, an enzyme key to microRNA maturation, and Ago2, a protein essential for microRNA stability. This study demonstrates that ionophoric zinc can induce cytotoxicity in cancer cells by globally regulating posttranscriptional events.

Introduction
Transient metals such as zinc are known to play a critical role in cellular biology by affecting many key cellular processes [1]. Transcriptional regulation of mammalian genes by zinc has been extensively studied [2-5]. It has been established that both high and low intracellular zinc levels alter gene transcription through the metal response elements present in the gene promoters.
For instance, high zinc availability leads to an up-regulation of MTF1 (MRE binding transcription factor 1), which in turn enhances expression of metallothionein-I and -II, and a zinc transporter 1 [6, 7]. On the other hand, zinc-induced suppression of gene transcription has also been described [8, 9]. Zinc was shown to affect mRNA stability of the ZnT5 gene [10], suggesting that this metal ion is involved in posttranscriptional regulation of gene expression. However, unlike transcriptional regulation, posttranscriptional regulation of gene expression by cytotoxic zinc has been less characterized. Since cellular zinc dyshomoeostasis is implicated in neurological disorders such as Alzheimer’s disease [11], and in malignant diseases such as prostate and breast cancer [12, 13], it is imperative to understand how this metal ion at cytotoxic concentrations affects posttranscriptional events in mammalian cell systems.

One of the recently discovered key events in posttranscriptional regulation of gene expression is microRNA-induced suppression of target gene expression [14, 15]. microRNAs are small non-coding RNAs that are initially transcribed by RNA polymerase II and processed in the nucleus into precursor-microRNAs. These precursor molecules are exported to the cytoplasm, where they are digested by Dicer (an RNase III) and become mature microRNAs composed of approximately 22 nucleotides. The mature single-strand microRNA is then incorporated into the RNA-induced Silencing Complex (RISC) and guides RISC to target the 3’-untranslated regions (3’-UTR) of the target transcripts, thereby causing repression of protein translation or promotion of mRNA degradation [16]. These later events are shown to take place in RNA processing bodies (p-bodies), which are assembled as small granules within the cytoplasm of mammalian cells [17].

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), an 8-hydroxyquinoline derivative, has been demonstrated to act as a zinc ionophore by transporting zinc into cells [18, 19]. We have recently reported that clioquinol plus zinc kills cancer cells through lysosome-dependent and -independent pathways [19, 20]. We therefore used clioquinol to achieve high intracellular zinc levels and examined the effects of this ionophoric zinc on posttranscriptional regulation in cancer cells, focusing on alterations in p-body assembly, microRNA profiling, and expression of several proteins involved in posttranscriptional regulation. We report here that high zinc availability enhances p-body assembly, globally down-regulates microRNA expression, and alters protein expression of argonaute2 (Ago2), Dicer, and eukaryotic translation initiation factor 4E (eIF4E).

Materials and Methods

Materials

Antibodies against Dicer, Drosha, Ago2, and eIF4E were purchased from Cell Signaling Technology Inc. (Danvers, MA). The antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Promab Biotechnologies, Inc. (Richmond, CA). Clioquinol, CuCl2, ZnCl2, N, N, N’, N’-tetrakis (2-pyridylmethyl) ethylenediamine-pentaethylen (TPEN), doxorubicin, paclitaxel and all other reagents were analytic grade and purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

MCF-7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in a humidified environment of 5% CO2 at 37°C, and propagated once a week.

Generation of MCF-7-GE-1-GFP, MCF-7-Dcp-1b-GFP, and MCF-7-RCK/P54-RFP cell lines

The expression plasmids for Dcp-1b-GFP and RCK/P54-RFP were kindly provided by Dr. Nahum Sonenberg (McGill Cancer Center, McGill University) and the GE-1-GFP construct was from Dr. Donald Bloch (Harvard Medical School). The plasmids (2 µg) of the GE-1-GFP, RCK/P54-RFP, or Dcp-1b-GFP were transfected into MCF-7 cells using Fugene HD transfection reagent (Roche, Indianapolis, IN). Cells were plated in 100 mm dishes at 2×10⁶ per dish 24 hours prior to transfection. The manufacturer’s protocol was exactly followed for the transfection. Cells were lifted 72 hours after transfection, and GFP- or RFP-expressing cells were sorted by flow cytometry (excitation 490nm, emission 525nm for GFP, and 580nm and 650nm for RFP, FACScan Analytic Flow Cytometer (BD-Bioscience, San Jose, CA)). The GFP- or RFP-expressing cells were further enriched by flow cytometry three weeks after the first sorting. GFP and RFP expression were confirmed by a confocal microscope.

Confocal microscopy

MCF-7-GE-1-GFP and MCF-7-Dcp-1b cells (1.5 × 10⁵) were seeded in a 6-well plate and incubated overnight. Cells were then washed three times with HBSS (Hanks balanced salt solution) and kept in 2 ml HBSS buffer. Clioquinol (10 µM) was added in combination with CuCl2 (10 µM) or ZnCl2 (50 µM); or cells were treated with 1 µM of doxorubicin or paclitaxel for indicated times. Cells were viewed and monitored over a 5-60 min period of time under a Leica TCS SP2 confocal microscope with LCS Lite imaging software (0.9 × 63 objective, with excitation at 490 nm and emission at 527 nm for GFP, and 580 nm and 650 nm for RFP respectively). Images were captured at indicated time points.

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**MicroRNA isolation**

MCF-7 cells (4×10^6) were seeded in 100 mm dishes that allowed cells to reach 80% confluence overnight. Cells were then treated with chloquinol (10 µM) plus ZnCl₂ (50 µM) for 4 hours. Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. RNA concentrations were determined by NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). MicroRNA was enriched from the total RNA using a microRNA isolation kit (SABiosciences, Frederick, MD). Total RNA (40 µg) was diluted to the final volume of 400 µl with the buffer provided, then 215 µl of 100% ethanol was added to each sample and mixed. The sample was loaded into a spin column and centrifuged for 30 seconds, after which 750 µl of 100% ethanol was added to the elute, mixed, and added to a second set of spin columns. The spin column was washed with 70% ethanol and the microRNAs were eluted with RNase-free H₂O. The concentrations of the microRNAs were determined by NanoDrop ND-1000.

**MicroRNA array**

For the microRNA array, 100 ng of the purified microRNA was reverse transcribed into cDNA using a kit from SABiosciences (Frederick, MD). microRNA was mixed with the RT-primer and the reverse transcriptase mix in a 10 µl reaction volume. The samples were mixed and incubated at 37°C for 2 hours. The reaction was stopped by incubating the samples at 95°C for 5 minutes. The samples were chilled on ice for 1 minute prior to addition of 90 µl of RNAse-free H₂O. The final 100 µl cDNA was subjected to microRNA real-time PCR array using a 96-well format (MAH-100A, SABiosciences, Frederick, MD) following the manufacturer’s instructions. In short, the PCR cocktail was prepared by mixing the SYBR Green PCR master mix and the 100 µl cDNA with an appropriate amount of H₂O. Then, 25 µl of the cocktail was loaded to each well of the 96-well plate. The assay was initiated by activation of the HotStart DNA polymerase at 95°C for 10 minutes using the ABPrism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The thermal cycling was as follows: denaturing at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 30 seconds, total 40 cycles. The microRNA array detects 88 microRNA species simultaneously. Fold changes (2^ΔΔCt) of the treated versus untreated control samples were calculated by normalizing to a housekeeping gene SNORD 48, as instructed by the manufacturer’s protocol. The following formula was used: \( \Delta\Delta C_t = \Delta C_t \text{ (sample) } - \Delta C_t \text{ (control) } \), where \( \Delta C_t = C_t \text{ (target miRNA) } - C_t \text{ (house-keeping miRNA) } \).

**Western blot**

Western blot was performed as we previously described [18, 21]. Briefly, cells were lysed with the lysis buffer, sonicated on ice, and insoluble materials were removed by centrifugation at 15,000 × g for 15 minutes. Cell lysate (30 µg) from each sample was loaded onto a 10% SDS PAGE gel, transferred to a PVDF membrane, and blotted with antibodies against human Dicer, Ago2, Drosha, eIF4E, and GAPDH.

**Real-time PCR**

Total RNA was isolated from MCF-7 cells using TRizol reagent as we previously described [22]. The first strand synthesis was achieved using the SuperScript II kit following the manufacturer’s protocol (Invitrogen, Carlsbad, CA). The cDNA was PCR-amplified to detect mRNA expression of GE-1, Dcp-1b, Ago2, eIF4E, and GAPDH. The ABPrism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for real time PCR amplification (45 cycles of 95°C for 15 seconds and 50°C for 60 seconds) and the SYBR-Green qPCR Master Mix was applied for detection. Primers used were as follows: Ago2 forward, 5'-GTC TGT ATT GAT TAT TG CAG GAC G-3'; Ago2 reverse, 5'-CAG AAT TCA CGG ACG TAT GG-3'; Dcp-1b forward, 5'-GAA CTG GAG CAG GAA TTT CC-3'; Dcp-1b reverse, 5'-CAT AGA TGG CAG AGG AAAC TG-3'; eIF4E forward, 5'-CAC GCA AGA TTT AGC CCT G-3'; eIF4E reverse, 5'-GTG GTTC ATT CGT TGC CAT TTC-3'; GE-1 forward, 5'-CAC AGC TGA ACC CTG ATG TG-3'; GE-1 reverse, 5'-TGA AGG CGT CAG GTG TCA TC-3'; GAPDH forward, 5'-TGG GGA AGG TGA AGG TCG G-3'; GAPDH reverse, 5'-GGG ATC TCG CTC CTG GAA G-3'. GAPDH was used as an internal control. Amplification was quantified using the formula described above.

**Cell viability assay**

The MTS reagent (CellTiter 96® Aqueous ONE Solution, Promega, Madison WI) was used to assess cellular viability. MCF-7 cells were plated in each well of a 96-well tissue culture plate with 100 µl of medium. Twenty-four hours after plating, the medium was replaced with 100 µl of fresh medium containing paclitaxel at indicated concentrations, and the cells were grown for three days. The MTS solution (20 µl) was added to each well, and cells were incubated at 37°C for 1 hour. The optical density (490 nm) of each well was then recorded. Data are presented as a percentage of the values obtained from cells cultured under the same conditions in the absence of paclitaxel.

**Results**

**p-body formation in MCF-7 cells**

Several mRNA de-capping enzymes are known components of p-bodies and are involved in mRNA turnover [23]. We therefore examined the protein expression of these enzymes, including that of Dcp-1b, GE-1, and RCK/P54, in MCF-7 cells. Constructs expressing GFP-tagged Dcp-1b and GE-1, and RFP-tagged RCK/P54 were stably expressed in MCF-7 cells. Typical cytoplasmic discrete granules representing p-bodies [17] were observed using confocal microscopy in cells expressing GFP- or RFP-tagged proteins, demonstrating the establishment of the model system to study p-body formation. When we transfected RFP-RCK/P54 constructs into the GFP-GE-1 expressing cell line,
expression of the two tagged proteins was only partially overlapped (Fig. 1), indicating that p-bodies are likely heterogeneous in mammalian cells, consistent with previous observations [24, 25].

*Clioquinol plus ZnCl\textsubscript{2} but not CuCl\textsubscript{2} enhances p-body assembly*

We previously reported that clioquinol acts as a zinc ionophore in Raji [18], DU-145 [19], and MCF-7 cells...
Treatment with zinc alone did not increase intracellular zinc levels in MCF-7 cells [26]. The combination of clioquinol and ZnCl₂ was then used throughout the study to achieve enhanced intracellular zinc levels. Previous studies have identified p-bodies as cytoplasmic RNA granules where mRNA degradation takes place [27]. We thus investigated p-body formation as an indication of posttranscriptional alterations. When the cells over-expressing GFP-GE-1 were treated with clioquinol plus zinc for 15-30 min, p-body formation was significantly enhanced (Fig. 2). This enhancement seems to be zinc specific, as clioquinol plus copper did not enhance the p-body assembly, and TPEN, a well-established zinc chelator [28], reversed the enhancement.

The enhanced p-body formation is also not likely a general stress response from the cells, as doxorubicin and paclitaxel, two classical chemotherapeutics, had no effect on p-body assembly in this model system (Fig. 3a), even when used at a highly cytotoxic concentration ([21], Fig.

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**Table 1.** Top six microRNA species whose expression was down-regulated by clioquinol plus zinc (10 μM clioquinol plus 50 μM ZnCl₂ for 4 hours).

| microRNAs     | Fold of reduction |
|---------------|------------------|
| miR-223       | 31               |
| miR-29a       | 17               |
| miR-29b       | 19               |
| miR-130a      | 17               |
| miR-13a       | 17               |
| miR-19b       | 16               |

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Fig. 6. Effects of clioquinol plus zinc on the expression of Drosha, Dicer, Ago2, and eIF4E in MCF-7 cells. A. MCF-7 cells were treated with different concentrations of clioquinol plus 50 µM ZnCl₂ for 4 hours. Cell lysate was prepared and Western blot performed using antibodies against Drosha, Dicer, Ago2, eIF4E, and GAPDH. Shown are representative images of three experiments. B. MCF-7 cells were treated with 10 µM clioquinol plus 50 µM ZnCl₂ for indicated times. Total RNA was isolated, reverse transcribed, and analyzed using real-time PCR for the expression of Ago2, eIF4E, and GAPDH. Data from duplicate samples of three experiments are expressed as fold of the levels detected in untreated control cells. (bar, SE). * p<0.05, compared with untreated control cells using one-way ANOVA followed by Dunnett’s analysis.

3b). Considering the heterogeneous nature of the p-bodies in MCF-7 cells, we also examined the effects of clioquinol plus zinc on GFP-Dcp-1b expression in MCF-7 cells. Again clioquinol plus zinc enhanced p-body assembly in a time-dependent manner, as shown in Fig. 4a. Five minutes of the treatment led to an enhanced p-body formation, which lasted for at least 60 min (Fig. 4a). Only 5 minutes are required for enhancing p-body formation by clioquinol plus zinc, implying that it is the p-body assembly process that is augmented. This was supported by the results showing that mRNA levels of GE-1 and Dcp-1 were not enhanced by the treatment (Fig. 4b). In fact, Dcp-1b mRNA expression was reduced by the treatment.

**Clioquinol plus zinc globally down-regulates microRNA expression**

p-bodies are known as the cellular compartments where microRNA-induced mRNA degradation takes place [17]. To understand more about zinc’s effects on posttranscriptional regulation, we further examined the effect of clioquinol plus zinc on microRNA expression levels. MCF-7 cells were treated with clioquinol (10 µM) and ZnCl₂ (50 µM) for 4 hours and cellular microRNAs were isolated, reverse transcribed, and profiled using a real-time PCR-based microRNA array. Clioquinol plus zinc globally down-regulated microRNA expression levels, ranging from - 3.1 to - 31.0-fold (Fig. 5). Table 1 shows the top six microRNA species that are down-regulated by clioquinol plus zinc.

**Clioquinol plus zinc alters expression of microRNA processing enzymes and p-body components**

The maturation and stability of microRNA is regulated by enzymes and proteins, such as Drosha, Dicer [29], and Ago2 [15, 30, 31]. To understand whether clioquinol plus zinc targets microRNA processing and stability, we examined the expression of Drosha, Dicer, Ago2, and eIF4E in MCF-7 cells that had been treated with clioquinol plus zinc. Western blot analysis indicated that protein levels of Dicer were significantly decreased in response to treatment with clioquinol plus zinc, while Drosha levels were only slightly down-regulated. Expression of Ago2, the core microRNA binding protein that protects microRNA from degradation [31], was also suppressed by clioquinol plus zinc at the protein level. In contrast, expression of eIF4E, a translation initiation factor usually found in p-bodies [32], was significantly elevated by clioquinol plus zinc (Fig. 6a). Real-time PCR analysis indicated that clioquinol plus zinc suppresses mRNA expression of Ago2 and enhances mRNA expression of eIF4E, observations consistent with the analysis of respective protein levels (Fig. 6b).

**Discussion**

While a cytotoxic level of zinc has been shown to regulate gene transcription [33], its involvement in posttranscriptional regulation of gene expression remains
to be elucidated. We demonstrated in the present study that intracellular zinc at cytotoxic concentrations modulates posttranscriptional events of gene expression in mammalian cells, which includes enhanced p-body formation, global microRNA down-regulation, and alterations of posttranscriptional regulatory protein expression.

The importance of posttranscriptional regulation of gene expression has been increasingly recognized [14, 34, 35]. In recent years, microRNAs have emerged as key posttranscriptional regulators controlling gene expression [36]. Because microRNA regulation of target mRNAs or proteins is associated with the p-body [17, 37], we first examined the effect of clioquinol plus zinc on the assembly of p-bodies in MCF-7 cells. The enhancement of p-body formation was detected in both GFP-Dcp-1b and GFP-GE-1 cells, demonstrating that high intracellular ionophoric zinc can lead to alterations of posttranscriptional regulation of gene expression. We further characterized the nature of the enhanced p-body formation in this model system. First, clioquinol plus copper did not alter p-body assembly, suggesting that the enhanced p-body formation is zinc specific. This was further supported by the observation that addition of TPEN, a well-established zinc chelator [28], reversed the effect of clioquinol plus zinc on p-body formation. Second, the enhanced p-body formation by clioquinol and zinc is not likely a stress response, as paclitaxel and doxorubicin, used at highly cytotoxic concentrations, did not cause changes of p-body formation in the same model system. Third, it is unlikely that the enhanced p-body assembly is due to an augmentation of the expression of the proteins examined, as p-body formation is increased as soon as 5 minutes after addition of clioquinol and zinc. This conclusion was further strengthened by the observation that mRNA expression of Dcp-1b and GE-1 was not enhanced in cells that had been treated with clioquinol plus zinc.

It is logical to predict that alterations of p-body formation are associated with microRNA levels, as microRNA functions in concert with p-bodies to degrade target transcripts [17]. It has been reported that when mRNA decay is inhibited, p-body formation is enhanced in a model system [37]. Our microRNA array data showed that clioquinol plus zinc globally suppresses microRNA levels in MCF-7 cells, indicating that a high level of intracellular ionophoric zinc negatively influences microRNA expression, which could lead to inhibition of mRNA degradation and therefore the enhancement of p-body formation. This is the first direct evidence showing that clioquinol plus zinc globally suppresses microRNA expression in mammalian cells.

In addition to transcriptional regulation, microRNA expression is also tightly regulated through its biogenesis process [31, 38], in which Drosha and Dicer play critical roles in microRNA maturation [29], while Ago2, the key microRNA binding protein, keeps microRNAs stable in cells [15, 30, 31]. Down-regulation or over-expression of Ago2 results in diminished or elevated mature microRNA levels, respectively [31]. Clioquinol plus zinc significantly decreased the expression of Dicer and Ago2, indicating that an increase in the intracellular zinc level alters both microRNA maturation and stability in mammalian cells. Interestingly, in contrast to Dicer and Ago2, expression of the translation initiation factor eIF4E is significantly enhanced at both mRNA and protein levels by clioquinol plus zinc. The enhanced expression of eIF4E indicates that a high level of ionophoric zinc alters posttranscriptional regulation of gene expression, as eIF4E functions in translation initiation [39] and is an established component of p-bodies [32]. The mechanism(s) of zinc-induced expression of eIF4E merits further investigation.

In conclusion, the present study demonstrates for the first time that cytotoxic intracellular zinc enhances p-body assembly, globally down-regulates microRNA expression, and alters the expression level of posttranscriptional regulatory proteins in our model system. These findings provide new insight into our understanding of ionophoric zinc cytotoxicity in cancer cells.

**Abbreviations**

3'-UTR (3'-untranslated regions); Ago2 (argonaute2); eIF4E (eukaryotic translation initiation factor 4E); GAPDH (glyceraldehyde 3-phosphate dehydrogenase); p-body (RNA processing body); RISC (RNA-induced silencing complex); TPEN (N, N, N', N', tetrakis (2-pyridylmethyl) ethylenediaminepentaethylene).

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