Abstract. Clioquinol, a metal-binding compound, has been shown to have anticancer activity in in vitro and in vivo model systems. This study investigated the effects of clioquinol on cyclin D1 gene expression in breast cancer cells. Treatment with clioquinol significantly reduced cyclin D1 protein levels in a concentration-dependent manner, effects being more pronounced in the presence of zinc. Clioquinol reduced cyclin D1 mRNA contents in cells that had been pre-treated with actinomycin D, indicating that this compound alters cyclin D1 mRNA stability, an event associated with post-transcriptional regulation. Using a cyclin D1 3'-UTR reporter construct (CCND1-3'-UTR), we confirmed that this 3'-UTR mediates the inhibitory action of clioquinol, likely through miR-302C. This study demonstrates for the first time that clioquinol targets post-transcriptional steps of cyclin D1 gene expression in cancer cells, adding new insight into our understanding of its mechanisms of anticancer action.

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline), an 8-hydroxyquinoline derivative, has been found to have anticancer activity in both in vitro and in vivo model systems (1). A phase I clinical trial is being conducted to test the potential of clioquinol as a cancer therapeutic agent (http://www.cancer.gov/search/ResultsClinicalTrials.aspx?protocolsearchid=8353329). The mechanisms of the anticancer action of clioquinol have been investigated by several studies (1-4). We demonstrated that clioquinol acts as a zinc ionophore, transporting zinc into cells and down-regulating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling (1). Others also reported that clioquinol inhibits proteasome activity in breast and prostate cancer, as well as in leukemia cells (4-6), an event known to result in the inhibition of the NF-κB signaling pathway (7). Clioquinol itself attenuates NF-κB activity in a dose-dependent manner and the addition of zinc dramatically enhances this attenuation (1). We therefore predicted that genes whose transcription is controlled by the NF-κB pathway are likely targeted by clioquinol. Among the NF-κB downstream target genes, cyclin D1 is of great interest because of its involvement in cell cycle regulation and its frequent overexpression in cancer cells (8). Indeed, cyclin D1 is an established therapeutic target for cancer treatment (9, 10).

It is important to note that gene expression in mammalian cells is controlled at both transcriptional and post-transcriptional levels. Transcriptional regulation of gene expression requires transcription factors that work in concert to control gene transcription process (11). Post-transcriptional regulation involves RNA-binding proteins (12), splicing factors (13), and microRNA targeting of the 3'-UTR of the transcripts (14), leading to finely tuned control of gene expression patterns. Relative to other steps of gene expression regulation, microRNA regulation is a newly defined cellular mechanism that negatively influences gene expression. It is well-established that these small non-coding RNAs, approximately 22 nucleotides long, negatively regulate target gene expression by acting on the 3'-UTR of the transcripts that promote mRNA degradation or repress protein translation (15, 16).

Both transcriptional and post-transcriptional regulation of cyclin D1 gene expression have been well-documented (17, 18), suggesting that interference with these steps of regulation could lead to a down-regulation of cyclin D1 levels and suppression of tumor progression. The present study investigated the effects of clioquinol and zinc on cyclin D1 gene expression in a breast cancer model system.

Materials and Methods

Materials. LB Broth powder, LB agar MILLER and SOB powder were purchased from EMD Chemicals Inc. (Gibbstown, NJ). DH5α competent cells were purchased from Invitrogen (Carlsbad, CA, USA). The CCND1 promoter reporter construct was a kind gift from Dr. Richard G. Pestell (Kimmel Cancer Center, Thomas...
Jefferson University). The pcDNA3.1LacZ-CCND1-3′-UTR vector was kindly provided by Dr. Katherine L.B. Borden (University of Montreal, Quebec, Canada). Clioquinol, CuCl₂, ZnCl₂, actinomycin D, ampicillin, and all other reagents were analytic grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. MCF-7 cells were purchased from American Type Culture Collection in 2009 (ATCC, Manassas, VA, USA). Cells were routinely cultured in ATCC-defined 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.

microRNA isolation. MCF-7 cells (4.5×10⁶) were seeded in 100 mm dishes and reached 80% confluency overnight. Cells were then treated with clioquinol (20 μM) and ZnCl₂ (50 μM) alone or in combination for 4 h. Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, cells were lysed with 1 ml TRizol and incubated for 5 min at room temperature, before addition of 200 μl chloroform. The lysate was vortexed and kept at room temperature for another 3 min. RNA separation was achieved by centrifugation at 12,000 × g for 15 min. The top clear phase was collected and precipitated by 800 μl isopropanol alcohol for 10 minutes at room temperature, before addition of 200 μl 75% ethanol, air dried, and dissolved in RNase-free H₂O. The RNA was pelleted, washed with 75% ethanol, air dried, and dissolved in RNase-free H₂O. RNA concentrations were determined using the Nano-Drop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). microRNA was enriched from the total RNA using a microRNA isolation kit (SABiosciences, Frederick, MD, USA). Total RNA (40 μg) was diluted to a final volume of 400 μl with the buffer provided, and 215 μl of 100% ethanol was then added. After mixing, the sample was loaded onto the spin column and centrifuged for 30 s. The elute contained microRNA, to which 750 μl of 100% ethanol was added, mixed well, and added to the second set of spin columns. The spin column was washed with 70% ethanol and the microRNA was eluted with RNase-free H₂O. The concentrations and quality of the microRNA were determined by Nano-Drop ND-1000.

microRNA array. 100 ng of the purified microRNA was reverse transcribed to cDNA using a kit from SABiosciences. In brief, microRNA was mixed with the RT primer and the reverse transcriptase mix in a 10 μl reaction volume. The samples were mixed well and incubated at 37°C for 2 h. The reaction was stopped by incubating the samples at 95°C for 5 min. The samples were chilled on ice for 1 min and 90 μl of RNase-free H₂O was added. The final 100 μl cDNA was subjected to microRNA real-time PCR array using a 96-well format (MAH-100A; SABiosciences) following the manufacturer’s instructions. First, 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 min using the ABPrism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermal cycling was set as the following: denaturing at 95°C for 15 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec, total 40 cycles. The microRNA array detects 88 microRNAs simultaneously. Fold changes (2^(-ΔΔCt)) of the treated versus untreated control samples were calculated by normalizing to protein contents in each sample.
**Cyclin D1 mRNA detection.** Cyclin D1 mRNA was analyzed with reverse transcription real-time PCR assay using the ABPrism 7700 Sequence Detection System (Applied Biosystems). The primers used for cyclin D1 mRNA amplification: forward, 5'-gatgccaacctcctcaac-3' (264-281, NM_053056); reverse, 5'-cgggtcacacttgatcac-3' (932-915, NM_053056). The primers for GAPDH were: forward, 5'-tggggaaggtgaaggtcgg-3'; reverse, 5'-gggatctcgctcctggaag-3'. Total RNA was isolated from MCF-7 cells using TRIzol reagent, reverse transcribed with SuperScript II Reverse Transcriptase, and subjected to real time PCR amplification using the SYBR Green PCR master mix. The samples were denatured at 95°C for 10 min. The thermal cycling was as follows: 95°C for 15 sec, and 50°C for 60 sec, for a total of 45 cycles. The cyclin D1 mRNA level was quantified following the manufacturer’s instructions and normalized by the GAPDH mRNA level in each sample. Data were expressed as percentages of the level in control cells.

**Western blot.** Western blots were performed as previously described (1, 22). In short, cells were lysed with the lysis buffer, sonicated on ice, and centrifuged at 15,000 × g for 15 minutes to remove insoluble material. Subsequently, 20 μg cell lysate from each sample was separated on a 10% SDS PAGE gel, transferred to a PVDF membrane, and blotted with antibodies against human cyclin D1, SPI (Cell signaling, Danvers, MA, USA), and β-Actin (Sigma, St. Louis, MO, USA).

**Statistical analysis.** All statistical analysis was performed with GraphPad Prism software version 4 for windows (GraphPad Software, San Diego, CA, USA). Differences among groups were assessed by one-way ANOVA followed by Dunnett’s post-test, with p<0.05 as the level of statistical significance.

**Results**

Clioquinol suppresses cyclin D1 gene expression in breast cancer cells. We recently demonstrated that clioquinol acts as a zinc ionophore and inhibits the NF-κB signaling pathway in cancer cells (1, 3). Since cyclin D1 is one of the major downstream targets of NF-κB (8), we examined the effects of clioquinol on cyclin D1 expression in MCF-7 cells. Cells were treated with increasing concentrations of
Clioquinol (1, 10, 30 μM) in the presence or absence of ZnCl₂ (50 μM) for 4 h and cyclin D1 protein levels were measured by Western blot analysis. Clioquinol alone suppressed cyclin D1 protein expression in a concentration-dependent manner. The addition of zinc significantly enhanced the inhibitory effects of clioquinol (Figure 1A), consistent with our previous reports (1, 23). To understand whether clioquinol inhibits NF-κB signaling in this cell line, we analyzed nuclear expression of p65, the most frequently detected NF-κB subunit (7, 24), after clioquinol treatment of MCF-7 cells. As shown in Figure 1B, clioquinol reduced nuclear p65 levels, an effect which is also more pronounced in the presence of zinc. The results are similar to our previous observations in other cell model systems (1, 3). Clioquinol plus zinc did not alter nuclear SP1 expression levels. These data indicate that clioquinol specifically inhibits NF-κB signaling and suppresses cyclin D1 expression in MCF-7 cells.
Clioquinol suppresses the cyclin D1 gene promoter and the cyclin D1 3′-UTR-mediated reporter gene activity. To understand whether transcriptional suppression of the cyclin D1 gene is solely responsible for the inhibitory effect of clioquinol, we tested its effects on cyclin D1 gene promoter activity using a well-established reporter construct (18), and cyclin D1 3′-UTR-mediated reporter activity, using the CCND1-3′-UTR constructs that we established over the course of this study. As predicted, clioquinol plus zinc, but not copper, suppressed cyclin D1 promoter activity in MCF-7 cells (Figure 2A), suggesting that this compound targets transcription of the cyclin D1 gene. The inhibitory effect of clioquinol plus zinc on the promoter activity was time- and concentration-dependent (Figure 2B). The CCND1-3′-UTR was cloned into the PGL3-promoter reporter construct in both sense (CCND1-3′-UTR-S) and antisense (CCND1-3′-UTR-A) orientations. The antisense 3′-UTR construct served as a control. The CCND1-3′-UTR enhanced the reporter gene activity, suggesting that this 3′-UTR positively influences cyclin D1 gene expression (Figure 3A). The 3′-UTR of the cyclin D1 transcript has been known to mediate cyclin D1 gene expression through post-transcriptional regulation involving several microRNA species (25-28). Interestingly, the CCND1-3′-UTR-S-mediated reporter activity in MCF-7 cells was also suppressed by clioquinol plus zinc but not copper (Figure 3B), in a time- and concentration-dependent manner (Figure 4), suggesting that post-transcriptional regulation of the cyclin D1 gene was targeted by clioquinol. To be certain that clioquinol affects post-transcriptional steps of cyclin D1 gene expression, we treated MCF-7 cells with actinomycin D (10 μg/ml), a transcription inhibitor (29, 30), in the presence and absence of clioquinol plus zinc. As shown in Figure 5, clioquinol plus zinc enhanced degradation of cyclin D1 mRNA, indicating post-transcriptional alterations of the cyclin D1 transcript by clioquinol. Thus, both transcriptional and post-transcriptional steps are targeted by clioquinol in our model system. As the mechanisms of zinc ionophore-induced inhibition of NF-κB signaling (clioquinol is a zinc ionophore) have been well-established (31, 32) and cyclin D1 gene transcription is well-known to be controlled by the NF-κB pathway (8), we then focused our effort on the effects of clioquinol on post-transcriptional regulation of the cyclin D1 gene.

![Figure 4](image4.png)  
Figure 4. Clioquinol plus zinc suppresses cyclin D1 3′-UTR-mediated reporter gene activity in a time- and concentration-dependent manner in MCF-7 cells. MCF-7 cells were transfected with the CCND1-3′-UTR-S and treated with CQ (10 μM) with or without ZnCl2 (50 μM) for various time periods (A) or treated with CQ with increasing concentrations of ZnCl2 for 4 h (B). Cells were lysed and luciferase activity was analyzed. Data (mean±SEM, n=3) are expressed as percentages of the luciferase activity detected in untreated control cells. *p<0.05 compared to untreated control.

![Figure 5](image5.png)  
Figure 5. Clioquinol plus zinc promotes cyclin D1 mRNA degradation in MCF-7 cells. Cells were treated with 10 μM actinomycin D (AcmD) for various times in the presence or absence of CQ (10 μM) plus ZnCl2 (50 μM). Total RNA was isolated, reverse transcribed, and amplified with real-time PCR. The cyclin D1 mRNA expression was normalized to that of GAPDH. Data (mean±SEM, from two experiments with triplicates each) are expressed as percentages of the mRNA level detected in control cells.
Clioquinol alters microRNA expression patterns in MCF-7 cells. Several microRNA species have been reported to regulate cyclin D1 gene expression by targeting its 3'-UTR, including miR-302C (15), miR-16, miR-17 (25), miR-20 (33), miR-19a (26), and let-7b (27) (Figure 6A). The potential involvement of these microRNAs in clioquinol-induced suppression of cyclin D1 expression was examined using microRNA array analysis. While expression of most of these microRNAs was suppressed by clioquinol plus zinc, miR-302C was up-regulated by 20-fold in this model system (Figure 6B). The up-regulation of miR-302C likely in part accounts for the suppression of cyclin D1 gene expression, as microRNAs often negatively regulate gene expression (14, 34). To confirm this assumption, we deleted the binding sites for miR-16 and miR-302C from the CCND1-3'-UTR-S reporter construct (Figure 7A). The deletion constructs along with the wild-type construct were transfected into MCF-7 cells and effects of clioquinol plus zinc on the reporter activity were examined. Deletion of the binding sites did not significantly alter the basal reporter gene activity (Figure 7B). While deletion of the miR-16 binding site had no effect on the compound-induced suppression of the luciferase activity, deletion of the miR302C binding site significantly attenuated the suppression (Figure 7C), strongly suggesting that miR-302C mediates the inhibitory effects of clioquinol on cyclin D1 gene expression.

Figure 6. Clioquinol alters microRNA expression pattern in MCF-7 cells. A: Schematic illustration of reported microRNA species that are involved in targeting cyclin D1 3'-UTR. B: MCF-7 cells were treated with CQ (20 μM) and ZnCl2 (50 μM) alone or in combination for 4 h. microRNAs were extracted and analyzed using the real-time PCR-based array. Data (mean±SD, from two individual arrays) are expressed as fold change relative to untreated control.
Discussion

Metal-binding compounds are increasingly believed to be an important group of anticancer agents. It is becoming apparent that individual metal-binding compounds kill cancer cells through different mechanisms of action (23, 35-37). We have previously reported that clioquinol inhibits NF-κB signaling and induces apoptosis of human cancer cells (1). As clioquinol is being tested in a clinical trial for the treatment of malignant diseases, a better understanding of its mechanisms of action in cancer cells will help further development of this compound into clinical practice. The novel finding from the present study is that clioquinol targets both transcriptional and post-transcriptional regulation of the cyclin D1 gene in human cancer cells. These effects are more pronounced when zinc is present, further confirming that clioquinol is a zinc ionophore. While suppression of gene transcription by clioquinol and other metal ionophores has been well established (1, 3, 31, 32), this is the first report on its intervention with post-transcriptional regulation of gene expression.

We started our experiments by investigating the inhibitory effect of clioquinol on cyclin D1 gene transcription. As we predicted, clioquinol down-regulated cyclin D1 promoter activity, which is most likely attributed to the inhibition of NF-κB signaling. The novel finding from the present study is that clioquinol targets both transcriptional and post-transcriptional regulation of the cyclin D1 gene in human cancer cells. These effects are more pronounced when zinc is present, further confirming that clioquinol is a zinc ionophore. While suppression of gene transcription by clioquinol and other metal ionophores has been well established (1, 3, 31, 32), this is the first report on its intervention with post-transcriptional regulation of gene expression.

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κB signaling in this model system. Clioquinol-induced transcriptional inhibition has been reported in different experimental systems (1, 3, 23). The possibility that clioquinol may also affect post-transcriptional regulation of the cyclin D1 gene was then examined. We provided convincing evidence in the present study indicating that clioquinol targets post-transcriptional regulation of the cyclin D1 gene. Using the reporter gene assay technique, we found that clioquinol down-regulated CCND1-3'-UTR-mediated luciferase activity in MCF-7 cells, indicating that this 3'-UTR is targeted by clioquinol. The most common gene expression regulation via targeting of a 3'-UTR involves microRNA binding to the 3'-UTR, leading to degradation of the RNA transcript or inhibition of the protein translation (14, 38). The results from our experiments with actinomycin D, an established transcription inhibitor (29, 30), indicated that clioquinol promotes mRNA degradation of the cyclin D1 gene. This effect of clioquinol on cyclin D1 mRNA degradation seems to be regulated by miR-302C, as clioquinol significantly enhanced miR-302C levels in MCF-7 cells, and deletion of the binding site for miR-302C from the 3'-UTR reversed the inhibition of clioquinol on CCND1-3'-UTR-mediated reporter gene activity.

It has been reported that cyclin D1 gene expression is post-transcriptionally regulated by microRNAs. Yu et al. reported that miR17/20 suppresses breast cancer cell proliferation by negatively regulating cyclin D1 translation, thereby inhibiting S phase entry of the cells (39). miR16 has been demonstrated to directly suppress multiple cell cycle genes, including cyclin D1, and induce G1 arrest (25). The let-7 family of microRNAs is significantly down-regulated in melanomas and over expression of let-7b significantly reduces cyclin D1 expression by targeting the 3'UTR (27). miR19a and miR-302c were also reported to negatively regulate cyclin D1 gene expression (26, 28). Therefore it is not surprising that clioquinol enhances miR-302C levels, which in turn suppress cyclin D1 gene expression in our model system. It is also interesting to see that clioquinol down-regulates expression of several microRNA species in MCF-7 cells. Some of the microRNAs, such as miR-17 and miR-19, are believed to act as oncogenes promoting tumor progression (40). Targeting certain microRNA species has been suggested as a way to kill tumor cells or to overcome tumor resistance to chemotherapy (41, 42). Whereas we do not know why miR-302C was up-regulated while the others were down-regulated by clioquinol in this model system, such a mixed reaction from different microRNA species to anticancer agents in cancer cells has been recently described (40, 43). Taken all together, our observations suggest that targeting microRNAs and the 3'-UTR of gene transcripts is part of the action of clioquinol in cancer cells.

The effects of clioquinol on post-transcriptional regulation of cyclin D1 gene expression imply that metal-binding compounds might affect gene expression at different regulatory levels, and that post-transcriptional regulation of gene expression may be a potential target for chemotherapy, a concept consistent with a recent report (44). These findings provide new insight into our understanding of the anticancer action of clioquinol in human cancer cells. As clioquinol has been reported to influence mammalian cells through several mechanisms (1, 2, 5, 6, 45), this compound seems to have multiple actions that lead to the suppression of tumor progression. The potential interconnections of these actions by clioquinol warrant further investigation and the proportional contributions of each action remain to be determined in cancer cell model systems.

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