Abstract. We have reported that clioquinol alters lysosome integrity, inhibits nuclear factor kappa B (NF-κB) activity, and induces apoptosis in human cancer cells. The present study investigated whether clioquinol targets both pathways dependently or independently in human prostate cancer DU 145 cells. Clioquinol inhibited NF-κB activity, an effect being more pronounced in the presence of zinc. This inhibition was mediated through a reduced nuclear level of p65, the most frequently detected NF-κB subunit. Clioquinol also induced alterations of lysosome permeability in a zinc concentration-dependent manner. Pretreatment of the cells with ammonium, a lysosome protection agent, attenuated clioquinol-induced disruption of the lysosomes, yet ammonium had no effect on clioquinol-induced inhibition of NF-κB signaling. MG132, an established NF-κB inhibitor, suppressed NF-κB activity without causing alterations of lysosome permeability. These findings indicate that clioquinol targets NF-κB and lysosome pathways independently, favoring further development of clioquinol as a novel anticancer agent.

5-Chloro-7-iodo-8-hydroxyquinoline (clioquinol) is a derivative of 8-hydroxyquinoline and was first prepared in Germany in the early part of the last century. Clioquinol is a bio-available metal chelator with different affinities for copper and zinc. Because of its weak binding affinity for metals, it was considered as a good candidate for chelating loosely-bound zinc in vivo (1). Its recent use in clinical trials of Alzheimer’s disease suggests that clioquinol can be safely administered in humans within a defined period of time (2, 3). Since metal-binding compounds are believed to be a novel group of anticancer agents (4), several recent studies investigated the anticancer activity of clioquinol in various model systems. Results from these studies support the conclusion that clioquinol has anticancer activity in cultured tumor cells and in animal models bearing human tumors (5-7). The mechanisms of this anticancer activity have been actively explored but remain incompletely defined. Reports from two research groups have demonstrated that clioquinol complexes with metals and acts as a proteasome inhibitor to induce apoptosis of tumor cells (6, 7). In our recent report, we have shown that clioquinol acts as a metal ionophore which brings metals into cancer cells, leading to apoptotic cell death (5). Furthermore, we demonstrated that clioquinol targets zinc to lysosomes, resulting in disruption of the lysosomal membrane, release of cathepsins, and induction of apoptosis (8).

Both proteasomes and lysosomes have been recognized as cellular targets for cancer therapy (9, 10). One of the consequences of proteasome inhibition is the suppression of NF-κB activity (11). Proteasome inhibitors are known to suppress NF-κB signaling (12), and clioquinol is a proteasome inhibitor (6, 7). To better understand the anticancer action of clioquinol, the present study investigated whether clioquinol targets lysosome and NF-κB pathways independently or dependently in DU 145 cells, a well-established human prostate cell model system.

Materials and Methods

Materials. The MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was purchased from Promega (Madison, WI, USA). Antibodies for p65 and IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); poly (ADP-ribose) polymerase (PARP) antibody was from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was obtained from ProMab Biotechnologies, Inc. (Albany, CA, USA); pNF-κB-Luc reporter construct was from BD Biosciences Clontech (Palo Alto, CA, USA); proteasome inhibitor MG132 was from EMD Biosciences Inc. (San Diego, CA, USA). Chemicals including clioquinol and zinc chloride were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).
Cell lines and cell viability assay. The human prostate cancer line, DU 145, was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultivated in DMEM supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cells were routinely grown under humid environment at 37˚C, 5% CO2, and split twice a week. Cells were grown in 100 mm dishes and were transfected with the NF-κB-Luc reporter construct using the lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), as previously described (5). After 24 hours of transfection, cells were plated into 24-well plates at 200,000/well. At 48 hours of transfection, cells were treated with clioquinol and zinc chloride at indicated concentrations and durations. The luciferase activity was assayed with the luciferase assay reagent (Promega, Madison, WI, USA). In short, the cells were lysed using reporter lysis buffer and the insoluble material was removed by a brief centrifugation. A total of 30 μl of luciferase assay reagent were mixed with 50 μl of protein extract and the luciferase activity was analyzed using a Turner TD/20E luminometer. The relative light units were normalized for the amount of protein in each extract, and the results are reported as percentages of the values obtained from untreated control cells.

DNA transfection and luciferase activity assay. DU 145 cells were grown in 100 mm dishes and were transfected with the NF-κB reporter construct using the lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), as previously described (5). After 24 hours of transfection, cells were lifted and plated into 24-well plates at 5,000 cells per well in 100 μl of medium. This ensured a cell density of 40% to 60% confluence after 24 hours of seeding. The medium was then removed, and fresh medium containing the testing agents was added to the cells. After the cells were grown for designated periods, 20 μl of the MTS medium solution was added to each well, and the incubation lasted for 1 hour at 37˚C to allow color development. The plate was then read at 490 nm and the readings were deducted by blanks. Each experimental group contained quadruplet readings. Data are expressed as percentages of the values obtained from untreated control cells.

Lysosome membrane permeability analysis. Acridine orange (AO) uptake and intracellular distribution were analyzed as described elsewhere (8). This was used to determine the changes in lysosomal membrane permeability (13, 14). In brief, DU 145 cells were plated in a 12-well plate and treated with different agents for 2 hours. AO was then added (final concentration, 2.5 μg/ml) and cells were incubated for another 30 min. Cells were washed twice with HBSS and examined with a fluorescence microscope (Nikon Eclipse TE2000-U). The excitation and emission wavelength of red fluorescence was 555/28 nm and 617/38 nm, respectively. A previous study demonstrated that AO concentrated in lysosomes emits a granular red fluorescence, whereas AO in the cytosol emits a diffuse green fluorescence. Following a change in lysosome permeability, a reduction in red fluorescence and an increased diffuse cytosolic green fluorescence are observed, indicating a relocation of AO from the lysosomes to the cytosol (14).

Western blot analysis. Western blot analysis was performed to analyze the expression level of p65, IκBα, GAPDH and PARP in DU 145 cells that had been treated with clioquinol and zinc chloride. The procedures for Western blot were as we previously described (5, 15). Briefly, cells were harvested at appropriate times, and cell lysates were centrifuged at 15,000 xg to remove insoluble material. A total of 50 μg of protein from each sample was loaded onto a 10% SDS PAGE gel, transferred to a PVDF membrane, and blotted with antibodies against p65, IκBα, GAPDH and PARP.

Statistical analysis. Graphpad Prism software (San Diego, CA, USA) was used for statistical analysis and graph making. One-way ANOVA with Dunnett’s post-test or Student’s t-test were used to determine differences among groups of data, with p<0.05 as the level of statistical significance.

Results

Clioquinol enhances lysosome permeability in a zinc concentration-dependent manner. We have recently reported that clioquinol targets zinc to lysosomes, which may contribute to alterations of lysosome permeability in DU 145 cells (8). To further support the involvement of zinc in this event, we applied different concentrations of zinc to the cells in the presence of a single dose of clioquinol. The lysosomal permeability was examined using AO staining (Figure 1). Significant increases in the diffuse cytosolic green fluorescence were observed in a zinc chloride-concentration dependent manner after clioquinol treatment, indicating that clioquinol-induced changes of lysosome permeability are associated with concentrations of zinc.

Clioquinol inhibits NF-κB activity. To understand whether clioquinol inhibits NF-κB activity in DU 145 cells, cells were transfected with the pNF-κB-Luc reporter construct and treated with 10 μM clioquinol for 4 hours in the presence or absence of 50 μM zinc chloride (Figure 2A). Clioquinol moderately suppressed NF-κB activity, an effect that was significantly enhanced by zinc. Consistent with this observation, clioquinol decreased total nuclear p65 (data not shown) and phosphorylated nuclear p65 level (Figure 2B). p65 is the most frequently detected NF-κB subunit (11, 16). An increased level of IκBα and p65 in the cytoplasm was also evident (Figure 2C), suggesting that clioquinol inhibits the degradation of IκBα, leading to the suppression of NF-κB signaling (16).

Ammonium attenuates clioquinol-induced lysosome disruption but not NF-κB inhibition. Ammonium is an established lysosome protection agent (17). Pretreatment of the cells with 10 mM ammonium for 1 hour significantly attenuated clioquinol/zinc-induced permeability changes of the lysosomes (Figure 3A). However, clioquinol/zinc-induced suppression of NF-κB activity was unchanged in cells pretreated with ammonium (Figure 3B), which strongly indicates that targeting lysosomes and NF-κB signaling by clioquinol are independent events in this model system. Interestingly, ammonium also failed to attenuate clioquinol/zinc-induced cytotoxicity (Figure 4).
Figure 1. Clioquinol enhances lysosome permeability in a zinc concentration-dependent manner. DU 145 cells were treated with 10 μM clioquinol (CQ) and different concentrations of ZnCl₂ for 120 min. The cells were stained with AO (2.5 μg/ml) for 30 min and examined under a fluorescence microscope (×200 magnification). Representative images of three independent experiments are shown.

Figure 2. Clioquinol down-regulates NF-κB signaling. DU 145 cells were transfected with the pNF-κB-Luc reporter construct, and treated with 10 μM clioquinol (CQ) for 4 hours in the presence or absence of 50 μM ZnCl₂. A: Cell lysates were prepared and luciferase activity assayed. Data are expressed as percentages of the values detected in untreated cells (n=3, bar, S.E.); **, p<0.01, *, p<0.05, Compared with control cells, using one-way ANOVA followed by Dunnett analysis. B and C: Cell lysates were prepared, loaded onto 10% SDS PAGE (50 μg per well), and blotted with antibodies against p65, PARP, IκBα, and GAPDH. Representative images of two independent experiments are shown.
MG132 inhibits NF-κB activity but does not induce changes in lysosome permeability. MG132 is a known proteasome inhibitor and has been extensively used in biomedical research (12, 18). To understand whether inhibition of NF-κB signaling and disruption of lysosomes are separate cellular events, DU 145 cells were treated with 10 μM MG132 for 4 hours. As expected, MG132 effectively suppressed NF-κB activity (Figure 5A), as analyzed with the reporter gene assay. However, the same treatment with MG132 did not alter the lysosome permeability, as evidenced with the AO staining. This indicates that inhibition of NF-κB signaling and disruption of lysosome integrity can be separate cellular events; this supports the idea that clioquinol targets both pathways independently.
Discussion

There are two major cellular systems for macromolecule degradation in eukaryotic cells: the proteasome pathway and the lysosome pathway. The proteasome pathway may degrade short-lived macromolecules, while the lysosomes degrade long-lived ones. Normal function of these systems is critical in maintaining cellular metabolism (19). It is well-established that inhibition of proteasome function and disruption of lysosome integrity induce apoptosis of cancer cells. Therefore both systems are considered to be cellular targets for cancer therapy (9, 10). One consequence of proteasome inhibition is the down-regulation of NF-κB activity in cancer cells, which promotes apoptotic cell death (11). On the other hand, disruption of lysosomes often leads to enhanced membrane permeability, the release of cathepsins from the lysosomes, and activation of the apoptotic pathway (10). The most interesting finding from the present study is that clioquinol targets both proteasome and lysosome pathways in chorus in our model system, which may explain the primary mechanisms of its anticancer action.

Simultaneously targeting both lysosome and proteasome pathways has been suggested as a potentially effective way to kill cancer cells (19). Experimental evidence from the present study indicates that clioquinol achieved this goal as a single agent. Clioquinol induced a disruption of lysosome integrity, which was dependent on zinc concentration. Clioquinol also significantly suppressed the NF-κB signaling, which was more pronounced in the presence of zinc. Theoretically, clioquinol could target both pathways sequentially or simultaneously. Our results suggest that inhibition of NF-κB and disruption of lysosomal integrity by clioquinol are independent cellular events. This is mainly supported by the fact that ammonium, an effective lysosome protective agent (17), attenuated clioquinol-induced alterations of lysosome permeability, yet failed to reverse clioquinol-induced inhibition of NF-κB activity. Thus, clioquinol initiated independent cellular events leading to the inhibition of NF-κB and disruption of lysosomal membranes. Furthermore, the proteasome inhibitor MG132 significantly suppressed NF-κB activity, yet it had no effect on lysosome membrane integrity. This indicates that targeting of NF-κB and lysosome pathways could occur independently. It is intriguing that ammonium failed to reverse or attenuate cytotoxicity of clioquinol/zinc in this model system. It is likely that clioquinol plus zinc is a powerful NF-κB inhibitor, and it induces apoptotic cell death in a manner that is independent of other pathways. However, the failure of ammonium in attenuating cytotoxicity of clioquinol may also be explained by the fact that ammonium alone was somewhat toxic to the cells, which could confound the viability analysis.

Combination therapy through targeting multiple signaling pathways has been suggested as a potentially effective treatment for cancer patients. This requires the use of more than one anticancer drug, which often leads to higher drug efficacy but also to more side-effects to the host (20). In this regard, one drug targeting multiple signaling pathways is likely to be superior to combination drug therapy acting through the same signaling pathways for cancer treatment. While both the lysosome and NF-κB pathways are established cellular targets for cancer therapy, no chemotherapeutics have been identified that effectively target both pathways independently or dependently. Thus, clioquinol is the first compound identified to act through both NF-κB and lysosome pathways, thereby exerting its anticancer action. These findings clearly favor the further development of clioquinol as a novel anticancer agent.

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References