

Clioquinol and docosahexaenoic acid act synergistically to kill tumor cells

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Abstract

Clioquinol, an 8-hydroxyquinoline derivative (5-chloro-7-iodo-8-hydroxyquinoline) with antimicrobial properties, has recently been found to have cytotoxic activity towards human cancer cell lines at concentrations achieved by oral administration. This study was initiated to determine whether clioquinol could potentiate the antitumor effects of two drugs, doxorubicin and docosahexaenoic acid (DHA), believed to act in part via the generation of reactant oxidant species. At low micromolar concentrations, clioquinol had little effect upon cell viability and did not potentiate doxorubicin's cytotoxicity. Clioquinol significantly enhanced DHA's cytotoxic effects, an interaction that was shown to be synergistic by isobolographic analysis. Clioquinol exhibited a synergistic interaction with DHA in reducing nuclear factor- κ B activity and inducing apoptosis, and the combination reduced the level of several molecules that promote cell survival, including Akt, p65, and Bcl-2. Interestingly, clioquinol neither induced lipid peroxidation itself nor increased peroxidation brought about by the addition of DHA. However, when cells were pretreated with antioxidant vitamin E, the synergism of clioquinol and DHA was blocked, indicating the essential role of lipid peroxidation for their action. These findings reveal a novel antitumor drug combination that synergistically targets major cell survival signaling pathways. [Mol Cancer Ther 2006; 5(7):1864–72]

Introduction

The chemotherapy of cancer is still hampered by tumor resistance and host toxicity. One approach to overcoming these problems is to use drug combinations. Combinations

of drugs with different modes of action may lead to enhanced antitumor effects without injuring the host. In the ideal case, a pair of drugs has a synergistic antitumor effect without increasing toxicity (1). Synergistic interactions of anticancer drugs have been reported in studies of tumor cell lines (2–4), animal models (5), and cancer patients (6). Such interactions include, but are not limited to, the combination of discodermolide and paclitaxel (2), capsicum and green tea concentrate (4), gemcitabine and docetaxel (6), docosahexaenoic acid (DHA) and paclitaxel (7, 8), and irinotecan and 5-fluorouracil (5).

Clioquinol chelates copper and zinc and functions as an ionophore for these metals (9). It has been used for many years as an antibiotic for the treatment of diarrhea and skin infection. Clioquinol was recently shown to be well tolerated and to induce some beneficial effects in the treatment of Alzheimer's disease in both a mouse model (10) and humans enrolled in clinical trials (11, 12). We have recently found (9) that clioquinol itself has antitumor properties at concentrations achieved in a recent clinical trial (12). Although clioquinol inhibits the activity of purified superoxide dismutase-1, presumably by chelating its essential copper and zinc, its cytotoxic effect did not seem to be due to inhibition of superoxide dismutase-1 (9).

DHA (22:6, n-3) is a polyunsaturated fatty acid with anticancer properties (reviewed in refs. 13, 14). Studies in cell culture and animal models have shown that DHA and eicosapentaenoic acid (20:5, n-3) inhibit tumorigenesis (15, 16) and the growth of rodent tumors (17, 18) and human breast cancer xenografts (19, 20). Importantly, n-3 polyunsaturated fatty acids selectively inhibit tumor cell proliferation and are significantly less toxic towards normal cells (21, 22). DHA increases lipid peroxidation within cells (23), and the anticancer effects of DHA are blocked by the antioxidant vitamin E, indicating that oxidation plays a role in DHA's mechanism of action. We have recently shown that DHA is differentially cytotoxic towards cancer cells, with human lymphoma lines being more sensitive than solid tumor lines, in part because of differential effects of DHA on the level of expression of the antioxidant enzyme superoxide dismutase-1 (24).

Others have shown an enhanced anticancer effect when DHA was given with conventional chemotherapeutic agents. DHA has been shown to enhance the effectiveness of paclitaxel in both experimental models (7) and a human clinical trial (8), although DHA was intended to serve as a tumor-targeting molecule rather than a therapeutic compound. DHA also enhanced the toxicity of other chemotherapeutic agents presumably because it enhanced the oxidative stress in cancer cells (25–27).

We report here that clioquinol exhibits a positive synergistic cytotoxic interaction with DHA but not doxorubicin. Surprisingly, the combination does not further

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enhance lipid peroxidation within cells; yet, lipid peroxidation initiated by DHA is required for their synergistic action. Exploration of this novel interaction shows that the combination results in decreases in nuclear factor- κ B (NF- κ B) signaling as well as in the expression of other cellular survival signaling proteins.

Materials and Methods

Materials

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was from Promega (Madison, WI). Antibodies were obtained from the following sources: caspase 3 from Transduction Laboratories (Lexington, KY); poly(ADP-ribose) polymerase from BIO-MOL Research Laboratories, Inc. (Plymouth Meeting, PA); Bcl-2, Akt, I κ B α , and p65 from Cell Signaling Technology (Beverly, MA). An ELISA-based kit for apoptosis analysis was from Roche Diagnostics Corp. (Indianapolis, IN). Other reagents including clioquinol were analytic grade and obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Cell Viability Assay

The human B-cell lymphoblastoid line Raji, the cervical cancer line SiHa, and the human breast cancer line MDA MB-231 were obtained from the American Type Culture Collection (Manassas, VA). The squamous carcinoma line SCC-38 was provided by Dr. Doris Benbrook (University of Oklahoma), and the ovarian cancer line A2780 was provided by Dr. Stephen Howell (University of California, San Diego). Cells were cultivated in appropriate media under a humid environment at 37°C, 5% CO₂. Cells were plated into 96-well plate at ~3,000 to 15,000 per well in 100 μ L medium, which enabled a 40% to 60% confluence of each individual cell line after 24 hours of plating. Cells were then treated with various compounds at indicated concentrations for 72 hours. Cell viability was assessed with a modified tetrazolium assay using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, following the manufacturer's protocol. In brief, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution was added to each well, and cells were incubated at 37°C for 1 to 2 hours before the absorbance of each well was recorded at 490 nm. Data are presented as a percentage of the values obtained from cells cultured under the same conditions in the absence of any added compounds.

Isobolographic Analysis

Isobolograms were constructed following published protocols (28, 29). In brief, the IC₅₀ values for clioquinol and DHA on cell viability were determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell viability assay. The ratio of these values was used to establish the molar ratio of clioquinol and DHA (1:7) subsequently used. The cells were then treated with this combination at increasing concentrations, and a new concentration-dependent curve was plotted based on 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

assay. The IC₅₀ values for each compound in the presence of the other coadministered drug were calculated. The isobologram was constructed by plotting the experimentally determined IC₅₀ values for clioquinol and DHA, when given alone, on the X- and Y-axes, respectively. The diagonal line connecting these values represents the theoretical additive line of these two compounds. The experimental IC₅₀ values of clioquinol and DHA, when coadministered, were graphed on the isobologram. An observed IC₅₀ value below the additive line indicates a synergistic interaction of these two compounds.

Apoptosis Assays

Western blot analysis of poly(ADP-ribose) polymerase cleavage and quantification of apoptosis by immunochemical detection of histone-DNA fragments using a commercial ELISA assay were done as described previously (9).

Quantification of Lipid Peroxidation

The generation of thiobarbituric acid reactive substances (TBARS) was measured following a published protocol (30) with minor modifications. Cells, grown in 75-cm² flasks, were treated with DHA for 6 or 20 hours. Harvested cells were suspended in 200 μ L PBS and disrupted by sonication. The sonicated material (150 μ L) was mixed with 1.5 μ L of 100 mmol/L butylated hydroxytoluene; equal volumes (75 μ L) of 15% trichloroacetic acid, 0.25 mol/L HCl, 0.25 mmol/L butylated hydroxytoluene, 0.375% 2-thiobarbituric acid, and 30 μ L of 8.5% SDS were added to the samples. The color was developed at 95°C for 60 minutes, after which time the samples were placed on ice for 10 minutes to stop the reaction. After centrifugation for 10 minutes at 1,500 \times g, 200 μ L of the supernatant from each tube were transferred to a 96-well plate. The absorbance at 540 nm was recorded with reference to a reagent blank and the TBARS concentration determined using a standard curve prepared with various concentrations of 1,1,3,3-tetraethoxypropane. The resulting TBARS value was normalized by reference to the protein concentration (determined by Bio-Rad protein assay reagent) of each sample.

DNA Transfection and NF- κ B Activity Assay

A NF- κ B-luciferase plasmid construct (pNF- κ B-Luc, BD Biosciences Clontech, Palo Alto, CA) was transfected into A2780 cells with LipofectAMINE reagent (Invitrogen, Carlsbad, CA) as previously described (9). After 48 hours of transfection, cells were treated with clioquinol and DHA for 4 hours. The cells were then lysed using reporter lysis buffer, and the luciferase activity was assayed using a Turner TD/20E luminometer with 30 μ L of luciferase assay reagent (Promega) mixed with 50 μ L of protein extract. The relative light units were normalized for the amount of protein in each extract, and the results were reported as relative changes in luciferase activity.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation assay was done following the manufacturer's protocol, using a kit from Upstate Biotechnology (Lake Placid, NY). A2780 cells were transfected with the pNF- κ B-Luc and grown in 75-mm flask. Two days after transfection, the cells were treated with

30 $\mu\text{mol/L}$ cloiquinol and 100 $\mu\text{mol/L}$ DHA for 4 hours. Cells were then fixed by adding formaldehyde directly to the medium (1% final concentration) at 37°C for 10 minutes. After washing cells twice with cold PBS, cells were removed from the dish, pelleted, and lysed for 10 minutes on ice in SDS lysis buffer. DNA was sheared by sonication to sizes ranging from 200 to 1,000 bp and precleared for overnight at 4°C with salmon sperm DNA-saturated protein A agarose. Chromatin solutions were precipitated overnight at 4°C using 8 μL of an anti-p65 polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immune complexes were collected with salmon sperm DNA-saturated protein A agarose. Input and immunoprecipitated chromatin were incubated at 65°C for 4 hours to reverse cross-links. After proteinase K digestion, DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA was analyzed by PCR amplification using primers specific to the promoter region of the pNF κ B-Luc construct. Forward primer (3-25), 5-TACCGAGCTCTTACGCGTGCTAG-3; reverse primer (379-357), 5-GCAATTGTTCCAGGAACCAGGGC-3. The thermo cycles included denaturation at 94°C for 1 minute, annealing at 51°C for 2 minutes, and elongation at 72°C for 2 minutes, for a total of 28 cycles. An additional 8-minute elongation step at 72°C was added after the cycling. The PCR products were visualized under UV light following electrophoresis in a 1% agarose gel containing ethidium bromide.

Determination of Changes in Free Cellular Zinc Levels Using the MRE- β Geo Reporter Gene Assay

Baby hamster kidney (BHK) cells carrying the MRE- β Geo reporter gene (line 3038; refs. 31, 32) were plated into 24-

well plates in DMEM supplemented with 10% fetal bovine serum and antibiotics. Cells were treated with ZnCl₂, cloiquinol, DHA, or a combination of cloiquinol and DHA for 18 hours. The β -galactosidase activity of each sample was then determined, using *o*-nitrophenyl- β -galactopyranoside as substrate, as described (31, 32). Cell survival was measured by exposing cells to drugs for 3 days in a 24-well format and then measuring the DNA content of each well.

Statistical Analysis

Differences among groups of data were assessed using one-way ANOVA followed by Dunnett analysis or Bonferroni's multiple comparison test. IC₅₀ was calculated through nonlinear regression using one site competition curve. All statistical analyses were done with the Prism 4 program (GraphPad Software, San Diego, CA).

Results

Synergistic Action of Cloiquinol and DHA

Treatment of the human B-lymphoblastoid line Raji with cloiquinol, DHA, or doxorubicin resulted in dose-dependent cytotoxicity. Addition of a low concentration of cloiquinol (5 $\mu\text{mol/L}$) along with doxorubicin or DHA to cells potentiated the toxic effect of DHA but not doxorubicin (Fig. 1). To determine if the potentiation was additive or synergistic, we analyzed subsequent studies by the isobolographic technique (28, 29). As shown in Fig. 2, cloiquinol interacts in a synergistic manner with DHA. In the presence of DHA, the IC₅₀ of cloiquinol dropped from 14 to 4.6 $\mu\text{mol/L}$, and the IC₅₀ for DHA dropped from 93.6 to 33 $\mu\text{mol/L}$. The synergistic effects of the cloiquinol/DHA combination were also found when other types of

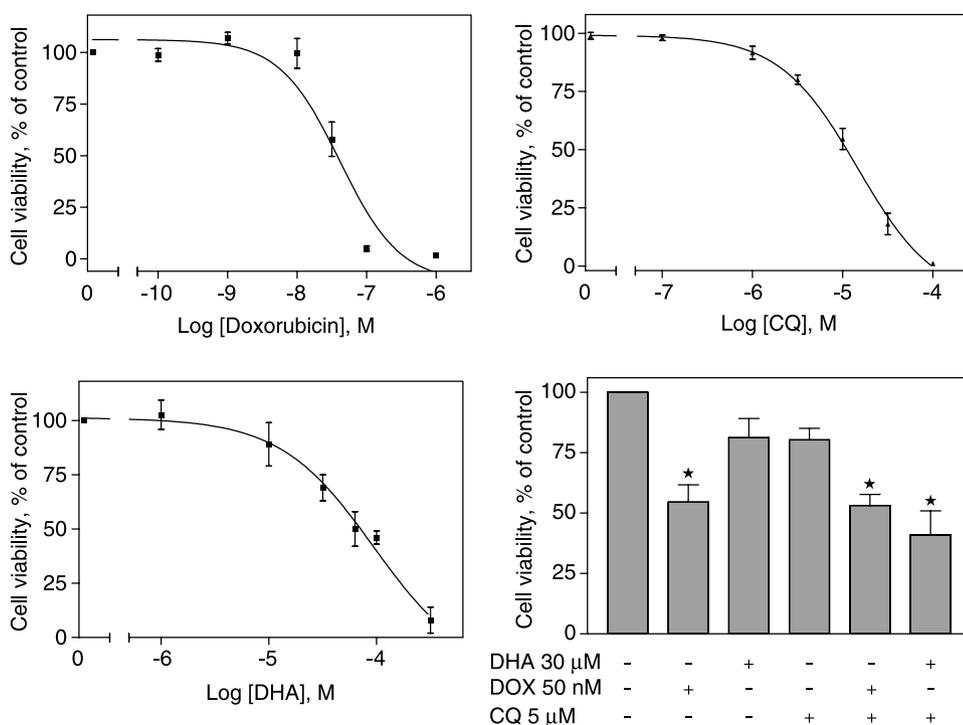


Figure 1. Effects of DHA, cloiquinol, and doxorubicin on the viability of Raji cells. Cells were grown in RPMI 1640 and treated with increasing concentrations of DHA, cloiquinol (CQ), and/or doxorubicin (DOX) for 72 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Points, mean percentage of untreated cells ($n = 3$); bars, SE. IC₅₀ values were calculated by nonlinear regression analysis. *, $P < 0.05$, compared with control cells, using one-way ANOVA followed by Dunnett's analysis.

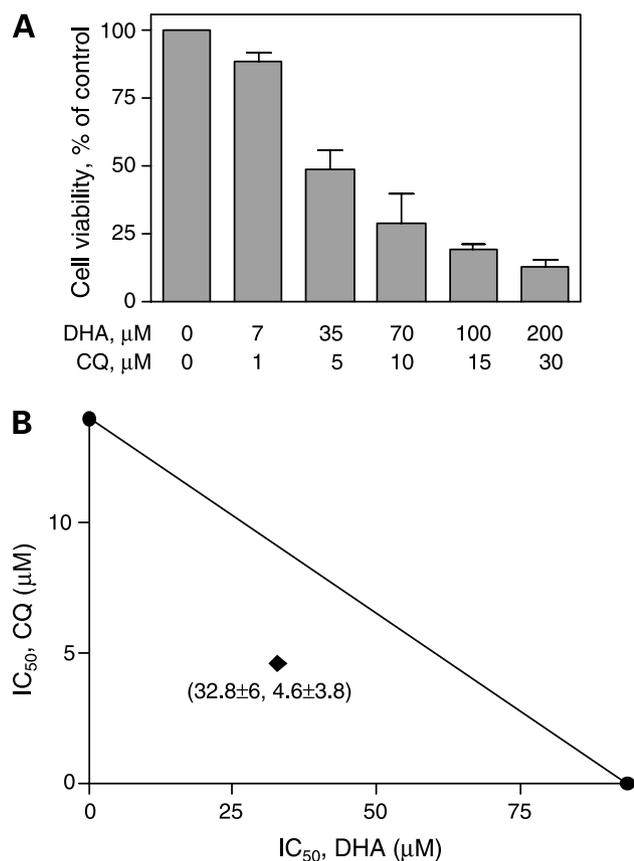


Figure 2. Synergistic effects of DHA and cloiquinol on the inhibition of Raji cells viability. **A**, coadministration of cloiquinol and DHA enhanced their cytotoxicity toward Raji cells. Cells were treated with increasing concentrations of cloiquinol and DHA at a fixed ratio of 1:7, determined by the IC_{50} values of each compound added to cells alone. Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. **B**, isobolographic plot using IC_{50} values obtained from data in **A** and Fig. 1. IC_{50} values of cloiquinol and DHA alone were plotted on the Y- and X-axes, respectively, and the line connecting them represents the theoretical additive line. Experimentally derived values from combination therapy lying beneath the line indicate a synergistic interaction, and those lying above the line indicate an antagonistic interaction. The IC_{50} values of each compound calculated from **A** provide the coordinates $(32.8 \pm 6, 4.6 \pm 3.8)$, indicating a synergy of these two compounds.

human cancer cell lines were studied. Results employing the human cervical cancer line SiHa, which has been shown to be relatively resistant to both compounds (9, 24), and the human breast cancer line MDA-MB-231 are shown in Fig. 3. Comparable effects were also obtained using the human squamous carcinoma line SCC-38 and the human ovarian cell line A2780 (data not shown). Replacement of cloiquinol with *N,N,N',N'*-tetrakis (2-pyridyl-methyl)ethylenediamine (TPEN), a high-affinity, cell-permeable zinc chelator (33), or DHA (22:6) with docosanoic acid (22:0), its saturated counterpart, resulted in a loss of synergistic cytotoxicity (Fig. 4), indicating the specificity of the interaction.

Treatment of Raji cells with either cloiquinol or DHA induced apoptosis, as shown using a specific apoptotic-

ELISA, and this effect was dramatically enhanced when the two compounds were added together (Fig. 5A). Western blot analysis revealed that the combination of drugs caused hydrolysis of procaspase-3 and cleavage of poly(ADP-ribose) polymerase (Fig. 5B). The levels of Akt, p65, and Bcl-2, proteins that mediate cell survival, were also reduced by coadministration of DHA and cloiquinol (Fig. 5B). Interestingly, time course study further revealed that cloiquinol and DHA reduced protein levels of both total Akt and phosphorylated Akt forms after 16 hours of treatment. Similar results were also found for the phosphorylated I κ B α proteins (Fig. 5C), suggesting the involvement of NF- κ B signaling in this event.

Alterations in the NF- κ B Signaling Pathway Brought by the Combination of Cloiquinol and DHA

We have previously shown that cloiquinol down-regulates NF- κ B activity in an ovarian cancer cell line A2780 (9). In the present study, we examined the effects of the cloiquinol plus DHA on NF- κ B signaling. Each compound by itself had moderate effects on NF- κ B activity, as

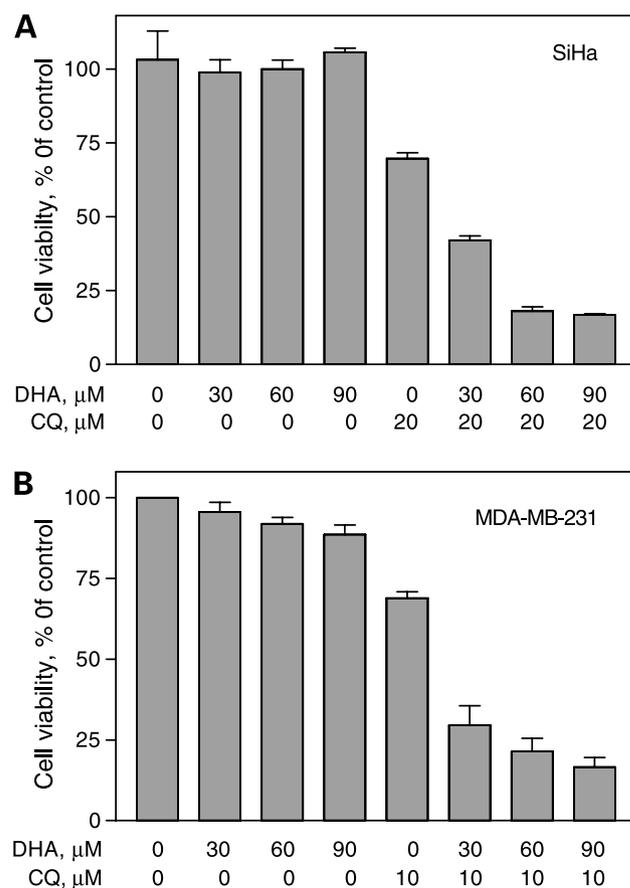


Figure 3. Effects of cloiquinol and DHA on the viability of SiHa and MDA-MB-231 cells. Cells were treated with cloiquinol and DHA at the indicated concentrations for 72 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. *Columns*, percentage of untreated cells ($n = 3$); *bars*, SE.

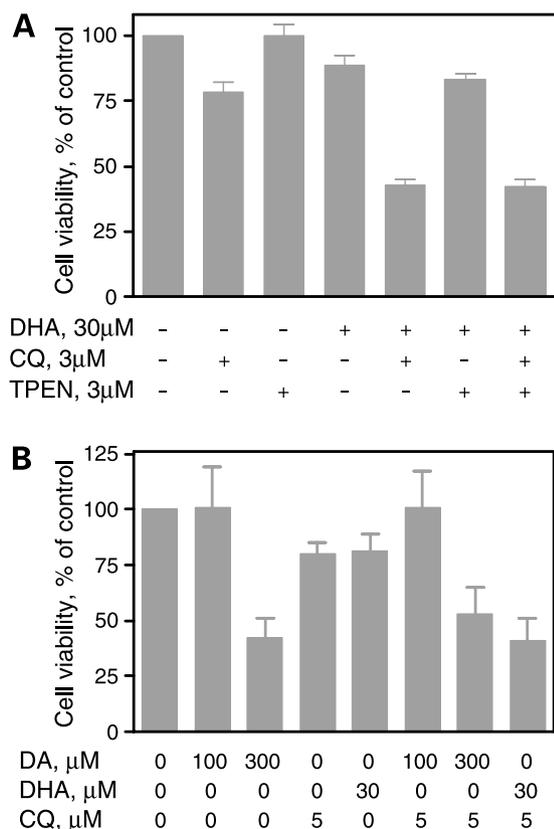


Figure 4. Effects of TPEN, DHA and cloiquinol, and docosanoic acid (DA) on the viability of Raji cells. Cells were treated with the combination of TPEN and DHA (**A**), or cloiquinol and docosanoic acid (**B**) at indicated concentrations for 72 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Columns, mean percentage of untreated cells ($n = 3$); bars, SE.

analyzed with a reporter gene assay (Fig. 6A), consistent with previous reports (9, 34). When cells were treated with both cloiquinol and DHA, a further significant reduction of NF- κ B activity was evident, paralleling the decrease in p65 protein level, as determined by Western blotting (Fig. 5B).

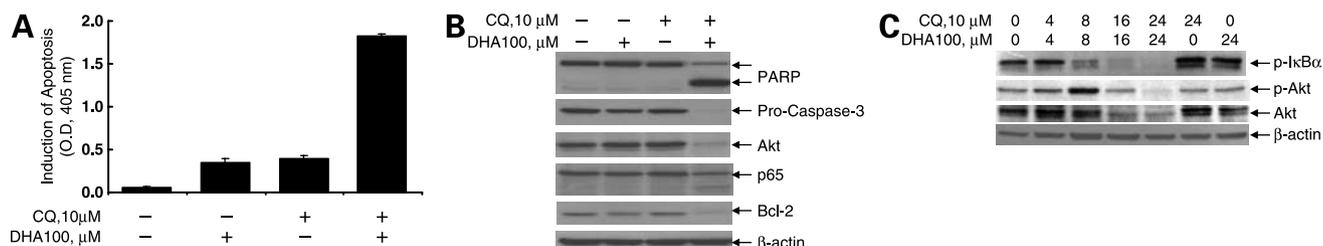


Figure 5. Effects of DHA and cloiquinol on induction of apoptosis and protein levels of Akt, p65, and Bcl-2 in Raji cells. **A**, induction of apoptosis by cloiquinol and DHA. Raji cells were treated with 10 μ mol/L cloiquinol and 100 μ mol/L DHA for 24 h. Cell lysates were prepared, and the extent of apoptosis was determined with an immunochemical measurement of released DNA-histone fragments, using an ELISA. Columns, mean actual readings ($n = 3$); bars, SE. **B**, an equal amount of protein from each sample prepared as in **A** was resolved by 8% SDS-PAGE followed by Western blotting analysis with specific antibodies against poly(ADP-ribose) polymerase (PARP), Akt, p65, Bcl-2, or β -actin. Representative of three experiments. **C**, time course of cloiquinol and DHA-induced alterations of phosphorylated I κ B α and Akt protein levels. Raji cells were treated with 10 μ mol/L cloiquinol and 100 μ mol/L DHA for 4 to 24 h. Cell lysates were prepared, and cellular proteins were separated on 8% SDS-PAGE followed by Western blotting analysis with specific antibodies against phosphorylated I κ B α (p-I κ B α), phosphorylated Akt (p-Akt), total Akt, and β -actin. Representative of two experiments.

To confirm the down-regulation of NF- κ B signaling by these two compounds, A2780 cells were analyzed by a chromatin immunoprecipitation assay designed to measure the binding activity of p65 to the promoter region of the pNF- κ B-Luc plasmid. Although neither 10 μ mol/L cloiquinol nor 100 μ mol/L DHA alone had a clear effect on the p65 binding activity in this assay, the combination of these two significantly inhibited p65 binding to the promoter (Fig. 6B). As a positive control, we tested zinc and the zinc ionophore pyrrolidine dithiocarbamate.

Cloiquinol Does Not Potentiate DHA-Induced Lipid Peroxidation

Because the cytotoxic effects of DHA have been shown to involve lipid peroxidation (23), we hypothesized that cloiquinol might potentiate DHA-induced lipid peroxidation. To test this hypothesis, Raji cells were treated with 10 μ mol/L cloiquinol, 100 μ mol/L DHA, or both for 6 to 24 hours. Generation of TBARS was used as a measure of lipid peroxidation (30). Cloiquinol did not increase TBARS levels by itself and did not enhance DHA-induced lipid peroxidation (Fig. 7A). Additional studies used the antioxidant vitamin E, which is known to inhibit DHA's cytotoxicity (24). A 15-minute preincubation with vitamin E (100 μ mol/L) blocked cytotoxicity induced by DHA but not cloiquinol. The addition of both cloiquinol and DHA to vitamin E-pretreated cells abolished the synergistic interaction (Fig. 7B).

Long-term Cytotoxicity of Cloiquinol Is Independent of Its Zinc Ionophore Activity

We have shown that cloiquinol acts as a zinc ionophore (9); thus, the synergistic interaction of cloiquinol and DHA might result in further increases of cytoplasmic zinc concentrations. To test this hypothesis, we used BHK cells that have been stably transfected with a β -galactosidase reporter construct sensitive to free intracellular zinc concentrations (31, 32). Figure 8A shows the induction of the zinc-sensitive reporter gene by cloiquinol or zinc. Half-maximal induction of the reporter by cloiquinol was about 16 μ mol/L compared with about 40 μ mol/L for zinc sulfate. The induction of β -galactosidase by cloiquinol was completely inhibited by 3.3 μ mol/L TPEN (Fig. 8A),

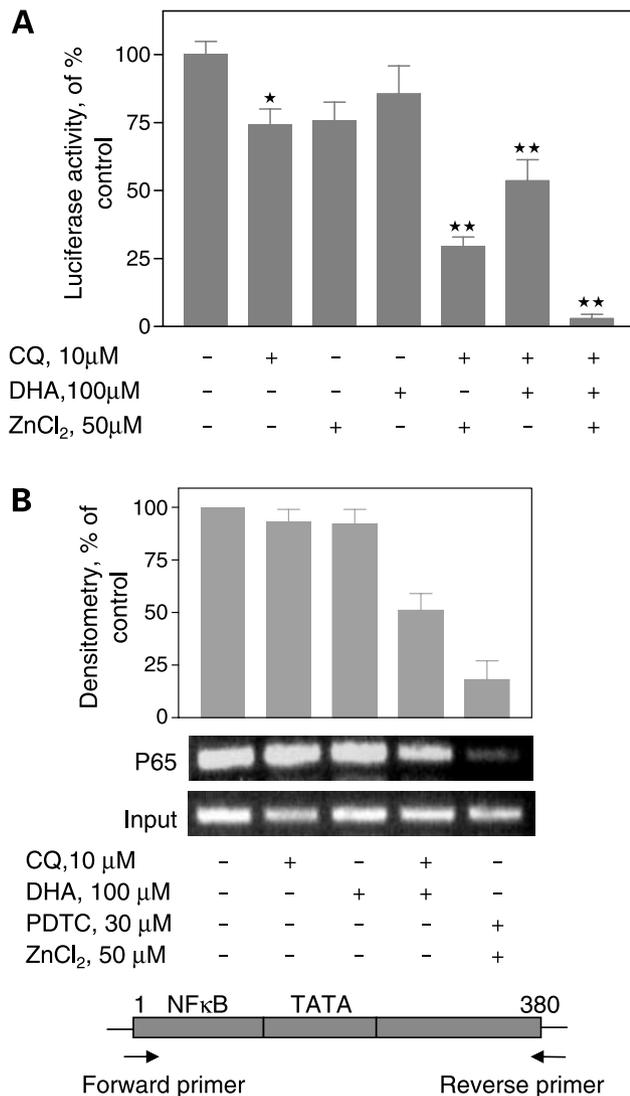


Figure 6. Effects of clioquinol and DHA on NF- κ B activity and p65 DNA binding. **A**, A2780 cells were transfected with the pNF- κ -B constructs and treated with 10 μ mol/L clioquinol, 100 μ mol/L DHA, 50 μ mol/L ZnCl₂, or in combinations for 4 h. Cell lysates were prepared, and luciferase activity was assayed as previously described (9). *Columns*, mean percentage of untreated control cells ($n = 3$); *bars*, SE. *, $P < 0.05$; **, $P < 0.01$, compared with control cells, using one-way ANOVA followed by Dunnett's analysis. **B**, A2780 cells were transfected with the pNF- κ -B constructs and treated with 10 μ mol/L clioquinol and 100 μ mol/L DHA for 4 h. Chromatin immunoprecipitation assay was done using a specific antibody against p65, as described in Materials and Methods. PCR amplification of the promoter region of the pNF- κ -B construct was done using primers spanning that region, and the reactions were separated on 1% agarose gel containing ethidium bromide. *Top*, densitometry of the amplified DNA bands; *middle*, PCR separation on agarose gel, representative of three experiments; *bottom*, a diagram of the amplified promoter region of the pNF- κ -B construct with the NF- κ B binding sites and TATA box indicated.

whereas this concentration of TPEN had no effect on induction by exogenous zinc (data not shown). Addition of increasing amounts of DHA to a low concentration of clioquinol that had little effect on its own significantly

increased expression of the reporter gene (Fig. 8B), and this effect was blocked by TPEN; 72 hours of exposure to clioquinol inhibits growth/survival of these BHK cells as shown in Fig. 8C; the IC₅₀ for survival of these BHK cells after 3 days in clioquinol was 10.5 μ mol/L. DHA (20 μ mol/L) alone had no effect on growth of these cells, but it reduced the IC₅₀ in the presence of clioquinol to 6 μ mol/L (Fig. 8C). The toxicity of 20 μ mol/L DHA in the presence of clioquinol was eliminated by the addition of 25 μ mol/L vitamin E (Fig. 8C). The addition of TPEN (3.3 μ mol/L) did not block the toxicity of clioquinol and had a minimal effect on reversing the combined toxicity of DHA and clioquinol (Fig. 8D). These results suggest that the toxicity of clioquinol alone or in the presence of DHA may not be due to its zinc ionophore effects.

Discussion

Previously, we reported that both clioquinol (9) and DHA (24) have anticancer properties. Here, we report that

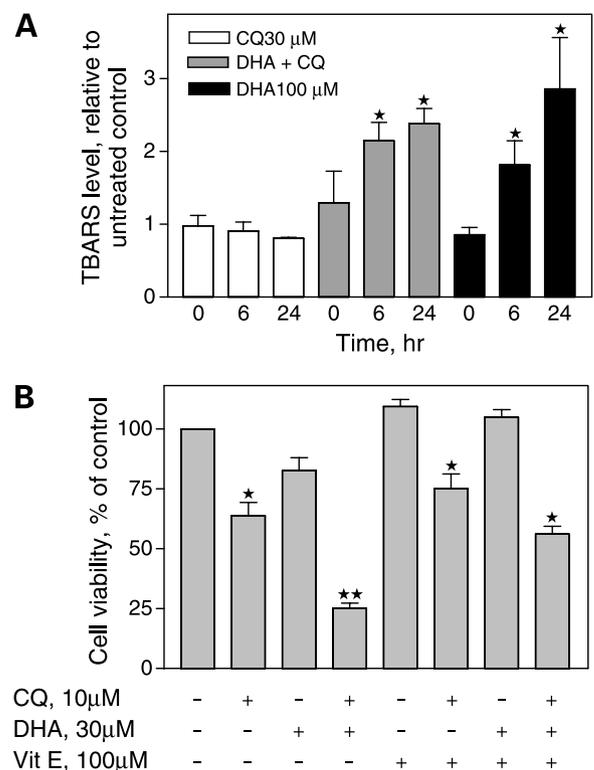


Figure 7. Clioquinol does not enhance lipid peroxidation in Raji cells. **A**, cells were grown in 75 cm² flasks and treated with 30 μ mol/L clioquinol or 100 μ mol/L DHA for 6 or 24 h. TBARS generation was assayed as described in Methods and Materials. *Columns*, mean TBARS concentrations relative to control cells ($n = 3$); *bars*, SE. **B**, effects of pretreatment with vitamin E (Vit E) on DHA- and clioquinol-induced cytotoxicity. Cells were pretreated with 100 μ mol/L vitamin E for 15 min before addition of 10 μ mol/L clioquinol or 100 μ mol/L DHA. After 72 h of treatment, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. *Columns*, mean percentage of untreated cells ($n = 3$); *bars*, SE. *, $P < 0.05$; **, $P < 0.01$, compared with untreated cells, using one-way ANOVA followed by Dunnett's analysis.

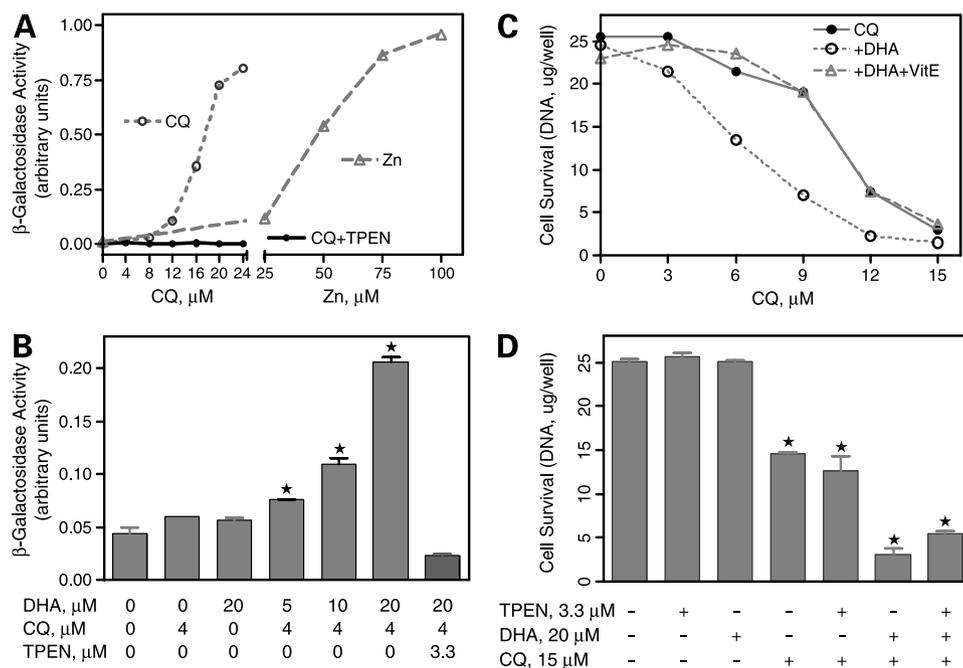


Figure 8. Responses of BHK cells to cloiquinol and DHA. **A**, cloiquinol acts as a zinc ionophore to induce expression of β -galactosidase from the zinc responsive reporter gene. BHK cells were treated with the indicated concentrations of cloiquinol \pm TPEN (3.3 $\mu\text{mol/L}$) or ZnSO_4 for 18 h, and β -galactosidase activity was measured as described in Materials and Methods. **B**, DHA facilitates the activation of the zinc-responsive reporter gene by cloiquinol. Cells were treated with the compounds shown for 18 h, and then β -galactosidase activity was measured; for reference, 16 $\mu\text{mol/L}$ cloiquinol resulted in 0.95 unit of activity in this experiment. **C**, growth and survival of BHK cells after a 3-d exposure to cloiquinol and DHA. An equal number of cells were plated in 24-well dishes and exposed to the indicated concentrations of cloiquinol, DHA (20 $\mu\text{mol/L}$), or vitamin E (25 $\mu\text{mol/L}$) for 3 d; then DNA content per well was measured. **D**, experiment was done as in **C**. *, $P < 0.001$, compared with untreated cells, using one-way ANOVA followed by Bonferroni's multiple comparison test.

cloiquinol interacts in a synergistic manner with DHA, but not doxorubicin, to induce apoptosis of cancer cells. Enhanced cell killing by this combination is dependent on lipid peroxidation initiated by DHA. NF- κ B pathway, a well-characterized survival signaling pathway in cancer cells, is a likely molecular target of the synergistic action of these two compounds. However, other signaling proteins, including Akt and Bcl-2, also seem to be involved.

The synergistic toxicity of cloiquinol and DHA was clearly evident by cell viability assay and apoptosis analysis. This synergy was observed among several different cell lines tested. Cytotoxicity of DHA is known to be due to lipid peroxidation (23, 24). We have shown that cloiquinol acts as heavy metal ionophore (9). Several potential cellular mechanisms might mediate the synergy of DHA and cloiquinol: (a) DHA might potentiate the ionophore activity of cloiquinol, thereby enhancing metal toxicity; (b) cloiquinol might enhance the lipid peroxidation of DHA leading to greater toxicity; or (c) the two compounds might act on different steps of NF- κ B signaling pathway, leading to a synergistic reduction in signaling by this critical pathway.

To determine whether the synergistic toxicity of DHA and cloiquinol was due to enhanced ionophore activity, we examined BHK cells carrying a zinc-responsive reporter gene. We showed that DHA does potentiate the ability of

cloiquinol to increase cytoplasmic zinc levels, although DHA does not affect intracellular zinc on its own. The mechanism of this effect is unknown. One possibility is that DHA facilitates uptake of cloiquinol by cells and hence cloiquinol/zinc complexes. Another possibility is that DHA-induced peroxidation lowers the cytoplasmic pool of reduced thiols available to bind cytoplasmic zinc, resulting in an additional increase in free zinc beyond that induced by cloiquinol. Regardless, we showed that the toxicity of cloiquinol alone or in combination with DHA is independent of its zinc ionophore activity by showing that the long-term toxicity is not blocked by TPEN, under conditions where TPEN completely prevents induction of the zinc-dependent reporter gene. Although long-term exposure to cloiquinol inhibits cell growth and survival ($\text{IC}_{50} \sim 10 \mu\text{mol/L}$) in a zinc-independent manner, the zinc ionophore activity can kill BHK cells at higher concentrations (e.g., overnight exposure of BHK cells to $>20 \mu\text{mol/L}$ cloiquinol is as deadly as exposure to $>150 \mu\text{mol/L}$ zinc).

The role of lipid peroxidation in the synergy of cloiquinol and DHA was examined in both human tumor cells and BHK cells. We showed that pretreatment with antioxidant vitamin E blocked the synergistic action in both model systems, suggesting that lipid peroxidation is essential for the synergy. Interestingly, the amount of lipid peroxidation (measured by TBARS assay) caused by DHA was not

enhanced by clioquinol, even at very high concentrations. These seemingly contradictory observations suggest that the combination of clioquinol and DHA act on a vitamin E-sensitive process that is not reflected in the TBARS assay. Such toxic effects may include enhanced damage of DNA or signaling proteins that do not lead to a further increase in lipid peroxidation levels but promote the death of tumor cells.

Because both DHA and clioquinol have been shown to down-regulate NF- κ B activity (9, 35, 36), and because NF- κ B signaling is a well-established molecular target for chemotherapy (37, 38), we explored the possibility that both compounds may be influencing NF- κ B signaling in a synergistic manner. Experimental evidence obtained through Western blot, reporter gene assay, and chromatin immunoprecipitation analysis indicate that DHA plus clioquinol induced significant down-regulation of NF- κ B signaling, effects that paralleled their toxicity. Specifically, expression of p65, one of the most frequently detected NF- κ B subunits (37), was reduced more by DHA plus clioquinol treatment than by either alone. How DHA plus clioquinol could act on NF- κ B signaling is unclear, but Akt, a signaling protein upstream of NF- κ B complex (37), seems to be involved because treatment with DHA plus clioquinol reduced Akt level in our model system more than either alone. Furthermore, time course study showed that the level of phosphorylated I κ B α is also reduced by clioquinol plus DHA, parallel to that of Akt, indicating that Akt and NF- κ B may act in concert in response to the treatment. These results are consistent with a previous study suggesting that DHA down-regulates NF- κ B activity by targeting Akt (35).

Clioquinol has been given systemically to humans and is currently undergoing evaluation as a treatment for neurologic disorders, notably Alzheimer's disease (11, 12). Importantly, the lowest blood level found when clioquinol was given once daily (750 mg, orally) was ~ 25 μ mol/L, and subjects did not experience any toxicity (12). Its effectiveness in animal models of other neurologic diseases suggests that it may be studied in other patient populations as well (39). DHA has also been documented to be tolerated by humans, at doses up to 18 g/d (40, 41). The present study shows that clioquinol, a metal binding compound, acts synergistically with DHA, an unsaturated long-chain fatty acid, to induce apoptosis of cancer cells. These findings provide the basis for further development of the novel drug combination for cancer therapy. As both compounds can be well tolerated by animals and human subjects (9, 12, 20, 40, 41), and as previous studies have shown that DHA selectively enhanced paclitaxel toxicity upon cancer cells *in vivo* (7, 8), exploration of the clioquinol plus DHA combination in animal models and human subjects seems warranted.

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