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# Clioquinol induces DNA double-strand breaks, activation of ATM, and subsequent activation of p53 signaling

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ABSTRACT

Clioquinol, a $Cu^{2+}/Zn^{2+}/Fe^{2+}$ chelator/ionophor, was used extensively in the mid 1900s as an amebicide for treating indigestion and diarrhea. It was eventually withdrawn from the market because of a link to subacute myelo-optic neuropathy (SMON) in Japan. The pathogenesis of SMON, however, is not fully understood. To clarify the molecular mechanisms of clioquinol-induced neurotoxicity, a global analysis using DNA chips was carried out on human neuroblastoma cells. The global analysis and quantitative PCR demonstrated that mRNA levels of $p21^{Cip1}$, an inhibitor of cyclins D and E, and of GADD45α, a growth arrest and DNA damage-inducible protein, were significantly increased by clioquinol treatment in SH-SY5Y and IMR-32 neuroblastoma cells. Activation of p53 by clioquinol was suggested, since clioquinol induced phosphorylation of p53 at Ser15 to enhance its stabilization. The phosphorylation of p53 was inhibited by KU-55933, an inhibitor of ataxia-telangiectasia mutated kinase (ATM), but not by NU7026, an inhibitor of DNA-dependent protein kinase (DNA-PK). Clioquinol in fact induced phosphorylation of ATM and histone H2AX, a marker of DNA double-strand breaks (DSBs). These results suggest that clioquinol-induced neurotoxicity is mediated by DSBs and subsequent activation of ATM/p53 signaling.


## 1. Introduction

Clioquinol (5-chloro-7-indo-8-quinolinol) was used extensively as an amebicide for treating indigestion and diarrhea in the mid 1900s. Later it was withdrawn from the market because its use was epidemiologically linked to the incidence of subacute myelo-optic neuropathy (SMON) in Japan (Cahoon, 2009). SMON is characterized by subacute onset of sensory and motor disturbances in the lower extremities and visual impairment (Nakae et al., 1973; Tsubaki et al., 1971). Although pathological studies demonstrated axonopathy of the spinal cord and optic nerves (Tateishi, 2000), the underlying mechanisms of clioquinol toxicity are yet to be elucidated. In PC12 cells, clioquinol suppressed nerve growth factor-induced Trk autophosphorylation and neurite outgrowth (Asakura et al., 2009). In cultured dorsal root ganglion neurons, clioquinol induced mechanical hyperalgesia and cold allodynia via activation of TRPA1 (Andersson et al., 2009). When injected into young rats, it attenuated dentate gyrus long-term potentiation (Takeda et al., 2010).

Recently, clioquinol has been reevaluated as a prototype for metal-protein-attenuating compounds that decrease oxidative stress and deposits of metalloproteins in patients with Alzheimer disease (Adlard et al., 2008; Cherny et al., 2001), Parkinson disease (Kaur et al., 2003), and Huntington disease (Nguyen et al., 2005). Especially for Alzheimer disease, the effectiveness of clioquinol and its derivative PBT2 was verified by phase II clinical trials (Faux et al., 2010; Lannfelt et al., 2008; Ritchie et al., 2003). These beneficial effects appeared to be mediated by prevention of protein aggregation via chelation of metal ions. Clioquinol was also reported to inhibit the aging-associated mitochondrial enzyme CLK-1 (Wang et al., 2009). Another line of investigation, however, demonstrated that cytotoxicity of clioquinol was mediated by oxidative stress (Benvenisti-Zarom et al., 2005), inhibition of the 20S proteasome (Mao et al., 2009), or induction of the cytoplasmic clearance of X-linked inhibitor of apoptosis protein (XIAP) (Cater and Haupt, 2011). These cytotoxic effects were also thought useful for cancer therapy (Ding et al., 2005).

Clioquinol has been recognized conventionally as a $Cu^{2+}/Zn^{2+}$ chelator (Cherny et al., 2001; Choi et al., 2006). It also works as a $Fe^{2+}$ chelator (Kaur et al., 2003; Wang et al., 2009). Furthermore, it was recently shown to work as a $Cu^{2+}/Zn^{2+}/Fe^{2+}$ ionophore that

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**Table 1**
Sequences of primers used in quantitative PCR.

| Gene | | Primer sequences |
|---|---|---|
| $p21^{Cip1}$ | Sense | 5′-aggggacagcagaggaaga-3′ |
| | Antisense | 5′-ggcttcctcttggagaagatcag-3′ |
| GADD45α | Sense | 5′-ccacattcatctcaatggaag-3′ |
| | Antisense | 5′-cagggagattaatcactggaa-3′ |
| HPRT | Sense | 5′-agactttgctttccttggtca-3′ |
| | Antisense | 5′-aggctttgtattttgcttttc-3′ |

drives these metal ions into the cell (Andersson et al., 2009; Ding et al., 2005). Thus, the characteristics of clioquinol and its effects on neuronal cells have been controversial. To clarify the molecular mechanisms underlying clioquinol-induced neurotoxicity, we carried out a global analysis using DNA chips in human neuroblastoma cells and demonstrated that clioquinol activates ATM and subsequent p53 signaling.

## 2. Materials and methods

### 2.1. Materials

Clioquinol, KU-55933 (InSolution ATM Kinase Inhibitor), U0126, and MnTBAP were purchased from Merck (Darmstadt, Germany). NU7026 was obtained from Cayman Chemicals (Ann Arbor, MI). SB239063 was purchased from Alexis Biochemicals (San Diego, CA). Antibodies against phospho-p53 (Ser15) and p53 (DO-1) were purchased from Cell Signaling Technology (Danvers, MA) and Medical and Biological Laboratories (Nagoya, Japan), respectively. The antibody against β-actin was obtained from Sigma (St. Louis, MO). Antibodies against phospho-ATM (Ser1981), ATM, histone H2AX (phospho-Ser139), and histone H2AX were purchased from Epitomics (Burlingame, CA).

### 2.2. Cell culture

Human SH-SY5Y cells, purchased from European Collection of Cell Cultures, were cultured in Ham's F12: Eagle's medium with Earle's salts (1:1) supplemented with non-essential amino acids and 15% fetal bovine serum (FBS). IMR-32 cells from the JCRB Cell Bank were cultured in Eagle's medium with Earle's salts supplemented with non-essential amino acids and 10% FBS.

### 2.3. Cell proliferation assay

SH-SY5Y or IMR-32 cells were grown in 96-well plates in the presence or absence of clioquinol for 24 h. Cell proliferation was quantitated using a CyQuant Direct Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

### 2.4. Microarray analysis

SH-SY5Y cells were grown in the presence or absence of 50 μM clioquinol for 24 h. Total RNA isolated using an RNeasy Plus Mini kit (Qiagen, Hilden, Germany) was prepared for labeling and hybridization to a human Oligo chip 25k (Toray, Tokyo, Japan) using standard methods. The GEO database accession code for the microarray data obtained is GSE32173.

### 2.5. Quantitative PCR

Total RNA was reverse transcribed to first-strand cDNA using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan), and quantitative PCR was performed in a StepOnePlus Real-Time PCR System (Life Technologies) using THUNDERBIRD SYBR qPCR Mix (TOYOBO). Gene expression was quantified using standard curves that were generated using serially diluted plasmid reference samples and normalized to the expression level of hypoxanthine phosphoribosyltransferase (HPRT). The specificity of the PCR products was confirmed by gel electrophoresis and a dissociation curve analysis. Primer sequences are shown in Table 1.

### 2.6. Western blotting

Whole cell lysate was prepared essentially as described previously (Fan et al., 2005; Katsuyama et al., 2005). Briefly, cells were lysed in a buffer containing 1% Triton, 0.5% sodium deoxycholate, 10 mM Tris–HCl (pH 6.8), 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan), 1 mM NaF, 20 mM β-glycerophosphate, and 1 mM $Na_3VO_4$. The lysate was centrifuged and the supernatant was used as whole cell lysate. Aliquots containing equal amounts of protein (10 μg) were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Hybridization of antibodies and washing were carried out essentially as described previously (Fan et al., 2005; Katsuyama et al., 2005). Representative autoradiographs from three experiments are shown in the figures.

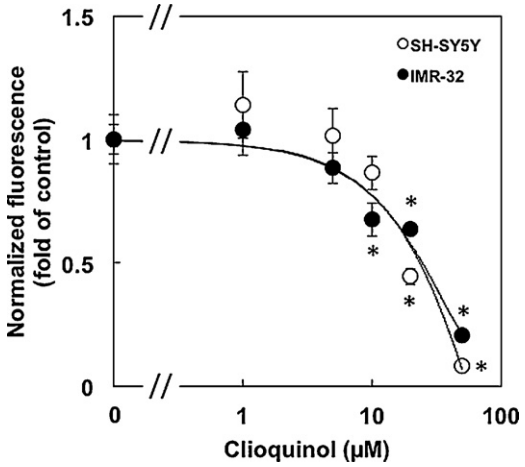


**Fig. 1.** Clioquinol suppressed proliferation of SH-SY5Y and IMR-32 neuroblastoma cells. SH-SY5Y and IMR-32 cells were grown in 96-well plates in the presence or absence of clioquinol for 24 h. Cell proliferation was quantitated using a CyQuant Direct Cell Proliferation Assay Kit. Open circles, SH-SY5Y; closed circles, IMR-32. $^*P < 0.05$ vs. control ($N = 8$).

### 2.7. Statistical analysis

Values were expressed as the mean ± SE. The statistical analysis was performed using Student's *t* test. For multiple treatment groups, one-way ANOVA followed by Bonferroni's *t* test was applied.

## 3. Results

### 3.1. Clioquinol suppressed proliferation of SH-SY5Y and IMR-32 human neuroblastoma cells

We first examined whether clioquinol affects the proliferation of neuroblastoma cells using the CyQuant Direct Cell Proliferation Assay Kit. As shown in Fig. 1, treatment with clioquinol for 24 h significantly suppressed proliferation of SH-SY5Y and IMR-32 cells at concentrations higher than 10–20 μM.

### 3.2. Clioquinol increased levels of mRNA for $p21^{Cip1}$ and GADD45α

A global analysis with DNA chips was carried out using SH-SY5Y cells grown in the presence or absence of 50 μM clioquinol for 24 h. Among approximately 25,000 genes, 2429 were up-regulated in their expression and 2727, down-regulated, by clioquinol (GEO database accession code: GSE32173). Notably, the expression of $p21^{Cip1}$, an inhibitor of cyclins D and E, and that of a growth arrest and DNA damage-inducible protein, GADD45α, were up-regulated by treatment with clioquinol. Both of these proteins are dependent on p53, a tumor suppressor. Up-regulation of the mRNA expression for these proteins was verified by quantitative PCR in SH-SY5Y and IMR-32 cells (Fig. 2).

### 3.3. Clioquinol induced phosphorylation of p53 at Ser15

The up-regulation of $p21^{Cip1}$ and GADD45α expression suggests that p53 is activated by clioquinol. We therefore examined whether phosphorylation at Ser15, an indicator of the activation of p53, is enhanced by clioquinol treatment. As shown in Fig. 3A, treatment with clioquinol for 24 h markedly induced phosphorylation of p53 in SH-SY5Y cells at concentrations higher than 10 μM. In IMR-32