

**Clioquinol promotes cancer cell toxicity through tumour necrosis factor  
alpha release from macrophages**

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**Abbreviations:** CQ: Clioquinol, BCS: bathocuproine sulphonate, PDTC: pyrrolidine dithiocarbamate,  
TNF $\alpha$ : tumor necrosis factor alpha

## **Abstract**

Copper has an important role in cancer growth, angiogenesis and metastasis. Previous studies have shown that cell permeable metal ligands including clioquinol (CQ) and pyrrolidine dithiocarbamate (PDTC) inhibit cancer cell growth in cell culture and in vivo. The mechanism of action has not been fully determined but may involve metal-mediated inhibition of cancer cell proteasome activity. However, these studies do not fully account for the ability of cell permeable metal ligands to inhibit cancer cell growth without affecting normal cells. In this study, we examined the effect of CQ on macrophage-mediated inhibition of HeLa cancer cell growth in vitro. When CQ was added to RAW 264.7 macrophage-HeLa cell co-cultures, a substantial increase in HeLa cell toxicity was observed when compared to CQ treatment of HeLa cells cultured alone. Transfer of conditioned medium from CQ-treated macrophages to HeLa cells also induced HeLa cell toxicity demonstrating the role of secreted factors in the macrophage-mediated effect. Further investigation revealed that CQ induced copper-dependent activation of macrophages and release of tumour necrosis factor alpha (TNF $\alpha$ ). In studies with recombinant TNF $\alpha$  we showed that the level of TNF $\alpha$  released by CQ-treated macrophages was sufficient to induce HeLa cell toxicity. Moreover, the toxic effect of conditioned medium from CQ-treated macrophages could be prevented by addition of neutralizing antibodies to TNF $\alpha$ . These studies demonstrate that CQ can induce cancer cell toxicity through metal-dependent release of TNF $\alpha$  from macrophages. Our results may help to explain the targeted inhibition of tumour growth in vivo by CQ.

## Introduction

Copper (Cu) has an important role in cancer development and progression. Increasing evidence identifies Cu as a critical growth factor in tumour angiogenesis (Brem, 1999; Brewer, 2001; Theophanides and Anastassopoulou, 2002; Daniel et al., 2004; Chen et al., 2007). High levels of Cu have been observed in a number of cancers including prostate, breast, colon, lung and brain (Chen et al., 2007; Habib et al., 1980; Huang et al., 1999) and metal chelators have been successfully used to suppress angiogenesis, tumour growth and metastasis (Brewer et al., 2000; Lovejoy and Richardson, 2003; Redman et al., 2003; Ding et al., 2005). Tetrathiomolybdate (TM), a Cu chelator used in treatment of Wilson disease, has been effective in reducing growth of mammary tumours in HER2/neu transgenic mice and inhibiting metastasis of lung tumours in mice (Pan et al., 2002; Khan et al., 2002; Daniel et al., 2005). A phase 1 clinical trial resulted in disease stabilization in five of six patients with metastatic cancer (Brewer et al., 2000; Daniel et al., 2005). These reports support the potential use of Cu chelators as anticancer therapeutics.

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline, CQ) is a lipophilic compound capable of complexing with Cu and zinc (Di vaira et al., 2004). Initially, CQ was used as an antibiotic for treatment of diarrhoea and skin infections (Yassin et al., 2000), however, its use was stopped in some countries after a possible association with subacute myelo-optic neuropathy in Japan (Richards, 1971; Yassin et al., 2000; Di Vaira et al., 2004). Subsequent epidemiological evidence does not support this link and it appears that vitamin B12 deficiency may have had a role in the syndrome (5, 19). Several recent studies have generated interest in CQ as a modulator of metal homeostasis in neurodegenerative disorders. CQ has been shown to reduce or prevent the formation of amyloid plaques in transgenic Alzheimer's disease (AD) mice (Cherny et al., 2001) and conferred benefit to AD patients in a small clinical trial (Ritchie et al., 2003). The compound has also shown positive effects in a Parkinson's disease mouse model and inhibited aggregation of huntingtin in a cell model of Huntington's disease

(Nguyen et al., 2005).

Due to the association between Cu and cancer growth and metastasis, a number of groups have investigated the potential of CQ as an anticancer drug. Ding et al. (Ding et al., 2005) demonstrated caspase-dependent apoptosis in a number of cancer cell-lines treated with CQ. This toxic effect was related to the ionophoric activity of CQ as CQ-mediated cellular Cu uptake resulted in increased cytotoxicity. Subsequent CQ treatment of mice with xenograft tumours revealed a substantial reduction in tumour size but without evidence of broad cytotoxicity. A potential role for the nuclear factor kappa B (NFkB) was investigated but only small changes in activity were observed after CQ treatment of cancer cells (Ding et al., 2005). Daniel et al. (Daniel et al., 2005) also reported specific anticancer effects of CQ. CQ and another Cu ionophore, pyrrolidine dithiocarbamate (PDTC) were found to induce metal-dependent inhibition of the proteasome in human breast cancer cells. Subsequently, Chen et al. (Chen et al., 2007) reported that CQ-Cu complexes targeted chymotrypsin-like activity of the proteasome.

While these studies have highlighted the potential for CQ as a therapeutic agent for treatment of cancer, little information is available on how CQ specifically targets cancer cells, particularly *in vivo*. The metal ionophore effect of CQ has been demonstrated in many different cell types (Treiber et al., 2004; Benvenisti-Zarom et al., 2005; White et al., 2006; Masuda et al., 2007). While CQ may specifically target the proteasome in some cancer cell-lines (Chen et al., 2007), it is uncertain whether this extends to all cancer cells and whether this is the primary anticancer mechanism *in vivo* (Ding et al., 2005). An alternative mode of action for CQ-mediated anticancer activity is through the immune system. To further investigate the potential role of immune cells in anticancer effects by CQ, we examined whether macrophage-mediated cancer inhibition could play a role in CQ activity. RAW 264.7 macrophages were co-cultured with HeLa cancer cells in the presence of CQ. We found that the presence of macrophages greatly potentiated the toxic effect of CQ towards HeLa cells. Further

examination showed that TNF $\alpha$  release from CQ-treated macrophages induced toxic effects on co-cultured HeLa cells. Continued investigation of how CQ induces macrophage activation may help to identify novel metal-mediated signal transduction pathways involved in anticancer responses.

## Methods

*Cell Culture:* Raw 264.7 macrophages were obtained from Dr David Thomas (Ian Potter Cancer Genomic Centre, Peter MacCallum Institute, Melbourne, Australia) and Dr Dmitri Sviridov (Baker Heart Research Institute, Melbourne, Australia). HeLa cells were a gift from Dr Patrick Sexton (Howard Florey Institute, Melbourne, Australia). Raw 264.7 macrophages were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 16 mM Hepes and 50 µg/ml penicillin/streptomycin. For experiments, cells were plated into 24 or 6 well plates or 100 mm dishes (Nunc) at a dilution of 1:4 in RPMI medium supplemented with 10% FCS. Cells were grown for 1-2 days until 80-90% confluent and used for experiments. HeLa cells were maintained in DMEM supplemented with 5% FCS, 2 mM glutamine, 10 mM Hepes and 50 µg/ml penicillin/streptomycin. Cells were passaged at 1:20 dilution and seeded at 1:10 into 24 well plates or onto glass coverslips for experiments. Cells were maintained 1-2 days until 80-90% confluent before experimental treatments.

*CQ treatment:* The medium used for the co-culture and transfer experiments was Opti-MEM (Gibco) without serum. For co-culture experiments, HeLa cells on coverslips were added to wells containing macrophages. CQ (10 mM stock in DMSO) and/or Cu(II) (10 mM in dH<sub>2</sub>O) was added to macrophage-HeLa cell co-cultures at indicated concentrations for 24 hr. Coverslips of HeLa cells were then removed and cell viability of macrophages and HeLa cells was determined separately using the MTT assay as previously described (White et al., 1999) or Trypan blue staining with 0.4% Trypan blue (Sigma). For experiments involving transfer of conditioned medium, macrophages were treated with CQ or metals at indicated concentrations for 2 or 24 hr and medium transferred to separate cultures of HeLa cells for a further 24 hr. This was followed by MTT or Trypan blue assay of cell viability. Where indicated, CQ was also added alone to HeLa cells for 24 hr. In some experiments, the Cu(I)-selective

metal ligand, bathocuproine disulphonate (BCS) was added at a concentration of 200  $\mu\text{M}$ . Experiments were also performed in media pre-treated with Chelex 100 resin to deplete residual Cu levels. We have reported previously that Chelex 100 treatment reduces Cu levels in the medium by approximately ten-fold (White et al., 2004).

*Nuclear factor kappa B (NFkB) assay:* Nuclear extracts of CQ treated and control macrophages were obtained using a nuclear extraction kit (Active Motif, Carlsbad, CA). NFkB activity was determined in extracts using the NFkB p50 transcription factor ELISA (Active Motif) as per kit instructions.

*Multiplex analysis of cytokine release:* Macrophages were treated with CQ (10  $\mu\text{M}$ ) for 6 hr and levels of cytokines in the conditioned medium were determined using the ChemiArray™ Mouse Inflammation Antibody Array I (Chemicon) as per kit instructions. Measurement of TNF $\alpha$  levels in conditioned medium was also measured using a murine TNF $\alpha$  ELISA (Assay Designs) as per kit insert. As a positive control, macrophages were treated with 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich) and TNF $\alpha$  levels determined in conditioned medium.

*Treatment with TNF $\alpha$ :* To determine the effect of TNF $\alpha$  on HeLa cell viability, cocultures were treated with recombinant murine TNF $\alpha$  (eBioscience, San Diego, CA) at a concentration of 10.0 ng/ml and 100 ng/ml for 24 hr. Cell viability was determined using the MTT assay.

*TNF $\alpha$  neutralization:* Neutralizing antibody to TNF $\alpha$  was purchased from eBioscience (San Diego, CA). Antibody was added to culture medium at 10  $\mu\text{g/ml}$  together with CQ (10  $\mu\text{M}$ ). HeLa cells were co-cultured with macrophages in medium containing TNF $\alpha$  antibody and the effect on HeLa cell viability was determined by MTT assay. As a control, cells were also treated with neutralizing antibodies to IL-12p40/p70 (eBioscience) at a concentration of 10  $\mu\text{g/ml}$ .

*Statistical analysis:* All data described in graphical representations are mean  $\pm$  standard error of the mean (SEM) from 3 to 6 separate tests unless stated. Results were analysed on raw data using a two-



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tailed students'  $t$ -test.

## Results

### **CQ stimulates macrophages to release soluble toxic factors that reduce HeLa cell viability**

Initially, we examined the effect of co-culturing macrophages with HeLa cells in the presence of CQ. Co-cultures of macrophages and HeLa cells were exposed to CQ for 24 hr and the viability of each cell-type was determined separately. In addition, HeLa cells were also grown separately in CQ-supplemented medium to determine the basal effect of CQ on this cell-type. A concentration range of 1-10  $\mu\text{M}$  CQ was used as higher concentrations (25-100  $\mu\text{M}$  CQ) were found to be highly toxic to HeLa cells cultured in the absence of macrophages (data not shown). As shown in Figure 1A, treatment of macrophages had little effect on the viability of this cell-type. A small (17%) but significant increase in macrophage viability was observed after treatment with 10  $\mu\text{M}$  CQ (Figure 1A). In contrast, HeLa cells grown alone in CQ-supplemented medium revealed decreased cell viability (Figure 1B) and this effect was significantly exacerbated when HeLa cells were co-cultured with macrophages (Figure 1B). CQ had no significant effect on macrophage viability in co-cultures (data not shown). We then confirmed that the loss of HeLa MTT reduction induced by CQ in macrophage co-cultures was due to a decrease in viability and not simply altered cell metabolism. Measurement of Trypan blue and MTT staining of HeLa cells from macrophage co-cultures revealed a close correlation in cell toxicity between both methods (Figure 1C). This confirmed the loss of HeLa cell viability induced by CQ in the co-cultures. Our data established that macrophages can increase the toxic effects of CQ towards HeLa cancer cells. We then examined whether the toxic effect of CQ-treated macrophages was mediated by secreted molecules. This was achieved by transferring conditioned medium from CQ-treated macrophages to HeLa cells. Macrophages were cultured for 2 or 24 hr in medium supplemented with 1-10  $\mu\text{M}$  CQ and then the conditioned medium was applied to HeLa cell cultures for a further 24 hr. Conditioned medium from macrophages treated with CQ for 2 hr had little

effect on HeLa cell viability but when conditioned medium was transferred from macrophages treated for 24 hr, it induced a significant toxic effect on the HeLa cells (Figure 1D). Conditioned media from macrophages treated for 24 hr with 10  $\mu$ M CQ reduced HeLa cell viability to less than 50% which was analogous to the co-culture effect in Figure 1B and C. This result indicates that treatment of macrophages with CQ results in increased secretion of factors that are inhibitory to HeLa cancer cell survival.

### **CQ induces Cu-dependent activation of macrophages resulting in loss of HeLa cell viability**

As CQ is a Cu ionophore (White et al., 2006), we examined whether the anticancer activity of CQ was mediated through Cu uptake into macrophages. To achieve this, co-cultures of macrophages and HeLa cells were treated with CQ (1 – 10  $\mu$ M) for 24 hr in the presence or absence of 200  $\mu$ M BCS, a Cu(I) selective cell impermeable metal chelator (White et al., 2003). BCS co-treatment almost completely blocked the anticancer effect of CQ in macrophage-HeLa cell co-cultures (Figure 2A). To confirm that this effect was due to inhibition of Cu uptake by CQ into macrophages, we treated macrophage cultures with BCS and CQ for 24 hr and then transferred the conditioned medium to HeLa cells. Again, the addition of BCS completely blocked the toxic effects of macrophage-conditioned medium (Figure 2B), indicating that CQ-mediated delivery of Cu into macrophages is required for the release of toxic soluble factors that inhibit HeLa viability.

To further investigate the role of Cu in macrophage-mediated HeLa cell toxicity, co-cultures of macrophages and HeLa cells were maintained for 24 hr in medium supplemented with increasing concentrations of Cu(II) (1-10  $\mu$ M). We found that 10  $\mu$ M Cu(II) induced a significant decrease in HeLa cell viability in the co-cultures (Figure 3A). This effect was macrophage-mediated as treatment of HeLa cells alone with 10  $\mu$ M Cu(II) in parallel had no effect on viability (Figure 3A). We also

examined if treatment of co-cultures with CQ and Cu promoted greater toxicity than CQ or Cu alone. Co-cultures were maintained for 24 hr in medium containing 1-10  $\mu$ M CQ and equal concentrations of Cu(II). These were compared to cultures maintained in CQ alone at the same concentrations. We found that addition of Cu(II) to cultures did not further enhance CQ-macrophage toxicity toward HeLa cells compared to CQ added alone (Figure 3B). This finding strongly suggested that CQ was able to induce macrophage activation and release of toxic secreted factors by inducing uptake of residual Cu from the culture medium. Addition of further Cu did not enhance this effect. To confirm this, we maintained cocultures of macrophages and HeLa cells in Chelex-treated (Cu-depleted) medium. Addition of CQ to Chelex-medium had no effect on HeLa cell viability, confirming that basal metal in the medium was required for the CQ effect on HeLa cells. Returning Cu(II) to the medium restored the toxicity of CQ in macrophage-HeLa cell co-cultures (Figure 3C). As little as 100 nM Cu(II) was found to induce significant toxicity of CQ towards HeLa cells in the macrophage-HeLa cell co-cultures (Figure 3C). 1  $\mu$ M Cu(II) was sufficient to fully restore the toxicity of 10  $\mu$ M CQ against HeLa cells. These data demonstrate that CQ induces release of anticancer soluble factors by macrophages through interaction with basal Cu in the culture medium.

### **CQ induces up-regulation of cytokine release from macrophages**

Next, we examined how CQ induced HeLa cell toxicity by macrophages. The metal ionophore activity of CQ is similar to PDTC (5) and PDTC is a potent inhibitor of NF $\kappa$ B signalling (Sherman et al., 1993). NF $\kappa$ B activity was measured in nuclear extracts of macrophages treated with 10  $\mu$ M CQ for 24 hr. However, we found no evidence of altered NF $\kappa$ B activity (Figure 4). As CQ promoted release of a soluble factor(s) from macrophages that induced HeLa cell toxicity, we next examined the levels of cytokines in conditioned macrophage medium. Macrophages were maintained in CQ-treated (10  $\mu$ M)

medium for 24 hr and the cytokine levels in the media were determined using a Mouse Inflammation Antibody Array I (Chemicon). This analysis revealed up-regulation of a number of cytokines in conditioned medium after CQ treatment (Table 1). This included a range of interleukins as well as tissue inhibitors of matrix metalloproteases, interferon and tumour necrosis factor alpha (TNF $\alpha$ ).

### **CQ induces TNF $\alpha$ release from macrophages and inhibition of HeLa cell viability**

One of the most highly elevated cytokines in CQ-treated macrophage medium was TNF $\alpha$  (Table 1) and this cytokine has well-known anticancer properties (Mocellin et al., 2007). Therefore, we examined the role of TNF $\alpha$  further in CQ-treated macrophages and macrophage-HeLa cell co-cultures. Treatment of macrophages with CQ or Cu(II) (10  $\mu$ M each) for 24 hr induced an approximate 900% increase in TNF $\alpha$  levels while Cu(II) induced over 1,200% increase (Figure 5A). This was in the same order of magnitude as the level of TNF $\alpha$  induced by treating macrophages with 100 ng/ml LPS (Figure 5A). A dose-response effect was also observed with increasing concentrations of CQ (1-10  $\mu$ M) inducing greater release of TNF $\alpha$  from macrophages (Figure 5B). Interestingly, the level of TNF $\alpha$  release induced by 10  $\mu$ M CQ was substantially higher when measured by ELISA (900%) than by antibody array (135%) (Table 1). The difference in the observed magnitude of TNF $\alpha$  release between the two techniques may indicate suboptimal binding conditions used in the array. The binding environment is a compromise for all the cytokines and cannot be optimized for an individual antibody-antigen interaction. However, these data nonetheless confirm that TNF $\alpha$  was significantly up-regulated in macrophage cultures treated with CQ. Interestingly, when macrophages were co-treated with CQ and Cu (10  $\mu$ M each), no further elevation in TNF $\alpha$  levels were observed compared to CQ alone (Figure 5A). This was consistent with our finding that addition of CQ and Cu had no greater toxic effect than CQ alone on HeLa cells in co-cultures (Figure 3B).

We then further examined the role of Cu in CQ-mediated TNF $\alpha$  release by macrophages. Co-treatment of macrophages with 10  $\mu$ M CQ and 200  $\mu$ M BCS for 24 hr revealed that chelation of extracellular Cu by BCS prevented CQ-mediated TNF $\alpha$  release (Figure 5A). Similarly, Chelex treatment (Cu-depletion) of medium also inhibited TNF $\alpha$  release by CQ (Figure 4A). Returning Cu(II) to the medium of Chelex-treated cultures restored the ability of CQ to induced TNF $\alpha$  release (Figure 5A), confirming that CQ requires extracellular Cu to mediate TNF $\alpha$  release from macrophages.

While these studies demonstrated that CQ induced TNF $\alpha$  release from macrophages, it was still uncertain whether this cytokine was responsible for the loss of HeLa cell viability in the co-cultures. Therefore, HeLa cell cultures were treated with recombinant human TNF $\alpha$  at a concentration analogous to the level of TNF $\alpha$  measured by ELISA in culture medium from CQ-treated macrophages (approximately 10.0 ng/ml). HeLa cultures maintained in conditioned medium from CQ-treated macrophages induced a high level of toxicity as before (approximately 45% viability, Figure 5B). Treatment of separate HeLa cell cultures with TNF $\alpha$  at 10.0 or 100 ng/ml also induced toxicity (Figure 5B). This toxicity was less than induced by 10  $\mu$ M CQ suggesting that one or more of the additional cytokines up-regulated by CQ could also contribute to the toxicity in co-cultures. Therefore, to further confirm that TNF $\alpha$  was inducing toxicity in HeLa cells co-cultured with macrophages, we treated co-cultures with CQ and neutralizing antibody to TNF $\alpha$ . Addition of the neutralizing antibody significantly inhibited the toxic effects of CQ against HeLa cells when cultured with macrophages. Again, the inhibition by the neutralizing antibody to TNF $\alpha$  was not complete and is consistent with a role for additional macrophage-released cytokines in HeLa cell toxicity. Antibodies to the unrelated cytokine, IL-12p40/p70, but which also showed increased levels in CQ-treated macrophages, had no effect on HeLa cell viability. This demonstrated that addition of antibody per se does not inhibit CQ toxicity. These findings demonstrate that CQ is able to induce TNF $\alpha$  release from macrophages, which

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in turn, is toxic to HeLa cells. The data may have important implications for understanding how CQ induces specific anti-tumour activity in mouse models of cancer and potentially in human cancer patients.

## Discussion

This report describes a novel mechanism for the anticancer activity of CQ. While previous reports have described the toxicity of several cancer cell-lines by CQ, this is the first to demonstrate that CQ can induce cancer toxicity through macrophage activation. Our studies revealed that CQ promotes the release of TNF $\alpha$  from macrophages *in vitro* with the TNF $\alpha$  subsequently inducing toxicity in HeLa cancer cells. Although we did not investigate the cell toxicity pathway in this study, it is known that TNF $\alpha$  can induce apoptosis in cancer cells. However, activation of the cell death pathway in HeLa cells normally requires inhibition of protein synthesis in addition to TNF $\alpha$  treatment (White et al., 1999). Protein synthesis inhibition was not required to induce the toxic effects observed in our cultures by CQ-macrophage conditioned media or recombinant TNF $\alpha$ . Therefore, it is likely that the inhibition of HeLa cell viability in our cultures (as assessed by the MTT and Trypan blue assays) did not reflect apoptosis, but instead was TNF $\alpha$ -mediated inhibition of cell growth.

Previous *in vitro* studies have demonstrated a specific targeting of some cancer cell-lines directly by CQ. In these studies, CQ induced metal-dependent inhibition of proteasome activity resulting in activation of cell death pathways and cancer cell apoptosis (Chen et al., 2007). CQ has also been demonstrated to induce anti-tumour effects *in vivo* (Chen et al., 2007; Ding et al., 2005), but the mechanism of action remains unclear. Our report suggests CQ-mediated cancer cell toxicity may involve Cu-dependent activation of macrophages resulting in specific and localized attack on cancer cells. This indirect CQ effect may occur in addition to reported direct effects of CQ on cancer cell proteasome activity. Whether this effect occurs with alternative cancer cell types is not known. Alternatively, TNF $\alpha$  could exacerbate proteasome inhibition induced by CQ as reported with other proteasomal inhibitors (Rice et al., 2001).

We found that the ability of CQ to induce macrophage activation and TNF $\alpha$  release was



dependent on extracellular Cu. CQ is a Cu ionophore (White et al., 2006; Chen et al., 2007) and is therefore able to facilitate the uptake of Cu into cells including macrophages. Depletion of Cu from the culture medium prevented CQ-mediated TNF $\alpha$  release and anti-HeLa cell activity while restoring even 100 nM Cu to the medium induced toxic effects to HeLa cells in the presence of macrophages and CQ. As Cu levels can reach micro-molar concentrations *in vivo* (Wataha et al., 1996), our findings suggest that CQ could induce similar uptake of extracellular Cu *in vivo*. Interestingly, little is known about the role of Cu in direct stimulation of inflammatory responses by macrophages (Sidoti-de Fraise et al., 1998). This is the first report that ligand-mediated metal uptake can induce release of TNF $\alpha$  and a number of interleukins from macrophages. Previously, Wataha et al. (Wataha et al., 1996) reported that Cu and other metals could potentiate LPS-mediated release of interleukin 1 (IL-1) in human THP1 macrophages but Cu had no effect on TNF $\alpha$  in these cells. In contrast, Cuderì (Cuderì, 1990) observed increased TNF $\alpha$  mRNA in peripheral blood monocytes treated with Cu. It is possible that metals may have various effects on different immune cell populations or could act in concert with other, unidentified molecules. Whatever the mechanism, our findings together with recent reports by Ding et al. (Ding et al., 2005) and Chen et al. (Chen et al., 2007) further support the use of CQ as an anticancer metal ionophore rather than a metal chelator. This mode of action contrasts with cell impermeable metal chelators such as tetrathiomolybdate and desferroximine that chelate extracellular Cu, thus preventing angiogenesis (Chen et al., 2007).

The mechanism of CQ-mediated Cu uptake and TNF $\alpha$  release is uncertain. We have previously reported that epithelial cells and neuronal cell-lines treated with CQ-Cu complexes revealed robust activation of cell signalling pathways involving phosphoinositol-3-kinase (PI3K) and down stream modulation of mitogen activated protein kinases such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal regulated kinase (ERK). CQ-Cu complexes also modified phosphorylation of glycogen synthase kinase 3 (GSK3) (White et al., 2006). However, similar kinase activation by CQ or

Cu was not observed in our macrophage cultures (data not shown). An alternative mechanism could involve metal-mediated modulation of TNF $\alpha$  release from cell membranes through increased metalloprotease activity as metalloproteases are responsible for cleavage of pro-TNF at the cell surface (Bala et al., 1992). This is supported by our studies in other cell types, which have shown that CQ can induce increased production of matrix metalloproteases (White et al., 2006). Alternatively, previous studies have shown that Cu can induce release of IL-2, IL-6 and IL-8 from cells in culture (Schmalz et al., 1998; Bar-Or et al., 2003; Ju et al., 2006). Our protein array study revealed that levels of IL-2 and IL-6 were increased in culture medium of macrophages treated with CQ (Table 1). Although we did not substantiate this finding by ELISA, it is possible that elevated levels of interleukins may induce TNF $\alpha$  release from the macrophages. If this does occur, it may be triggered through Cu-dependent modulation of transcription factors that result in elevated cytokine production (Qiao et al., 2007). The specific pathway(s) activated by Cu may be dependent on which cellular compartment the metal complexes are localized to. This may also vary between different metal-ligand complexes such as CQ-Cu and PDTC-Cu. Such differences may explain why CQ did not significantly modulate NF $\kappa$ B activity in this study or a previous report (Ding et al., 2005) despite similarities in cellular metal uptake to the NF $\kappa$ B inhibitor, PDTC (Daniel et al., 2005).

Importantly, our findings have significant implications for the development of anticancer agents based on metal ligands. Our studies have demonstrated that CQ is able to enhance pro-inflammatory responses via a metal ionophore effect. While this may potentially allow more localized targeting of tumours through macrophage-dependent tumour cell toxicity, our data also suggests that metals could have an important and as yet uncharacterised role in inflammation. The level of metal found to stimulate release of TNF $\alpha$  from macrophages in this study was well within levels found in biological fluids (Wataha et al., 1996). Whether there are protective mechanisms *in vivo* that modulate metal-mediated inflammation is not known. Further studies are necessary to

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characterize the role of metals such as Cu in pro-inflammatory responses and whether metal ionophores can be developed into specific anticancer agents that target tumours through immune cell stimulation.

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**Legends for Figures**

**Figure 1:** Macrophages promote CQ-mediated inhibition of HeLa cell growth. **A:** Raw 264.7 macrophages were treated with CQ (1-10  $\mu$ M) for 24 hr and cell viability was determined using the MTT assay. CQ slightly but significantly increased macrophage cell viability at 10  $\mu$ M (\*  $p < 0.05$ ). **B:** HeLa cells were co-cultured with macrophages for 24 hr in the presence of 1-10  $\mu$ M CQ. HeLa cells were also treated with CQ without macrophages for 24 hr. Viability of HeLa cells was determined by MTT assay and this revealed a significantly greater loss of viability when HeLa cells were cultured with CQ and macrophages compared to CQ alone (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). **C:** HeLa cells were co-cultured with macrophages for 24 hr in the presence of 1-10  $\mu$ M CQ for 24 hr. The viability of the HeLa cells was determined by Trypan blue exclusion assay and compared to viability obtained with MTT assay. The Trypan blue and MTT assays both produced very similar measures of cell viability (\*  $p < 0.05$ , \*\*  $p < 0.01$  compared to untreated control). **D:** HeLa cells were cultured for 24 hr in media that had been pre-incubated on macrophage cultures for 2 or 24 hr with CQ (1-10  $\mu$ M). A significant decrease in HeLa cell viability was observed when cells were treated with macrophage-conditioned medium that had been exposed to CQ for 24 hr (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

**Figure 2:** Macrophage-mediated CQ effects on HeLa cells are Cu-dependent. **A:** HeLa cells were co-cultured with RAW 264.7 macrophages for 24 hr in the presence or absence of CQ (1-10  $\mu$ M) or CQ and BCS (200  $\mu$ M). Treatment with BCS significantly decreased HeLa cell toxicity (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). **B:** HeLa cells were cultured for 24 hr in media that had been pre-incubated on macrophage cultures for 24 hr with CQ (1-10  $\mu$ M) or CQ and BCS (200  $\mu$ M). BCS treatment significantly inhibited HeLa cell toxicity (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

**Figure 3:** Cu promotes HeLa cell toxicity by macrophages **A:** HeLa cells were treated with 1-10  $\mu\text{M}$  Cu(II) or co-cultured with macrophages and 1-10  $\mu\text{M}$  Cu(II) for 24 hr. HeLa cells co-cultured with macrophages and 10  $\mu\text{M}$  Cu(II) revealed a significant increase in cell toxicity compared to HeLa cells treated with 10  $\mu\text{M}$  Cu(II) alone (\*\*  $p < 0.01$ ). **B:** HeLa cells were co-cultured with macrophages and CQ alone (1-10  $\mu\text{M}$ ) or CQ and Cu(II) (1-10  $\mu\text{M}$  each) for 24 hr. Treatment with CQ-Cu induced the same level of HeLa cell toxicity as CQ alone. **C:** HeLa cells were cultured with macrophages in Chelex 100 treated-medium (Chelex medium) for 24 hr with CQ and increasing concentrations of Cu(II). Depletion of Cu using Chelex 100 prevented HeLa cell toxicity from CQ and macrophages. Addition of Cu(II) to the medium restored the toxic effects CQ in the co-cultures (\*\*  $p < 0.01$ ).

**Figure 4:** NF $\kappa$ B activity in macrophages treated with CQ. RAW 264.7 macrophages were treated with CQ, Cu(II) or CQ plus Cu(II) (10  $\mu\text{M}$  each) for 24 hr and NF $\kappa$ B activity was measured in nuclear extracts. No significant changes to NF $\kappa$ B levels were observed after any treatment.

**Figure 5:** CQ induces TNF $\alpha$  release from macrophages, which induces HeLa cell toxicity. **A:** RAW 264.7 macrophages were treated with CQ, Cu(II) or CQ plus Cu(II) (10  $\mu\text{M}$  each) for 24 hr and TNF $\alpha$  levels were determined in conditioned media by ELISA. LPS (100 ng/ml) was added as a positive control. Treatment of macrophages with CQ or Cu induced a significant increase in TNF $\alpha$  levels in the conditioned medium. Treatment with CQ and Cu(II) together did not induce greater levels of TNF $\alpha$  than CQ alone. Co-treatment with BCS (200  $\mu\text{M}$ ) or treatment in Chelex medium significantly inhibited TNF $\alpha$  release compared to CQ treatment alone in normal medium. Addition of Cu(II) (10  $\mu\text{M}$ ) to Chelex medium restored TNF $\alpha$  release after treatment with CQ (\*\*  $p < 0.01$  compared to

untreated cultures, \*\*\*  $p < 0.01$  compared to CQ-treated macrophages in normal medium). **B:** RAW 264.7 macrophages were treated with 1-10  $\mu\text{M}$  CQ for 24 hr and levels of  $\text{TNF}\alpha$  were measured in conditioned medium. All concentrations of CQ increased  $\text{TNF}\alpha$  levels relative to control (\*  $p < 0.05$ , \*\*  $p < 0.01$  compared to untreated control). **C:** HeLa cells were treated with conditioned medium from macrophages cultured with 10  $\mu\text{M}$  CQ for 24 hr or cultured in fresh medium containing 10.0 or 100 ng/ml recombinant  $\text{TNF}\alpha$ . After 24 hr, HeLa cell viability was determined and revealed that  $\text{TNF}\alpha$  induced a similar level of cytotoxicity compared to the conditioned medium (\*\*  $p < 0.01$  compared to untreated controls). **D:** HeLa cells were co-cultured with macrophages and 10  $\mu\text{M}$  CQ for 24 hr with or without neutralizing antibody to  $\text{TNF}\alpha$  or IL-12p40/p70 (10  $\mu\text{g}/\text{ml}$ ). Antibody to  $\text{TNF}\alpha$  significantly increased HeLa cell viability compared to treatment with CQ alone in co-cultures (\*\*  $p < 0.01$ ).

**Table 1:** Cytokine levels in medium of RAW 264.7 macrophages treated with CQ (10  $\mu$ M) for 24 hr. Cytokine levels were measured by protein array analysis.

	<b>Chemokine</b>	<b>Fold change (relative to control)</b>
1	Interleukin-6 IL-6	1.39
2	Tissue inhibitor of metalloproteinases-1 TIMP-1	1.38
3	Interleukin-3 IL-3	1.37
4	Tumour necrosis factor- $\alpha$ TNF- $\alpha$	1.35
5	Tissue inhibitor of metalloproteinases-2 TIMP-2	1.34
6	Interleukin-1 $\beta$ IL-1 $\beta$	1.34
7	Interleukin-2 IL-2	1.32
8	Interleukin-4 IL-4	1.31
9	Interleukin-9 IL-9	1.31
10	Interleukin-10 IL-10	1.31
11	Interleukin-12p70 IL-12p70	1.31
12	T cell activation protein- 3TCA-3	1.31
13	Granulocyte colony stimulating factor GCSF	1.30
14	Interleukin-12p40/p70 IL-12p40/p70	1.29
15	Lymphotoctin	1.29
16	Macrophage colony stimulating factor MCSF	1.29
17	Interleukin-1 $\alpha$ IL-1 $\alpha$	1.27

18	Interleukin-17 IL-17	1.27
19	Thymus expressed chemokine TECK	1.26
20	Soluble tumour necrosis factor receptor II sTNF RII	1.25
21	Interferon- $\gamma$ IFN- $\gamma$	1.24
22	Stromal cell derived factor-1 SDF-1	1.23

Figure 1

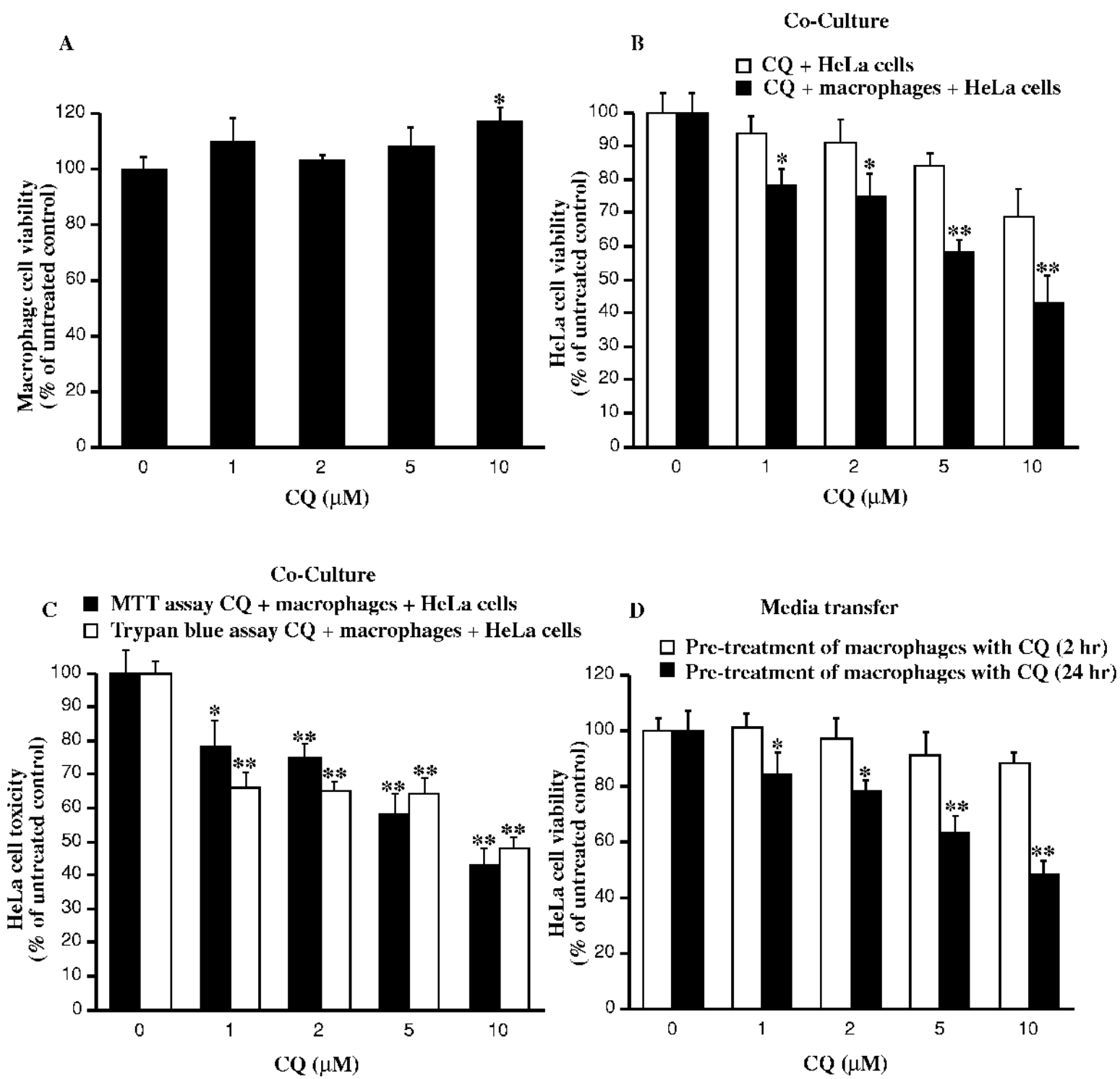




Figure 2

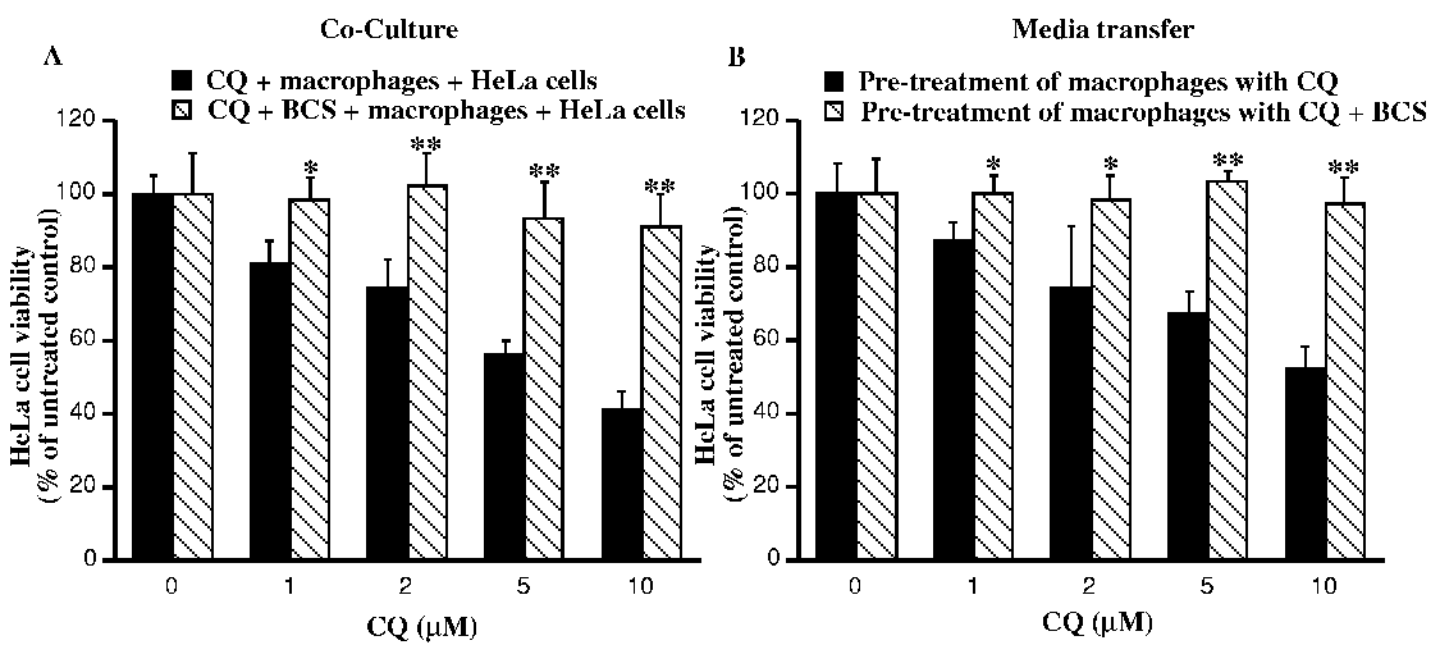
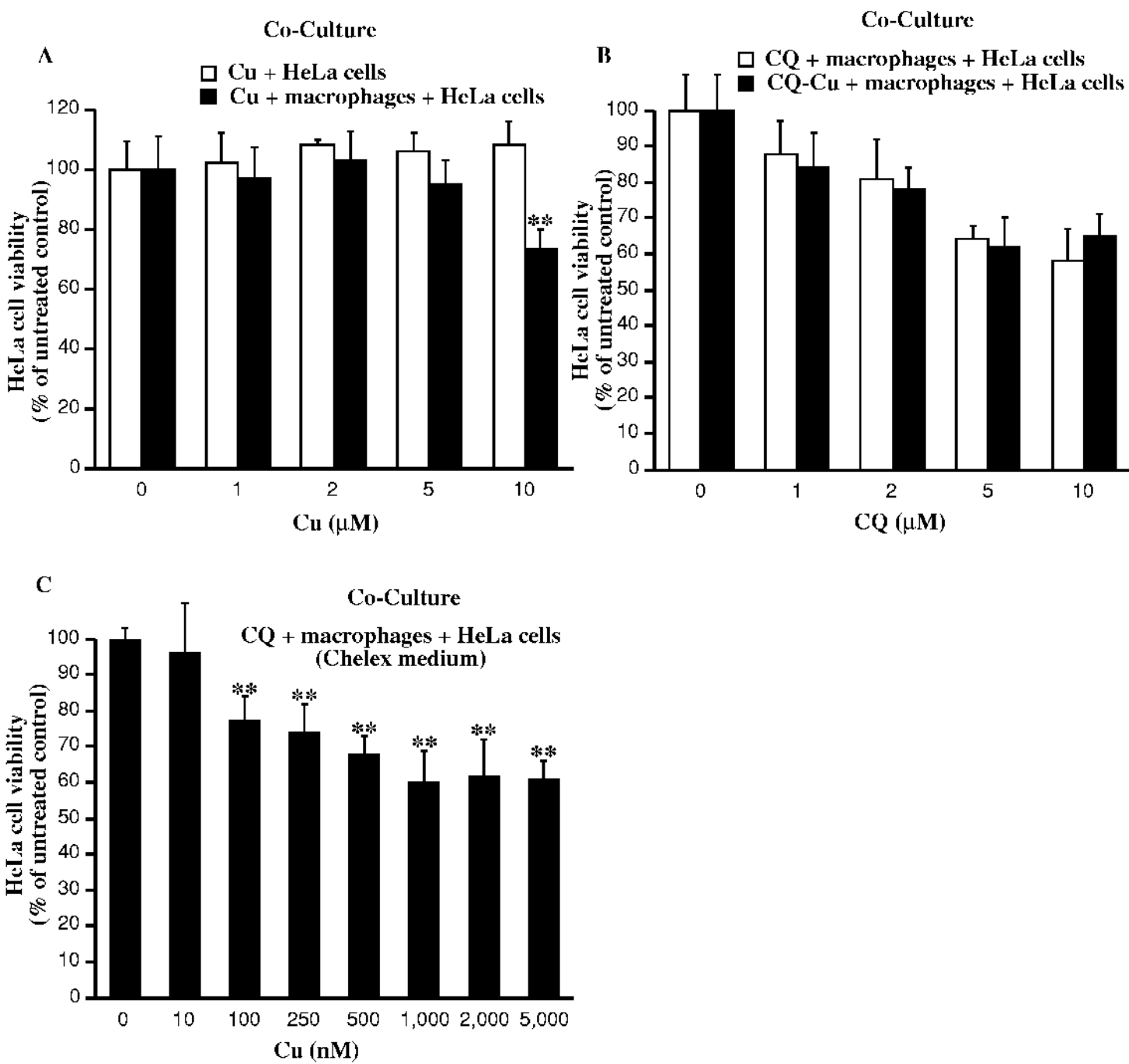
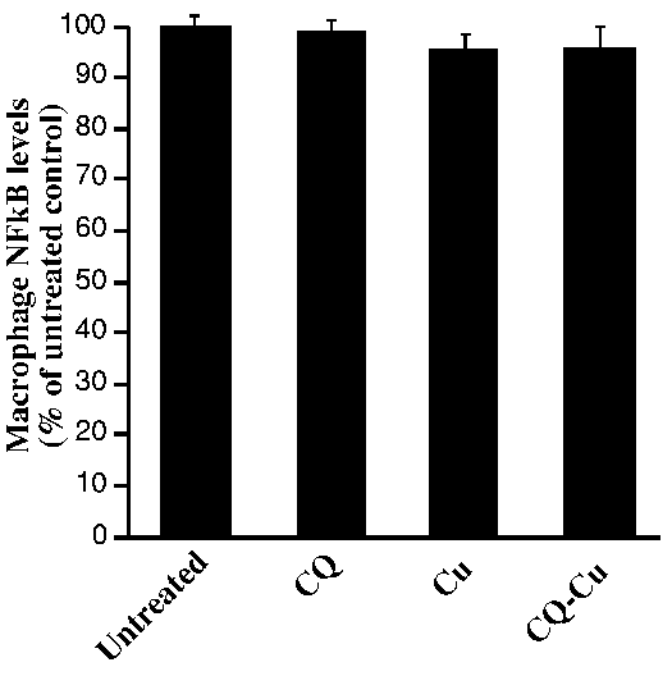


Figure 3



**Figure 4**



**Figure 5**

