REACTIVE OXYGEN SPECIES IS ASSOCIATED WITH CRYPTOLEPINE CYTOTOXICITY

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ABSTRACT

Cryptolepine (CLP), the major alkaloid of the West African anti-malarial plant Cryptolepis sanguinolenta is a known in vitro mammalian cytotoxin and a DNA intercalator. We have been studying the potential toxicity of CLP and the aqueous anti-malarial formulation (CSE) to mammalian cells in vitro. The objective of the present study was to determine if the cytotoxicity of CLP is as a result of metabolic activation and reactive oxygen species (ROS) generation. Involvement of metabolic activation was assessed by studying the differential toxicity of CLP to MCL-5 and cHoL, two human lymphoblastoid cell lines differing only in their metabolic competencies. ROS determination was performed using the fluorescent probe 2', 7'-dichlorofluorescein-diacetate (DCFH-DA), a dye commonly used to measure intracellular changes in ROS. In a Trypan blue exclusion assay, there was no difference between the growth curves of the two cell lines after exposure to CSE (5-100μg/ml) or CLP (0.5-5.0μM) for 24 hours under similar experimental conditions. Further, in an enzyme inhibition assay, CLP at 2.5μM reduced the viability of MCL-5 cells by 30% but the effect of CLP in the presence of inhibitors did not differ significantly from that of CLP alone. In contrast, both CSE and CLP caused a dose-dependent increase in ROS production, which reduced significantly following pre-treatment with N-acetylcysteine, an anti-oxidant. The results together suggest that CLP is a direct cytotoxin that readily generates damaging ROS.

Keywords: *Cryptolepine; cytotoxicity, metabolic activation, reactive oxygen species*

INTRODUCTION

Cryptolepine (CLP), the major alkaloid of the West African medicinal plant *Cryptolepis sanguinolenta* (Periplocaceae) is a known anti-malarial agent (Noamesi *et al.*, 1991; Kirby *et al.*, 1995; Wright *et al.*, 1996), a potent *in vitro* mammalian cytotoxin (Ansah and Gooderham, 2002) and a DNA intercalator (Lisgarten *et al.*, 2002). The aqueous extract of the roots (CSE), officially reported to be an anti-malarial in Ghana over twenty years ago (Boye and Ampofo, 1983) is now a popular anti-malarial remedy flooding the Ghanaian market under several trade names. We reported previously that the aqueous extract is equally cytotoxic to mammalian cells *in vitro* (Ansah and Gooderham, 2002). We had therefore reasoned on the basis of the

potent cytotoxicity of the aqueous extract that the routine use of CSE for malarial chemotherapy might be potentially harmful. To date however, there is very little understanding on the toxicology of CLP and the possible molecular mechanisms involved.

The cytotoxic effects of many chemicals are mediated through reactive intermediates following metabolic transformation. The reactive intermediate may be a metabolite of the parent compound or a reactive oxygen species (ROS) such as superoxide anion (O_2-), hydroxyl radical (.OH) or hydrogen peroxide (H_2O_2), generated by the parent compound or its metabolite. ROS formation leading to oxidative stress can cause considerable damage to biological macromolecules and has been implicated in aging and a variety of human diseases including certain cancers, neurodegenerative and cardiovascular diseases (Floyd, 1990; Jenner, 1994)

Whether cryptolepine undergoes metabolism prior to cytotoxicity or is reactive in the unmetabolised form is currently unknown. The present work aims at investigating whether the cytoxicity of CLP and the traditional formulation, the aqueous extract of the roots (CSE) is mediated through metabolic activation via the chemical formation of reactive oxygen species (ROS)..

Materials and Methods

Chemicals and Reagents

The preparation of the aqueous extract (CSE) to simulate the local method of preparation has been described previously (Ansah and Gooderham, 2002) and CLP was a kind donation from Dr J Addae-Kyereme of the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Minimum essential medium (MEM), Foetal calf serum (FCS), L-glutamine, penicillin/streptomycin and trypsin/ethylenediamine tetra-acetic acid (trypsin/EDTA) were purchased from Invitrogen Corporation (Paisley, Scotland, UK). Roswell Park Memorial Institute 1640 medium (RPMI 1640) was obtained from Gentest (Woburn, MA, USA). All other chemicals unless indicated in the text were obtained from Sigma Chemical Company (Poole, England) and were of Analar grade or the best equivalent.

Cell culture and Conditions

MCL-5, a human lymphoblastoid cell line transfected with two plasmids encoding multiple cytochrome P450s (CYP450) (CYP1A2, 2A6, 2E1, 3A4) and epoxide hydroxylase genes, and also has inducible/constitutive CYP1A1 (Crespi *et al.*, 1991) was obtained from Gentest (Woburn, MA, USA). These were grown in Gentest RPMI 1640 media (without histidine and with 2mM histidinol for plasmid selection), supplemented with 9% horse serum, 2mM L-glutamine and 100units of penicillin/streptomycin. Cells were grown to a density of 10^6 cells/ml and sub-cultured to 2.5 x 10^5 cells/ml every 48 hours. Hygromycin B (100μg/ml) was added at each sub-culturing for plasmid maintenance. cHoL are the same human lymphoblastoid cell line as MCL-5 cells, but do not have the CYP expressing plasmid DNA and are therefore metabolically non-competent. These were grown in the MCL-5 medium without hygromycin B.

HepG2, a human hepatoma cell line was propagated in MEM supplemented with 10% FCS, 2mM L-glutamine and 100 units of penicillin/streptomycin. Routinely, cells were maintained at 37°C in a humidified atmosphere of 95%/5% air/CO_2, and harvested by trypsinization with trypsin-EDTA. MCL-5 and cHoL cells were suspension cells and therefore did not require trypsin-EDTA for sub-culturing.

Possible role of metabolic activation in the Toxicity of CSE and CLP

The possible involvement of metabolic activation in the toxicity of CLP was investigated by studying the differential toxicity of CSE and CLP on cHoL and MCL-5 cells. Exponentially growing cells (2 x 10^6) were treated with varying

concentrations of CSE and CLP for 24 hours after an overnight incubation at 37°C. The cells were then harvested by centrifugation (room temperature at 200 g for 5 minutes), stained with 0.4% Trypan blue and counted manually under light microscope using a haemocytometer.

The effect of metabolic inhibitors on CLP toxicity in MCL-5 cells was also studied. In this study, human lymphoblastoid MCL-5 cells were grown and maintained as described. Exponentially growing cells were seeded overnight at 1 x 10^5 cells/well in 24-well tissue culture plates. The cells were then treated with a single dose of 2.5μM of CLP with or without specific inhibitors of CYP enzymes at concentrations known to cause substantial inhibition of CYP activity. The following inhibitors were used, 25μM ketoconazole (KT), a CYP 3A4 inhibitor (Gibbs *et al.*, 2000); 100μM diethyldithiocarbamate (DED), a CYP 2A6 inhibitor (Chang *et al.*, 1994)); 25μM alpha-naphthoflavone (αN), a CYP1A inhibitor (Chang *et al.*, 1994) and 25μM 3-amino-1, 2, 4-triazole (ATZ), a CYP2E1 inhibitor (Koop, 1990). After 24 hours incubation at 37°C, cell viability was assessed using the Alamar blue assay.

Viability of cells by the Resazurin (Alamar blue™) reduction assay

The toxicity of CSE and CLP was tested on HepG2 cells using the Resazurin reduction assay (Alamar blue assay) (Field & Lancaster, 1993, O'Brien *et al.*, 2000). Briefly, 100μl of 1% Alamar blue in water was added to each of the treated wells and the cell viability (fluorescence) was determined by means of a plate reader Fluostar® (BMG Labtechnologies, Offenburg, Germany) at 530nm excitation and 590 nm emission.

Analysis of reactive oxygen species (ROS) in HepG2 cells

ROS determination was performed using 2', 7'-dichlorofluorescein-diacetate (DCFH-DA), a dye commonly used to measure intracellular changes in ROS (Chen *et al.*, 1998). DCFH-DA diffuses readily into cells and is hydrolyzed by intracellular esterases to yield $DCFH_2$ (non-fluorescent), which is trapped within cells. ROS produced by cells oxidizes $DCFH_2$ to the highly fluorescent compound 2', 7'-dichlorofluorescein (DCF). Thus the fluorescent intensity is proportional to the amount of ROS produced by the cells.

Exponentially growing HepG2 cells (1.5 x 10^6) in 5ml culture medium were treated with vehicle (culture medium), CSE, CLP, H_2O_2 either alone or in combination with the anti-oxidant N-acetylcysteine (NAC) for a period of 4 hours. Immediately after treatment, cell cultures were dosed with 100 μ M DCFH-DA and incubated at 37°C for 1 hour. After washing the cultures twice, cells were trypsinized and resuspended in 1ml of cold phosphate buffered saline (PBS). The green fluorescence of DCF was excited by using an argon laser and was detected using a 525-nm band pass filter by flow cytometry analysis. Ten thousand (10,000) cells were analyzed per sample.

Statistical analysis

Significant differences were calculated using analysis of variance (ANOVA) or the students' t-test and p-values of <0.05 were considered significant.

Results and Discussion

Over the years, there has been a gradual diminution in the effectiveness of chloroquine as a first line anti-malarial medication due to the development of drug resistance. Further, side effects including pruritus, gastro-intestinal disturbances, headache and retinopathy have recently led to a decline in compliance with chloroquine usage amongst both the rural and urban communities as a cost-effective remedy for the management of malaria. These have led to the development of alternative anti-malarial medications such as halofanthrine and artesunate, which appear to be more effective than chloroquine but are neither

available nor affordable to the rural population who constitute about 70% of the population in Ghana. These factors have contributed immensely to the popularity of the aqueous root extract of *Cryptolepis sanguinolenta* (CSE) as a reliable and cost-effective approach to the management of malaria. Though, we are not aware of any adverse reports associated with the use of CSE, the traditional malarial therapy, this is not suggestive of safety since in general adverse reaction monitoring on herbal medications is far lower compared to orthodox medications

Recent reports on the DNA intercalating properties of the major alkaloid of CSE, CLP (Bonjean *et al.*, 1998; Lisgarten *et al.*, 2002), raised the possibility that the agents may evoke toxicity in host cells. These considerations prompted us to study the toxicology of this popular anti-malarial formulation. We had reported previously that both CSE and CLP showed potent toxicity to a variety of mammalian cells *in vitro* (Ansah and Gooderham, 2002).

In the present study we sought to determine if CSE and CLP require metabolic activation to exhibit their toxicity. We therefore selected two cell lines, which differed only in their metabolic competency. MCL-5 cell line is a human lymphoblastoid cell line transfected with two plasmids encoding multiple CYP450 and epoxide hydrolase genes and also possess constitutive/inducible CYP 1A1 (Crespi *et al.*, 1991). The cHoL cells are genetically identical to MCL-5 cells but lack the transfected plasmids coding for the drug metabolizing enzymes and are therefore metabolically non-competent. Cytochrome P450 oxidative enzymes (CYP's) are mostly involved in Phase I biotransformation, converting pro-toxic chemicals to more reactive species. A requirement for metabolic activation prior to CLP toxicity should therefore reflect in the toxicity profiles of the two cell lines. In a Trypan blue exclusion assay, there was no difference between the growth curves of the two cell lines after exposure to CSE (Figure 1a) or CLP (Figure 1b) for 24 hours under similar experimental conditions. In an enzyme inhibition assay, the CLP at 2.5μM reduced the viability of the MCL-5 cells by about 30%, but none of the specific inhibitors used in isolation showed any

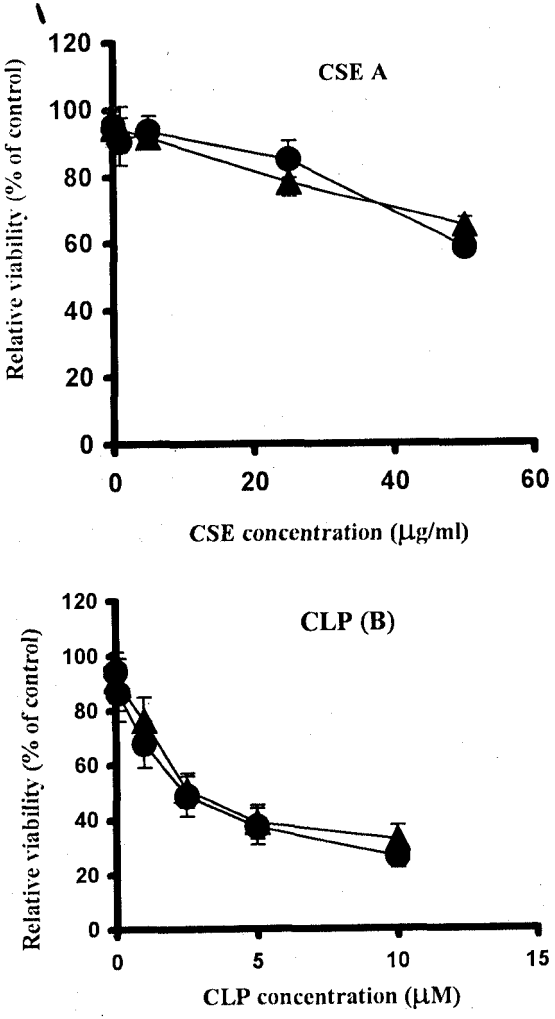

Figure 1

Viability of cHoL (▲) and MCL-5 (•) cells after treatment with CSE (A) and CLP (B) for 24 h as measured by Trypan blue exclusion. Treated cells were centrifuged out of drug medium and washed twice with PBS. They were then counted manually using a haemocytometer under a light microscope after staining with 0.4% Trypan blue. Indicated points are means of three separate experiments and bars are standard deviations.

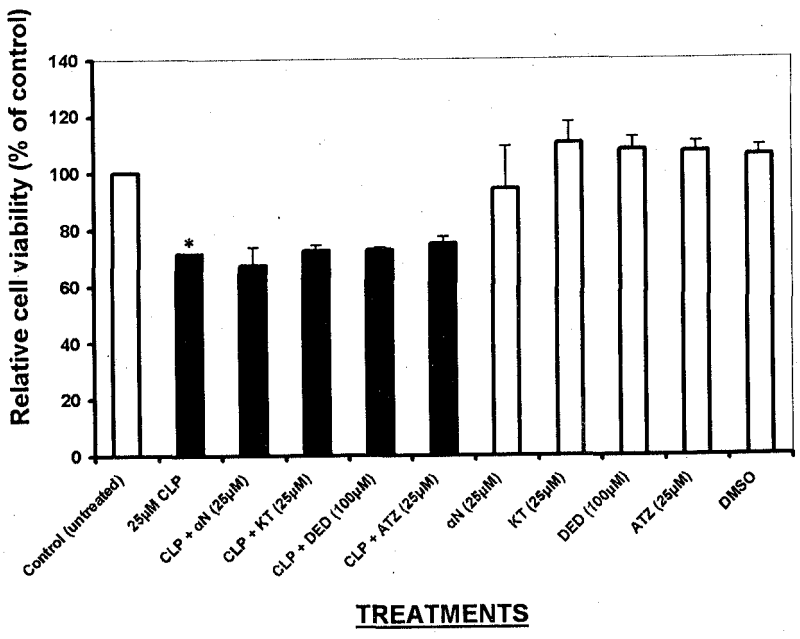

Figure 2

*Effect of CYP450 inhibitors on the toxicity of CLP. MCL-5 cells were cultured as described in the methods. Cultures were then treated with 2.5μM CLP with or without the enzyme inhibitors (ketoconazole (KT), a CYP 3A4 inhibitor; diethyldithiocarbamate (DED), a CYP 2A6 inhibitor; alpha-naphthoflavone (αN), a CYP1A inhibitor and 3-amino-1, 2, 4-triazole (ATZ), a CYP2E1 inhibitor) for 24 hours after which cell viability was assessed by the Alamar blue assay. Vehicle and inhibitor only treated cultures served as controls. Bars are the means ±SD of three separate experiments. * Significant compared to untreated control, $p<0.001$ (t-test).*

significant effect on the viability of the cell line compared to the untreated control (Figure 2). Interestingly, the effect of CLP in the presence of the inhibitors did not differ significantly from that of CLP alone (Figure 2). These results suggest that none of the inhibitors used appear to modulate the toxicity of CLP and that CLP is a direct-acting cytotoxin.

ROS production and the associated oxidative stress can alter cellular calcium homeostasis and induce dissipation of the membrane potential (Polla *et al.*, 1996), disruption of the mitochondrial membrane and a rapid demise of the cell. We therefore investigated the possible involvement of ROS in CSE-/CLP-induced cell death on the rapid and potent cell-kill effects of CSE and CLP.

For this purpose we chose HepG2 cells, which although have very limited metabolic capability are known to possess efficient protective mechanisms towards oxidative injury. First, we determined the toxicity of CSE and CLP to this cell line using the Alamar blue assay. HepG2 cells were treated with CSE and CLP continuously for 24 and 48 hours. CSE and CLP reduced the viability of HepG2 cells in a dose- and time-dependent manner (Figures 3a and b). This result confirms the toxicity of CSE and CLP to this human hepatoma cell line, in agreement with our

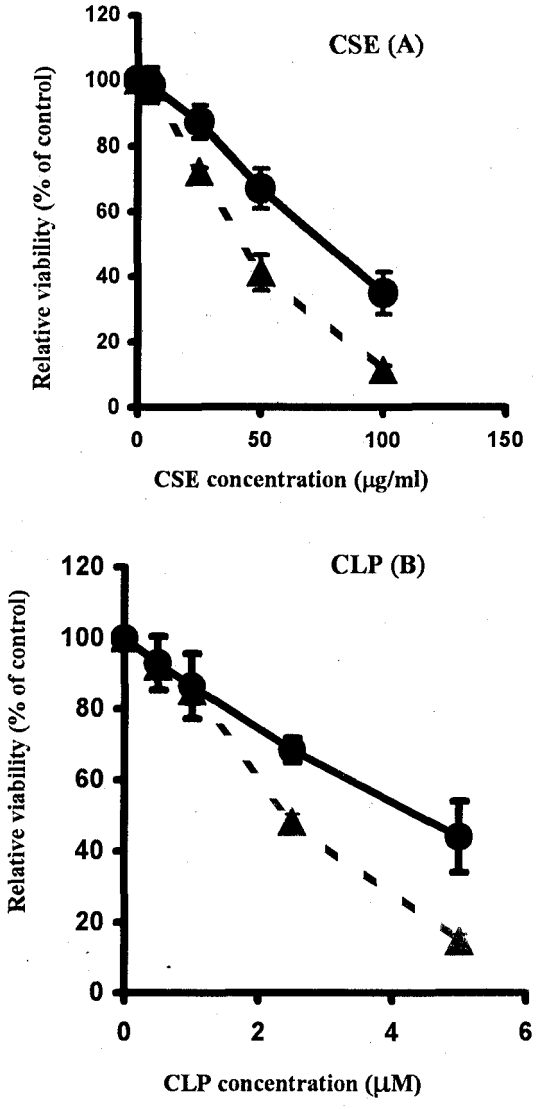

Figure 3

Effect of CSE (A) and CLP (B) on viability of HepG2 cells treated for 24 hours (•) or 48 hours (▲) using the Alamar blue method. Cells were continuously exposed to the indicated concentrations of the agents for 24 or 48 hours; then viability assessed by the Alamar blue assay. Values are the means of three independent experiments each in quadruplicate and bars are standard deviations.

previous finding of broad cytotoxic activity of the agents to mammalian cells (Ansah and Gooderham, 2002).

Levels of ROS were then determined in HepG2 cells treated with CSE and CLP as described in the methods using DCFH-DA. DCFH-DA is hydrolysed by intracellular esterases to $DCFH_2$, which is then oxidized to the fluorescent compound DCF in the presence of ROS. HepG2 cells with or without pre-treatment with the anti-oxidant N-acetylcysteine (NAC) for 1h were exposed to vehicle, H_2O_2, CSE or CLP continuously for 4h. Samples were then analyzed for ROS levels by flow cytometry. Indeed, both CSE and CLP caused a significant dose-dependent increase in ROS production compared to the vehicle-treated (culture medium) control (Figure 4 and 5). At the highest doses used, CSE (50μg/ml) and CLP (5.0μM) were more potent in generating ROS than H_2O_2 (100μg/ml). Pre-treatment with NAC reduced significantly ROS generation by CSE, CLP and H_2O_2 suggesting that the toxicity of the agents to HepG2 cells could at least in part be due to increased ROS production. To our knowledge this is the first report on the possible involvement of ROS in the toxicity of CSE and CLP.

ROS generation leading to oxidative stress is implicated in a variety of diseases including AIDS, Parkinson's disease, amyotrophic lateral sclerosis, *diabetes mellitus*, atherosclerosis, schizophrenia, cancer, Alzheimer's disease and retinal degenerative disorders (Floyd, 1990; Jenner, 1994; Mahadik and Scheffer, 1996). The brain is highly vulnerable to the effects of oxidative stress as the anti-oxidant defences of the central nervous system (CNS) are relatively low with almost no catalase and low levels of glutathione (Slivka *et al.*, 1987). The generation of high levels of ROS as observed in the present study could therefore be important if CLP is available to brain tissue. ROS is also known to cause chemical alterations in lipids, proteins, carbohydrates and nucleic acids (Wiseman and

Halliwell, 1996) and induce DNA damage in cells (Schraufstatter *et al.*, 1988; Halliwell and Aruoma, 1991). The generation of high-level ROS by CSE and CLP could therefore contribute to DNA damage in exposed cells.

Our data clearly demonstrate that the traditional anti-malarial formulation, CSE induces ROS generation in HepG2 cells in a similar manner to CLP, its major alkaloid. These findings suggest that the agents have the propensity to provoke oxidative stress, which is implicated in a wide variety of diseases. , Further studies aimed at

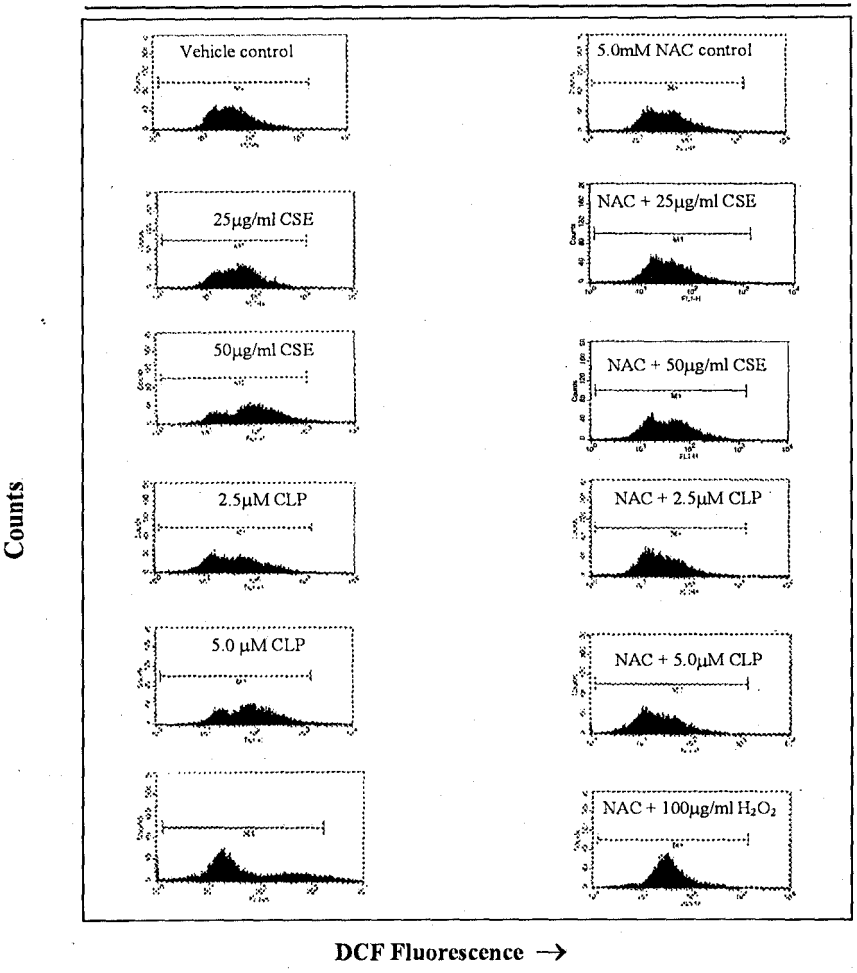

Figure 4

Induction of ROS in HepG2 cells treated with CSE and CLP for 4 hours using the DCFH-DA assay. After treatment, HepG2 cells were washed twice with PBS to remove the extracellular CSE or CLP. Samples were then loaded with 100µM DCFH-DA for 1 hour as described in the materials and methods. The fluorescent intensity (DCF fluorescence) was then measured by flow cytometry analysis. Mean fluorescent intensities from three independent experiments were used to generate the histogram in Figure 5. Representative fluorescent intensities are shown.

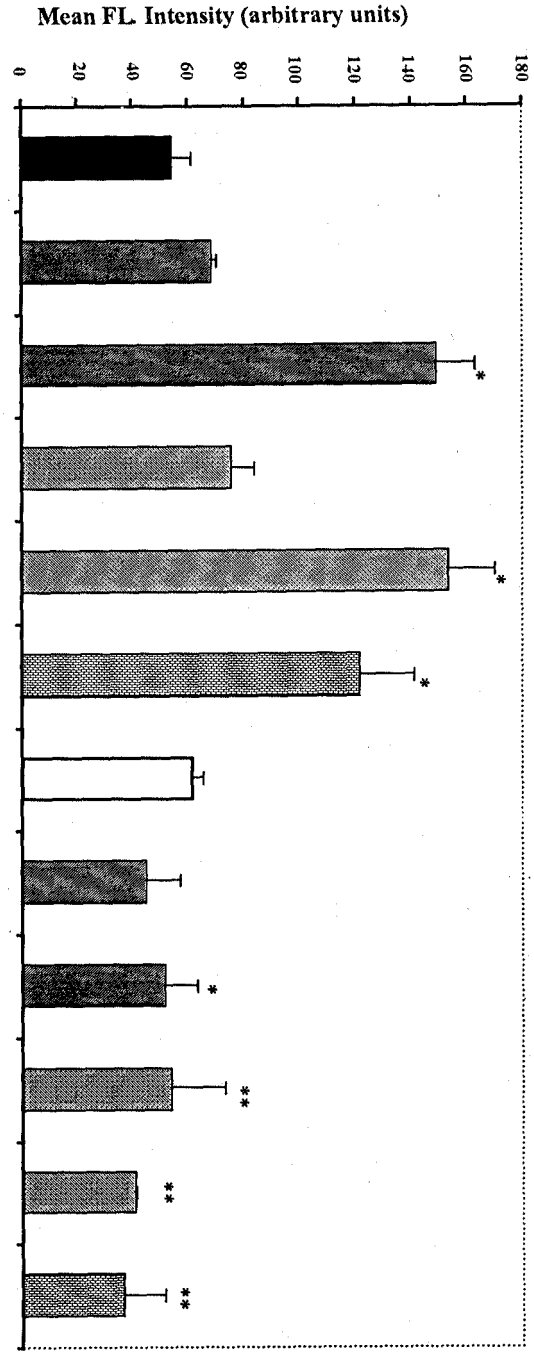

Figure 5

*Generation of ROS in HepG2 cells treated with CSE and CLP for 4 hours using the DCFH-DA assay. After treatment, HepG2 cells were washed twice with PBS to remove the extracellular CSE or CLP. Samples were then loaded with 100µM DCFH-DA for 1 hour as described in the methods. The fluorescent intensity (DCF fluorescence) was then measured by flow cytometry analysis. Each value is presented as the mean ± SD of mean fluorescent intensities of three independent experiments one of which is presented in Figure 4. * Significantly different from control; and ** CSE/CLP with NAC significantly different from CSE/CLP without NAC ($p < 0.001$) as measured by analysis of variance (ANOVA).*

determining whether ROS generation occurs *in vivo* in a similar manner as observed in the current studies are clearly warranted.

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