รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาผลของยาฆ่าแมลงคลอร์ไพริฟอสต่อการเจริญเติบโต อยู่รอด และเคลื่อนแผ่กระจายของเซลล์มะเร็งลำไส้และตับ: บทบาทของระบบนอนนิวโรนอลคอลิเนอร์จิก

โดย

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มิถุนายน 2558

สัญญาเลขที่ TRG5680007

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สถาบันวิจัยจุฬาภรณ์

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และสถาบันวิจัยจุฬาภรณ์

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ผู้วิจัยขอแสดงความเคารพและขอขอบพระคุณนักวิจัยที่ปรึกษา รศ.ดร.จุฑามาศ สัตยวิวัฒน์ หัวหน้าห้องปฏิบัติการเภสัชวิทยา สถาบันวิจัยจุฬาภรณ์ หัวหน้าสาขาวิชาพิษวิทยาสิ่งแวดล้อม สถาบันบัณฑิตศึกษาจุฬาภรณ์ และผู้ช่วยประธานสถาบันวิจัยจุฬาภรณ์ ฝ่ายธุรการวิทยาศาสตร์ ซึ่งการวิจัยนี้จะไม่สำเร็จลุล่วงได้เลยหากไม่ได้รับการชี้แนะที่มีประโยชน์อย่างยิ่งจากท่าน นอกจากนี้ ผู้วิจัยขอขอบพระคุณ ดร.ปิยจิต วัชรศิษย์ นักวิจัยชำนาญการ ห้องปฏิบัติการเภสัชวิทยา สถาบันวิจัยจุฬาภรณ์ ที่ให้คำแนะนำให้การปรับปรุงบทความสำหรับการเผยแพร่ ขอขอบคุณนายภูมิ เตชชาติวนิช นส.กาญจนา ชัยยศ นส.ดารณี วิสิฐนนทชัยและนักวิจัยห้องปฏิบัติการเภสัชวิทยาทุกท่านที่ช่วยสนับสนุนการดำเนินการวิจัยนี้ให้สำเร็จลุล่วงได้

สุดท้ายนี้ผู้วิจัยขอขอบพระคุณสำนักงานกองทุนสนับสนุนการวิจัยและสถาบันวิจัยจุฬาภรณ์ที่มอบทุนส่งเสริมนักวิจัยรุ่นใหม่ให้กับผู้วิจัยเพื่อใช้ในการศึกษาวิจัยนี้

Abstract

Project Code : TRG5680007

Project Title : The effects of chlorpyrifos and its metabolite on colon and liver cancer cell growth, survival and migration: Role of non-neuronal cholinergic system

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Project Period : 03/06/2013 – 03/06/2015

Apart from the effects on neuronal cholinergic system, the epidemiological study suggests the association of chlorpyrifos exposure and cancer risk. This *in vitro* study examined the effects of chlorpyrifos (CPF) and its toxic metabolite, chlorpyrifos oxon (CPF-O), on the growth of human colorectal adenocarcinoma H508, normal colon epithelial CCD841, liver hepatocellular carcinoma HepG2, and normal liver hepatocyte THLE-3 cells. The results showed that CPF (0.1-100 μM) not CPF-O concentration dependently increased viability of H508 and CCD841 cells in serum free condition, and this increasing effect was not found in HepG2 and THLE-3 cells. Meanwhile, CPF-O (50-100μM) reduced the viability of all cell lines. The cell cycle analysis showed the induction of cell in the S phase, and the EdU incorporation assay revealed the induction of the DNA synthesis in CPF-treated H508 cells. Even though, the inhibitory effect on the acetylcholinesterase activity and the stimulating effect on the generation of reactive oxygen species (ROS) were observed in CPF treatment but atropine which is a non-selective muscarinic acetylcholine receptor antagonist, and N-acetylcysteine (NAC), which is an antioxidant, did not reverse the growth promoting effect of CPF. Furthermore, CPF increased the phosphorylation of epidermal growth factor receptor (EGFR) and its downstream effector, extracellular signal regulated kinase (ERK1/2) in H508 cells. Moreover, AG-1478, a specific EGFR tyrosine kinase inhibitor, and U0126, a specific MEK inhibitor, completely mitigated the growth promoting effect of CPF. All together, these results suggest that CPF promotes the growth of colorectal adenocarcinoma H508 cells through the activation of EGFR/ERK1/2 signaling pathway.

Keywords : Chlorpyrifos, chlorpyrifos oxon, colon cancer, cancer cell growth, EGFR, H508 cells

บทคัดย่อ

รหัสโครงการ: TRG5680007

ชื่อโครงการ: การศึกษาผลของยาฆ่าแมลงคลอร์ไพริฟอสต่อการเจริญเติบโต อยู่รอด และเคลื่อนแผ่กระจายของเซลล์มะเร็งลำไส้และตับ: บทบาทของระบบนอนนิวโรนอลคอลิเนอร์จิก

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นอกเหนือไปจากความเป็นพิษต่อระบบประสาทคอลิเนอร์จิกแล้ว การศึกษาทางระบาดวิทยายังชี้ให้เห็นถึงความสัมพันธ์กันระหว่างการได้รับสัมผัสยาฆ่าแมลงคลอร์ไพริฟอสและความเสี่ยงของการเป็นมะเร็ง การศึกษาวิจัยในหลอดทดลองนี้มีวัตถุประสงค์เพื่อศึกษาผลของคลอร์ไพริฟอสและอนุพันธ์ที่มีพิษของมันซึ่งได้แก่ คลอร์ไพริฟอสออกซอน ต่อการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่ชนิด H508 เซลล์มะเร็งตับชนิด HepG2 เซลล์ผนังลำไส้ใหญ่ปกติชนิด CCD841 และเซลล์ตับปกติชนิด THLE-3 จากผลการทดลองพบว่า คลอร์ไพริฟอสที่ความเข้มข้น 0.1-100 ไมโครโมลาร์ สามารถเพิ่มจำนวนการมีชีวิตของเซลล์ลำไส้ใหญ่ H508 และ CCD841 ได้ในสภาวะที่ไม่มีซีรัมในอาหารเลี้ยงเซลล์ โดยการตอบสนองนี้จะแปรผันตรงกับความเข้มข้นของคลอร์ไพริฟอส ทั้งนี้ผลของคลอร์ไพริฟอสต่อการเพิ่มจำนวนการมีชีวิตของเซลล์นี้ไม่สามารถตรวจพบได้ในเซลล์ตับ HepG2 และ THLE-3 ในขณะที่คลอร์ไพริฟอสออกซอนจะทำให้เกิดการลดลงของการมีชีวิตของเซลล์ทั้ง 4 ชนิดที่ใช้ในการศึกษานี้ จากการศึกษาวัฏจักรของเซลล์แสดงให้เห็นว่ามีการเพิ่มขึ้นของเซลล์ที่อยู่ในช่วงแบ่งเซลล์ และยังพบว่ามีการสร้างสายพันธุกรรมใหม่เพิ่มขึ้นในเซลล์มะเร็งลำไส้ใหญ่ H508 ที่ได้รับคลอร์ไพริฟอสที่ความเข้มข้น 50-100 ไมโครโมลาร์ และแม้ว่าจะตรวจพบว่าคลอร์ไพริฟอสสามารถยับยั้งการทำงานของเอมไซม์อะซิติวโคลีนเอสเตอร์เรสและกระตุ้นการสร้างสารอนุมูลอิสระได้ แต่อะโทรปีนซึ่งเป็นสารปิดกันการทำงานของตัวรับสัญญาณมัสคารินิกและสารเอ็นอะซิติวซีสทีอีนซึ่งเป็นสารต้านอนุมูลอิสระไม่สามารถที่จะยับยั้งฤทธิ์ในการกระตุ้นการเจริญเติบโตของคลอร์ไพริฟอสได้ นอกเหนือไปจากนี้ยังพบว่าคลอร์ไพริฟอสสามารถกระตุ้นการทำงานของตัวรับสัญญาณ EGFR และโปรตีนรับสัญญาณ ERK1/2 ได้อีกด้วย และพบว่าสารยับยั้งการทำงานของตัวรับสัญญาณ EGFR ซึ่งได้แก่ AG-1478 และสารยับยั้งการทำงานของโปรตีน MEK ซึ่งได้แก่ U0126 สามารถยับยั้งฤทธิ์ในการกระตุ้นการเจริญเติบโตของคลอร์ไพริฟอสได้ กล่าวโดยสรุปได้ว่าคลอร์ไพริฟอสสามารถกระตุ้นการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่ชนิด H508 ได้โดยผ่านทางการกระตุ้นของตัวรับสัญญาณ EGFR และการส่งต่อสัญญาณของ ERK1/2

คำหลัก : ยาฆ่าแมลง คลอร์ไพริฟอส เซลล์มะเร็งลำไส้ คอลิเนอร์จิก

เนื้อหางานวิจัย

Introduction

Chlorpyrifos [O,O-diethyl-O-(3,5,5-trichloro-2-pyridyl)-phos-phorothioate] (CPF) is the most extensively used broad-spectrum organophosphate insecticide that has been widely applied to agricultural crop over more than 100 countries, such as Unite State, Canada, the United Kingdom, Spain, France, Italy, Australia, and Thailand (Colt et al., 2004; Panuwet et al., 2008). Although, the U.S. Environmental Protection Agency banned CPF for residential pest control uses in 2001, however many countries still use CPF for that purpose. The primary target of CPF toxicity is both the central and peripheral cholinergic neural systems, due to its ability to inhibit the acetylcholinesterase (AChE) activity (Mileson et al., 1998). CPF itself is a weak anti-AChE compound and in order to exert this inhibitory effect, CPF has to undergo an oxidation desulfuration to its oxygen (oxon) analogue, chlorpyrifos oxon (CPF-O), by the cytochrome P450 monooxygenase system, which is highly prevalent in the liver (Sultatos et al., 1984). It has been reported that CPF-O inhibited AChE activity up to 28 and 180 orders of magnitude more potent than the parent compound CPF in the immature and differentiated brain cells, respectively (Monnet-Tschudi et al., 2000). As a result of the irreversibly binding of CPF and CPF-O to the active site of AChE, the enzyme ability to hydrolyze neurotransmitter acetylcholine (ACh) is defected which causes an accumulation of ACh at the neuronal cholinergic synapses, over-activation of cholinergic signaling, and results in cholinergic toxicity (Howard et al., 2007).

It is well established that non-neuronal cholinergic system is functionally present on certain types of cancer cells including of lung (Song and Spindel, 2008), colon (Cheng et al., 2008b; Novotny et al., 2011; Pettersson et al., 2009), liver (Zhao et al., 2011), prostate (Rayford et al., 1997), cervical (Parnell et al., 2012) and breast cancers (Espanol et al., 2007; Negroni et al., 2010). The non-neuronal cholinergic system plays a key role in the regulation of important cell functions including proliferation, differentiation, migration, secretion, organization of the cytoskeleton, cell-to-cell communication, and other features critical for cancer progression (Paleari et al., 2008; Schuller, 2009; Shah et al., 2009). Recent study has shown that the

expression of AChE is often down-regulated in hepatocellular carcinoma and it functions as a tumor growth suppressor in regulating cell proliferation and increases the drug sensitivity via its enzymatic activity (Zhao et al., 2011). In addition, for example, human colon cancer cell can increase physiological responses; invasion, migration and proliferation via cholinergic muscarinic receptor activation (Belo et al., 2011; Cheng et al., 2008a). It has been demonstrated that the expression of anti-apoptotic protein, Bcl-2, can be induced by cholinergic muscarinic receptor signaling resulting in elevating the cell viability and hindering cell death (Budd et al., 2003).

The evidences that CPF contributes to cancer are still limited. Up to now, the International Agency for Research on Cancer (IARC) has not evaluated the carcinogenicity of CPF (IARC, 2010). Furthermore, the weight of biological evidences reviewed by the Environmental Protection Agency of the United State (U.S.EPA) and Canadian Pest Management Regulatory Agency (PMRA) do not suggest that CPF is a carcinogenic pesticide (Health Canada, 2003; Smegal, 2002). However, the epidemiological studies related to occupational pesticide exposures and cancer incidences in the Agricultural Health Cohort Study (AHS) showed that pesticide applicators with the highest lifetime exposure-days for CPF had increased colorectal and lung cancer risk with a significant exposure-response relationship relative to non-exposed applicators (Lee et al., 2007; Lee et al., 2004). Furthermore, a recent study demonstrated the action of CPF as an environmental breast cancer risk factor due to its effects on the mechanisms that modulate breast cancer cell proliferation (Ventura et al., 2012). However, it is difficult to conclude at this time regarding the causal nature of these associations, therefore, further studies are required.

It is well known that CPF causes ACh accumulation in the neuronal cholinergic synapses leading to over stimulation of cholinergic receptors (Howard et al., 2007). Together with cholinergic receptor activation, CPF causes non-neuronal cholinergic cancer cell proliferation especially colorectal and liver cancer (Cheng et al., 2008a; Paleari et al., 2008; Zhao et al., 2011). We hypothesize that as a result of AChE inhibiting action of CPF and CPF-

O, an accumulation of ACh in cancer cell is occurred and further causes cancer cell growth through an activation of cholinergic signaling.

Objectives

This *in vitro* study examined the effects of CPF and CPF-O on the growth of human colorectal adenocarcinoma H508, normal colon epithelial CCD841, liver hepatocellular carcinoma HepG2, and normal liver hepatocyte THLE-3 cells. Role of the non-neuronal cholinergic signaling and oxidative stress in the growth promoting effect of CPF were studied. The mechanistic effect of CPF in the growth promoting effect was also investigated.

Materials and Methods

Chemicals and reagents

Chlorpyrifos (diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate) (CPF; purity 99.9%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chlorpyrifos oxon (CPF-O; purity 98.9%) was ordered from Chem Service (West Chester, PA, USA). Epidermal growth factor (EGF) was obtained from BD Biosciences (Bedford, MA, USA). Carbamoylcholine chloride (carbachol), atropine sulfate, mecamylamine hydrochloride, dimethylsulfoxide (DMSO), and N-acetylcysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A specific EGFR tyrosine kinase inhibitor, tyrphostin AG-1478, was purchased from Calbiochem (Germany). A selective MEK1/2 inhibitor, U0126, was ordered from Cell Signaling Technology (Beverly, MA, USA). The stock solutions of CPF and CPF-O were prepared in ethanol (Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 100 mM. Carbachol, mecamylamine, atropine, EGF, and NAC were freshly prepared by dissolved in sterile water at the concentrations of 1 M, 100 mM, 10 mM, 100 μg/ml, and 100 mg/ml, respectively. AG-1478 and U0126 were prepared as a stock solution in DMSO at the concentration of 30 mM and 10 mM, respectively.

Cell lines

Cell lines including Hep-G2 cell line (a human epithelial hepatocellular carcinoma), THLE-3 cell line (a human normal liver epithelial-immortalized with SV40 large T-antigen), NCI-H508 cell line (a human epithelial colorectal adenocarcinoma), and CCD-841-Con cell line (a human normal colon epithelial-immortalized with SV40 large T-antigen) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Hep-G2 cells were cultured in minimum essential medium (MEM) (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), 1.0 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA), 100 unit/ml penicillin, 100 μg/ml streptomycin (Gibco, Carlsbad, CA, USA), and 10% (vol/vol) fetal bovine serum (FBS) (JR Scientific, Woodland, CA, USA). THLE-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 25 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 100 unit/ml of penicillin and 100 μg/ml streptomycin. NCI-H508 cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 4.5 g/l glucose (Sigma-Aldrich, St. Louis, MO, USA), 100 unit/ml of penicillin, 100 μg/ml streptomycin and 10% FBS. CCD-841-Con cells were cultured in DMEM supplemented with 10% FBS, 100 unit/ml of penicillin and 100 μg/ml streptomycin. All cells were maintained at 37ºC in a saturated humidity atmosphere containing 95% air and 5% CO_2.

Cell viability assay

Cell viability were measured by PrestoBlue™ cell viability assay (Molecular Probes, Invitrogen, Carlsbad, CA, USA) showing metabolically active cells and a quantitative colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) showing the mitochondrial activity of living cells. Cells were seeded in a 96-well plate (1×10^4 cells/well) and cultured overnight for attachment. On the next day, cells were starved by incubating in serum free medium for 24 hr. The starving cells were treated with various concentrations of CPF or CPF-O (0.1-100 μM) for 48 hr. The final concentration of ethanol in the medium was 0.1% which did not affect the cell viability in control plates. At the

end of the respective incubation period, 10 µl of PrestoBlue™ cell viability reagent was added to each well, and cells were further incubated at 37ºC for 30 min. The fluorescence intensity was determined at 560 nm for excitation and 590 nm for emission using microplate scanning spectrophotometer (SpectraMax® M3, Molecular Devices, Sunnyvale, CA, USA). After that, medium with PrestoBlue™ reagent was removed, and then 100 µl of MTT (500 µg/ml MTT in serum free medium) was added to each well. Cells were incubated further for 4 hr at 37^{o}C for color development, then MTT medium was removed and cells were lysed with dimethylsulfoxide (DMSO) (Sigma-Aldrich, ST. Louis, MO, USA). Following solubilization, the absorbance at 570 nm with reference wavelength at 650 nm was measured using microplate scanning spectrophotometer.

Cell cycle assay

The cells (5×10^{6} cells) were seeded in a 100 mm plate and processed as previous cell viability assay. After 48 hr incubation with CPF, the cells were analyzed for the distribution of G1, S, and G2/M phases of cell cycle by flow cytometer with propidium iodide (PI) staining. Briefly, the medium was removed, and the then cells were harvested with trypsin (Gibco, Carlsbad, CA, USA) and the supernatant was removed by centrifugation at 500 × g, 4°C for 5 min. Cell pellets were washed with phosphate buffer saline (PBS) (Gibco, Carlsbad, CA, USA) and fixed in 70% ethanol overnight at -20^{o}C. Then the cells were washed with cold PBS and stained with PI solution containing 50 µg/ml of PI (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 µg/ml of RNAse (Sigma-Aldrich, St. Louis, MO, USA) at ambient temperature for 15 min. The cell cycle stages were measured by flow cytometer (BD FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA) and the data was analyzed by Modfit LT software (Verity House Software, Topsham, ME, USA).

Cell proliferation assay

Cell proliferation was determined by the incorporation of 5-ethynil-2-deoxyuridine (EdU) into newly synthesis DNA stand using Click-iT® EdU microplate assay (Molecular probes, Invitrogen, Carlsbad, CA, USA). Briefly, cells were processed as previous cell viability assay. At

the end of the respective incubation period, a working stock of 10X EdU in pre-warmed complete media was added to each well at final concentration of 10 μM and further incubated at 37°C for 3 hr. The incorporated EdU in DNA was coupled with Oregon Green-azide dye, and then subsequently incubated with horseradish peroxidase-labeled anti-Oregon Green antibody, Amplex® UltraRed, and N-acetyl-3, 7-dihydroxyphenoxazine. The fluorescence intensity was determined at 490 nm for excitation and 585 nm for emission using microplate scanning spectrophotometer.

Enzymatic activity of acetylcholinesterase assay

Acetylcholinesterase (AChE) enzymatic activity in cells were determined by the Ellman method (Ellman et al., 1961) adapted for use with microplate. Briefly, cells were processed as previous cell cycle assay. After 48 hr incubation with CPF, cells were washed with cold PBS and lysed in lysis buffer containing 10 mM Tris-HCl pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA), 150 mM NaCl (Sigma-Aldrich, St. Louis, MO, USA), 1% triton X-100 (Bio-Rad, Hercules, CA, USA), 0.1 mM PMSF (Sigma-Aldrich, St. Louis, MO, USA), 1 mM Na_3VO_4 (Sigma-Aldrich, St. Louis, MO, USA), 20 mM NaF (Sigma-Aldrich, St. Louis, MO, USA), and protease cocktail inhibitor (Calbiochem, Germany). Cell lysates were sonicated and incubated at 4°C for 30 min then centrifuged at 16,000 × g for 15 min at 4°C. The concentration of protein was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Then, 100 μl of protein sample containing 300 μg proteins was mixed with 50 μl of dithiobisnitrobenzoate (DTNB) solution (1.25 mM DTNB (Sigma-Aldrich, St. Louis, MO, USA), 0.1875 mg/ml $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M PBS pH 8.0). The mixture was allowed to stand for 5 min at room temperature, and then 50 μl of acetylcholine iodine (ATCI) substrate solution (1.87 mM ATCI (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M PBS pH 8.0) was added. The product absorbance increase was monitored for 2 min intervals for 12 min at 410 nm, 25°C using microplate scanning spectrophotometer. In each case the rate of absorbance increase was corrected by subtracting the rate observed for a reagent blank. AChE activity was extrapolated from standard curve of standard AChE (Sigma-Aldrich, St. Louis, MO, USA).

Western immunoblotting assay

The cells were processed as previous cell cycle assay. . At the end of the respective incubation period, the total protein cell lysates were prepared as previous AChE enzymatic activity assay. Then, the protein (50 μg) was mixed with Laemmli loading buffer (Bio-Rad, Hercules, CA, USA) and boiled at 95°C for 5min. The proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis in a Mini-PROTEAN II system (Bio-Rad, Hercules, CA, USA). The separated protein bands were transferred onto a nitrocellulose membrane (GE Healthcare, United Kingdom). The membrane was incubated in blocking buffer containing 5% non-fat dry milk in TBST buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, and 0.05% Tween-20) for 1 h at room temperature followed by overnight incubation at 4°C with the primary antibody. The antibodies against AChE, phosphorylated EGFR at tyrosine 1173 residue, and total EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against phosphorylated ERK1/2, total ERK1/2, and β-actin were obtained from Cell Signaling Technology (Cell Signaling, Beverly, MA, USA). After washing with TBST buffer, the membrane was incubated with horseradish-peroxidase conjugated secondary antibodies (GE Healthcare, United Kingdom) for 2 hr at room temperature. The protein bands stained by the antibody were visualized by using enhanced chemiluminescence (GE Healthcare, United Kingdom) followed by exposure to x-ray films (Pierce-Perbio, Brazil). Relative protein expressions were calculated from band intensities using computerized densitometry with ImageQuantTL software (GE Healthcare, United Kingdom).

Reactive Oxygen Species (ROS) Assay

The cells were seeded in a 6-well plate (7×10^5 cells/well) and cultured overnight for attachment. After serum withdraw for 24 hr, the growth medium was removed and the cells were treated with 10-100 μM of CPF for 24-48 hr. The cells were incubated with 25 μM of H2DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min before the end of treatment. The treated cells were trypsinized and centrifuged at 2,300 × g for 5 min to remove the treatment medium and the excessed H_2DCF-DA dye. Then cells were re-suspended with cold PBS. The

fluorescence was measured using BD LSRFortessaTM Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Results

1. Chlorpyrifos increases the viability of normal colon epithelial CCD841 cells and colorectal adenocarcinoma H508 cells

After treatment with CPF and CPF-O in a serum free condition for 48 hr, cell viability was subsequently assessed by PrestoBlue™ and MTT metabolic activity assays. The results of MTT assay showed that 5 and 10 μM of CPF significantly increased the viability of normal colon epithelial CCD841 cells but this increasing response slightly drops down at higher concentrations of CPF (Fig. 1A). Interestingly, CPF (0.1-100 μM) concentration dependently increased the viability of colorectal adenocarcinoma H508 cells (Fig. 1B). Note that the significant difference from the control was started at 10 μM of CPF treated group. Furthermore, at the tested concentration range (0.1-100 μM), the viability of normal hepatocyte THLE3 cells and hepatocellular carcinoma HepG2 cells did not be affected by CPF (Fig. 1C&D). Meanwhile, CPF-O at the two highest tested concentrations (50 and 100 μM) dramatically reduced the cell viability of all tested cell lines, while CPF-O at lower concentrations (0.1-10 μM) did not affect the cell viability. Note that, the liver cell lines (THLE-3 and HepG2) are likely more sensitive to the toxic effect of CPF-O than the colon cell lines (CCD841 and H508).

For comparison, the results of PrestoBlue™ assay showed similar pattern of MTT assay with a higher sensitivity in all cell lines except H508 cells (Fig. 2). PrestoBlue™ cell viability assay showed that CPF-O (0.1-100 μM) concentration dependently increased the viability of H508 cells. Surprisingly, at the two highest tested concentrations of CPF (50 and 100 μM), MTT assay showed the reduction of H508 cell viability whereas PrestoBlue™ assay showed the opposite results. Notably, an observation in phase contrast microscopy revealed that cells treated with these high concentrations of CPF-O (50 and 100 μM) were detached from the plate and reduced in cell size (data not shown). Altogether, these results suggested that CPF increased CCD841 and H508 cell viability in serum free condition.

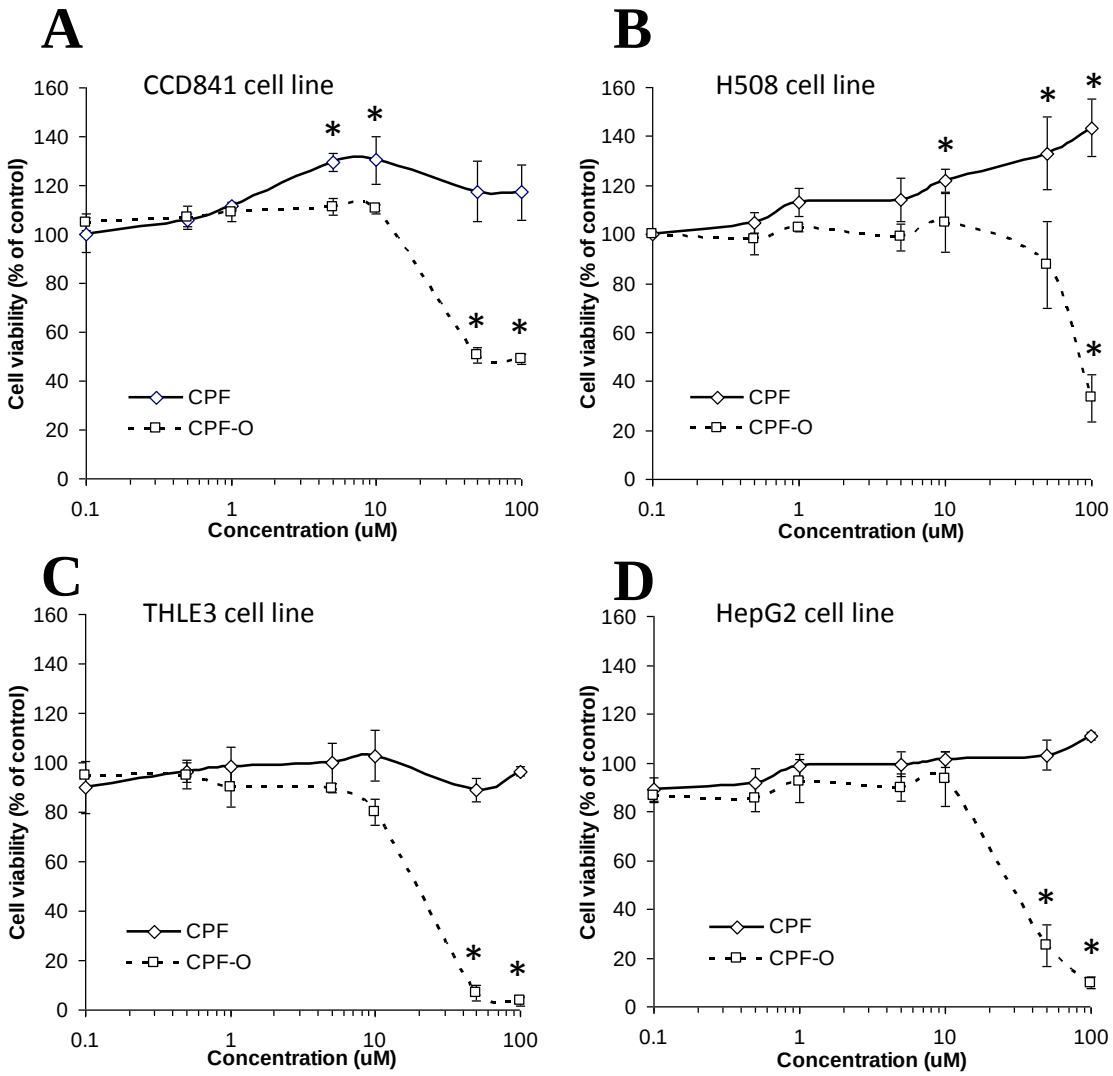

Figure 1: Effects of chlorpyrifos (CPF) and chlorpyrifos oxon (CPF-O) on the viability of (A) normal colon epithelial CCD841 cells, (B) colorectal adenocarcinoma H508 cells, (C) normal hepatocyte THLE3 cells, and (D) hepatocellular carcinoma HepG2 cells. Cells were starved in serum free condition for 24 hr, and then treated with 0.1- 100 μM of CPF or CPF-O in serum free condition for another 48 hr. Cell viability was assessed by MTT assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control (0.1% ethanol) at $P< 0.05$.

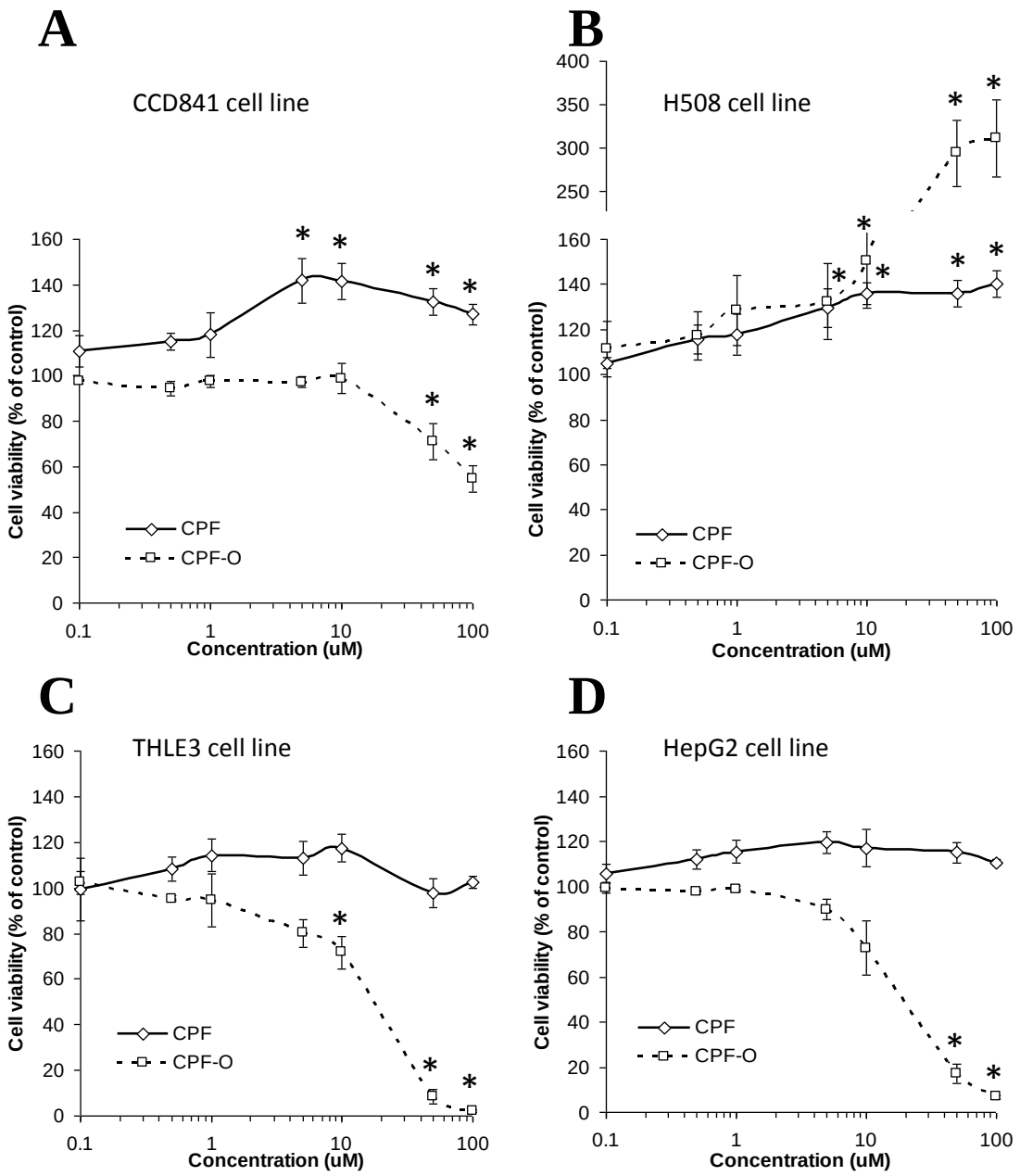

Figure 2: Effects of chlorpyrifos (CPF) and chlorpyrifos oxon (CPF-O) on the viability of (A) normal colon epithelial CCD841 cells, (B) colorectal adenocarcinoma H508 cells, (C) normal hepatocyte THLE3 cells, and (D) hepatocellular carcinoma HepG2 cells. Cells were starved in serum free condition for 24 hr, and then treated with 0.1- 100 μM of CPF or CPF-O in serum free condition for another 48 hr. Cell viability was assessed by PrestoBlue™ cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control (0.1% ethanol) at $P< 0.05$.

2. Chlorpyrifos stimulates growth of colorectal adenocarcinoma H508 cells

Our previous results revealed that CPF increased H508 cell viability in serum free condition. Further study was conducted to investigate the effect of CPF on cell cycle and DNA synthesis of H508 cells. Cell cycle was measured by flow cytometry with propidium iodide (PI) staining. The results indicated that CPF concentration dependently increased the percentage of cells in the S phase (Fig. 3). The percentage of H508 cells in the S phase increased from 8.44% as the control to 9.92-17.10% for cells treated with 1-100 μM of CPF and the significant differences from the control were observed at 50 and 100 μM of CPF treated groups. Furthermore, the increases of cells in S phase were accompanied with the decreased percentage of cells in G0/G1 phase. Note that in the positive control group (10 ng/ml of EGF), there was a significantly increased in the percentage of cells in the S and G2/M phases with the decreased percentage of cells in G0/G1 phase.

Next, the EdU incorporation assay was utilized to determine the cell proliferation by measuring the rate of DNA synthesis during S phase of cell cycle. CPF at concentrations of 10 and 50 μM induced cell proliferation by activation of DNA synthesis, significantly higher than the control about 108.7 and 164.7%, respectively (Fig. 4). Notably, the positive control (10 ng/ml of EGF) also dramatically activated cell proliferation by 372.4% compared to the control. Together, these results suggested that CPF stimulated colorectal adenocarcinoma H508 cell growth.

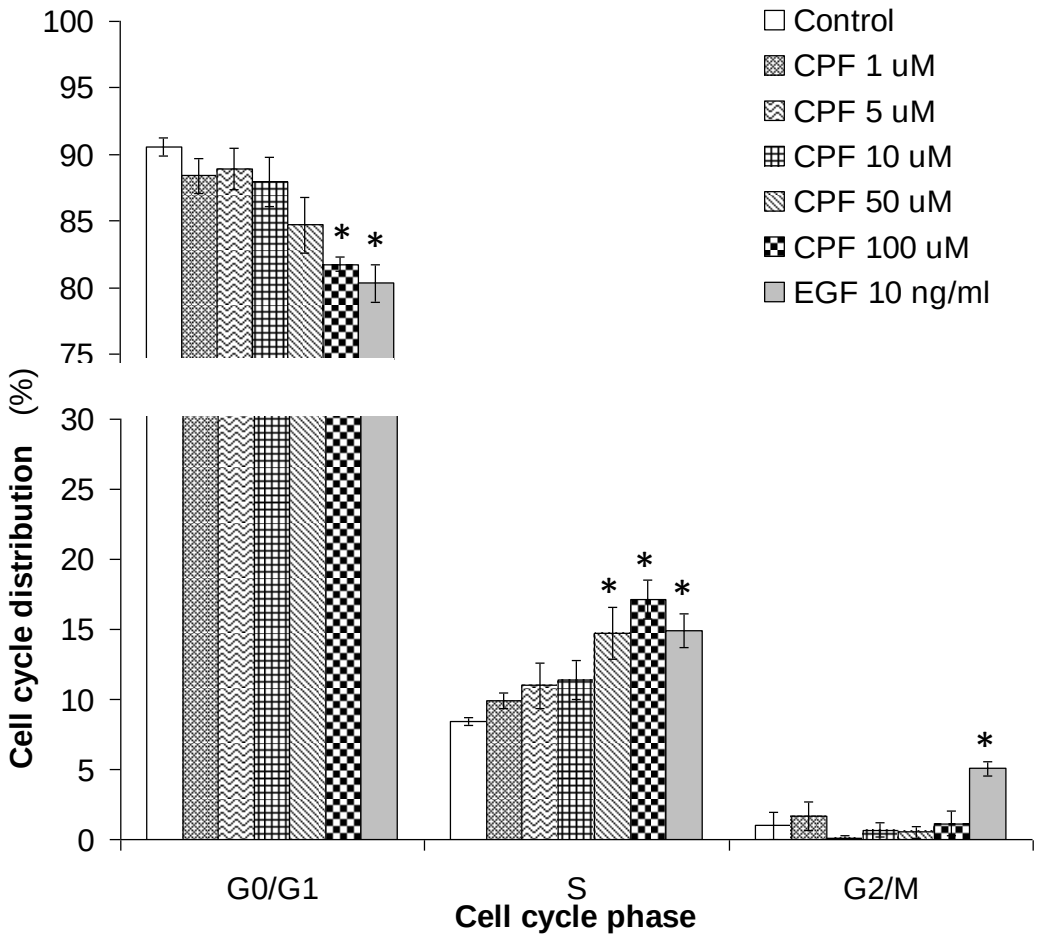

Figure 3: Effects of chlorpyrifos (CPF) on colorectal adenocarcinoma H508 cell cycle. Cells were starved in serum free condition for 24 hr, and then treated with 1-100 μM of CPF or 10 ng/ml of epidermal growth factor (EGF) (positive control) in serum free condition for another 48 hr. Cell cycle phase distribution was measured by flow cytometry with propidium iodide staining. The data are the percentage mean of each cell cycle phase±standard error of three independent experiments. * represents statistically significant difference from the control (0.1% ethanol) at $P< 0.05$.

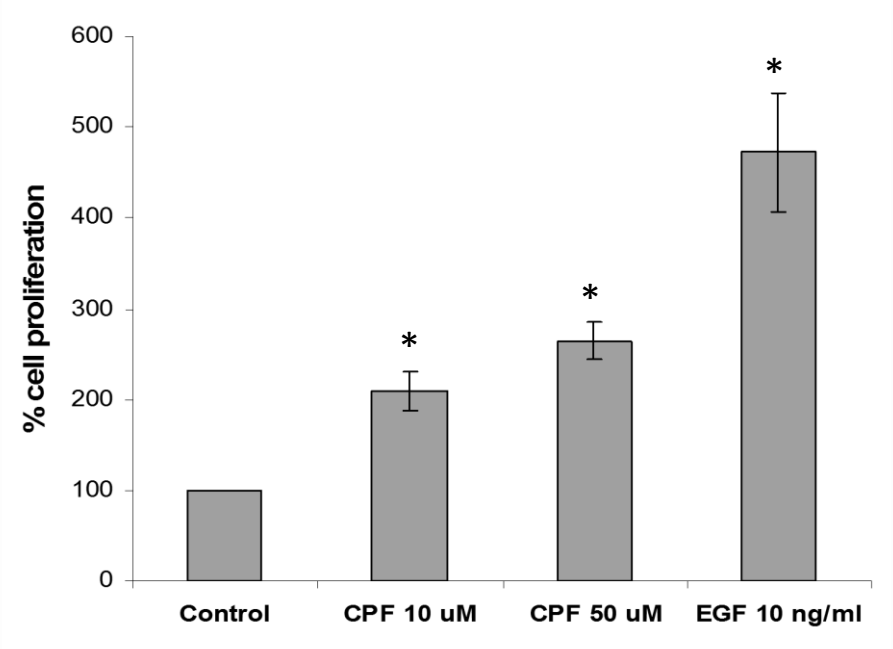

Figure 4: Effects of chlorpyrifos (CPF) on the proliferation of colorectal adenocarcinoma H508 cells. Cells were starved in serum free condition for 24 hr, and then treated with 10 or 50 μM of CPF or 10 ng/ml of epidermal growth factor (EGF) (positive control) in serum free condition for another 48 hr. Cell proliferation was measured by Click-iT® EdU cell proliferation assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control (0.1% ethanol) at $P< 0.05$.

3. Non-neuronal cholinergic system does not involve in chlorpyrifos-stimulated growth of colorectal adenocarcinoma H508 cells

It is well established that non-neuronal cholinergic system plays an important role in colon epithelial tumorigenesis (Novotny et al., 2011). Next, we studied the role of non-neuronal cholinergic system in CPF-induced H508 cell growth. Initial experiment was performed to measure the effects of CPF on the protein expression and enzymatic activity of acetylcholinesterse (AChE). Cells were treated with 1-50 μM of CPF in serum free medium for 24 and 48 hr, the protein expression of AChE was measured by Western immunobloting assay and AChE enzymatic activity in cells was determined by the modified Ellman method. The results showed that CPF did not alter the protein expression level of AChE either 24 or 48 hr (Fig 5A), meanwhile it decreased AChE enzymatic activity in a concentration dependent manner (Fig. 5B). Note that the significant differences from the control were observed at 10 and 50 μM of CPF-treated groups.

To examine whether the growth stimulating effect of CPF is related to the non-neuronal cholinergic system, cells were pretreated for 30 min with specific cholinoceptor antagonists, including 10 μM of atropine (a non-selective muscarinic acetylcholine receptor antagonist) or 100 μM of mecamylamine (a non-selective nicotinic acetylcholine receptor antagonist), and then cells were further incubated with 1-50 μM of CPF or 1 mM of carbachol (a cholinoceptor agonist) in serum free medium for 48 hr. The results of PrestoBlue™ assay showed that either mecamylamine or atropine failed to attenuate the growth promoting effect of CPF (Fig. 6). Notably, the positive control, carbachol, significantly stimulated growth of H508 cells and atropine but not mecamylamine completely blocked this growth promoting effect.

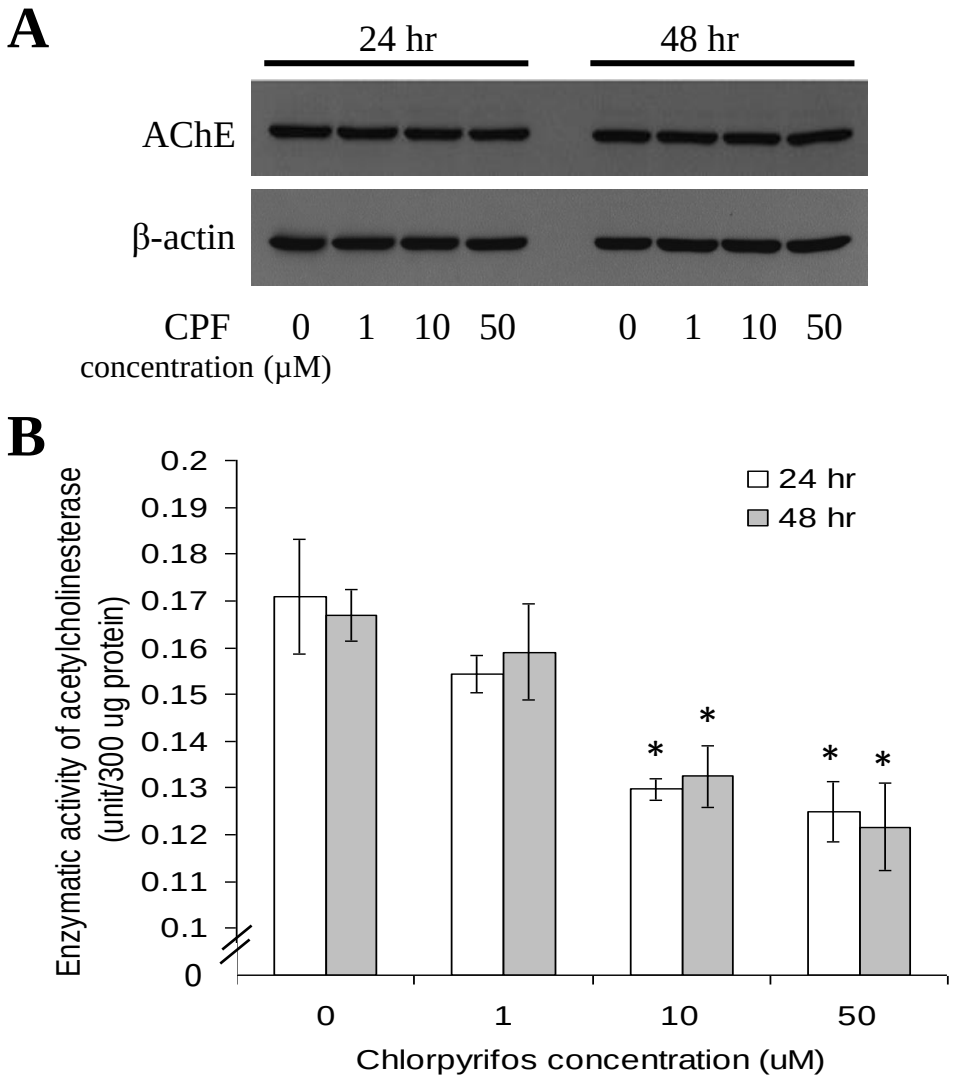

Figure 5: Effects of chlorpyrifos (CPF) on the expression and enzymatic activity of acetylcholinesterse (AChE). Cells were starved in serum free condition for 24 hr, and then treated with 1, 10 or 50 μM of CPF in serum free condition for another 24-48 hr. Protein expression of AChE was measured by Western immunobloting assay and AChE enzymatic activity was determined by the modified Ellman method. (A) Representative immunoblots band of AChE. The β-actin was used to ensure equal amount of loaded protein. (B) Enzymatic activity of AChE. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control (0.1% ethanol) at $P< 0.05$.

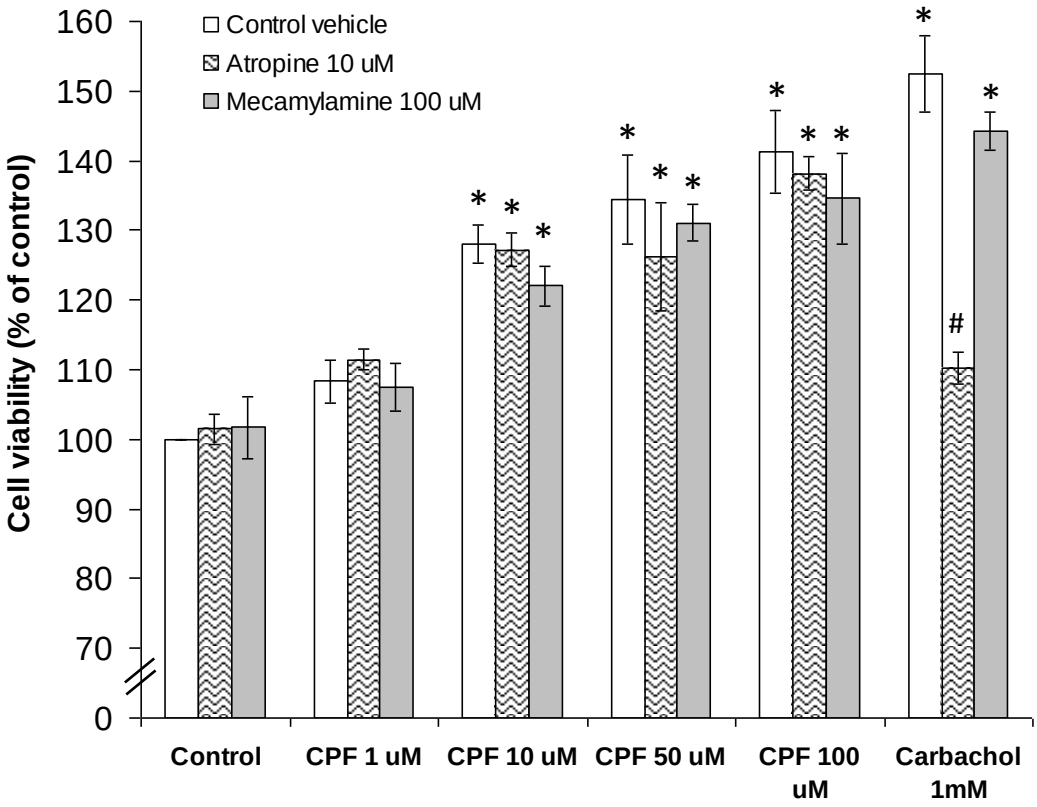

Figure 6: Effects of cholinoceptor antagonists on the growth promoting effect of chlorpyrifos (CPF). Colorectal adenocarcinoma H508 cells were starved in serum free condition for 24 hr. Starved cells were pre-treated for 30 min with 10 μM of atropine or 100 μM of mecamylamine and then further incubated with 1-50 μM of CPF or 1 mM of carbachol (positive control) in serum free medium for 48 hr. Cell growth was assessed by PrestoBlue™ cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control (0.1% ethanol + 0.1% water). * represents statistically significant difference from the control and # represents statistically significant difference from the control vehicle (0.1% ethanol + 1mM carbachol) at $P< 0.05$.

4. Chlorpyrifos induces colorectal adenocarcinoma H508 cell growth via an activation of EGFR/ERK1/2 signaling pathway

The epidermal growth factor receptor (EGFR) is over-expressed in colorectal cancer patient populations and it is recognized as an important player in colon cancer initiation and progression (Sasaki et al., 2013; Spano et al., 2005). We determined the expression level of EGFR in colon and liver cell lines. The results showed that the background levels of the total and active/phospholylated forms of EGFR were varying among colon and liver cell lines. H508 and THLE-3 cells highly expressed the total form of EGFR when compared to HepG2 and CCD841 cells (Fig. 7). Interestingly, the basal background level of the active/phosphorylated form of EGFR at tyrosine 1178 in colon cells especially H508 cells were higher than in the tested liver cells.

Next, we studied the effects of CPF on EGFR signaling in H508 cells. Cells were treated with 1-50 μM of CPF in serum free medium for 24 and 48 hr, the activation of EGFR and its downstream signaling cascade were determined by Western immunobloting assay. The results of 24 hr incubation period showed that CPF increased the expression level of active/phosphorelated EGFR at tyrosine 1173 residue in a concentration dependent manner with a significant difference from the control started at 10 μM of CPF (Fig. 8B). On the contrary, the results of 48 hr incubation period showed that the expression level of active EGFR concentration dependently reduced by CPF treatment. Extracellular signal regulated kinase (ERK1/2) which is one of the most important downstream effectors of EGFR activation, was slightly activated by CPF treatment at 24 hr exposure period (Fig. 8C). Furthermore, the activation of ERK1/2 was dramatically increased by CPF treatment at 48 hr exposure period.

In order to determine the role of EGFR/ERK1/2 signaling in CPF-induced H508 cell growth, cells were pretreated with 0.1-1 μM of AG-1478 (a specific EGFR tyrosine kinase inhibitor) or 1-5 μM of U0126 (a specific MEK inhibitor) for 30 min before co-treatment with CPF for 48 hr. The PrestoBlue™ viability assay revealed that the growth promoting effect of CPF was completely mitigated by pre- and co-treatment with 1 μM of AG-1478 (Fig. 9A). For MEK inhibitor, the growth stimulating effect of CPF was also completely attenuated by pre- and co-treatment with 5 μM of U0126 (Fig. 9B). Note that both of 1 μM of AG-1478 and 5 μM of U0126

by themself did not affect the viability of H508 cells but they significantly attenuated the growth stimulating effect of 10 and 100 ng/ml of EGF, respectively.

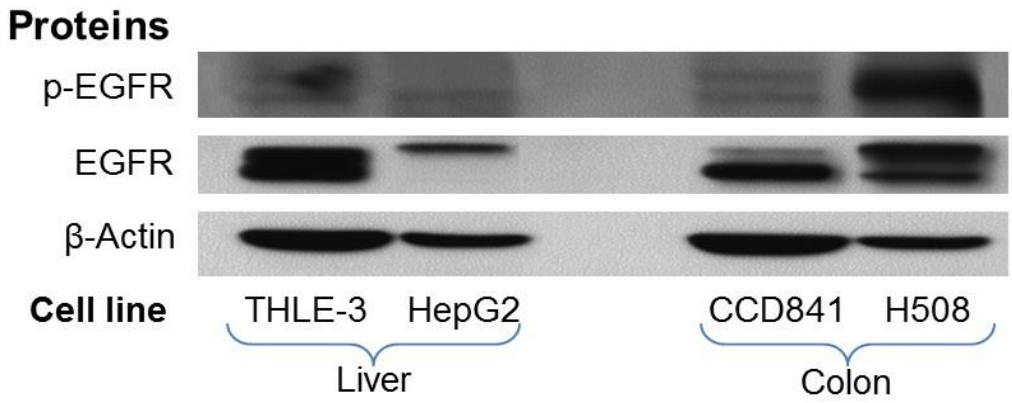

Figure 7: Background expression levels of the total and active/phosphorylated forms of epidermal growth factor receptor (EGFR) in liver (THLE-3 and HepG2) and colon (CCD841 and H508) cell lines. Expression of active/phosphorylated EGFR at tyrosine 1173 (p-EGFR), and total EGFR were measured by Western immunoblotting assay.

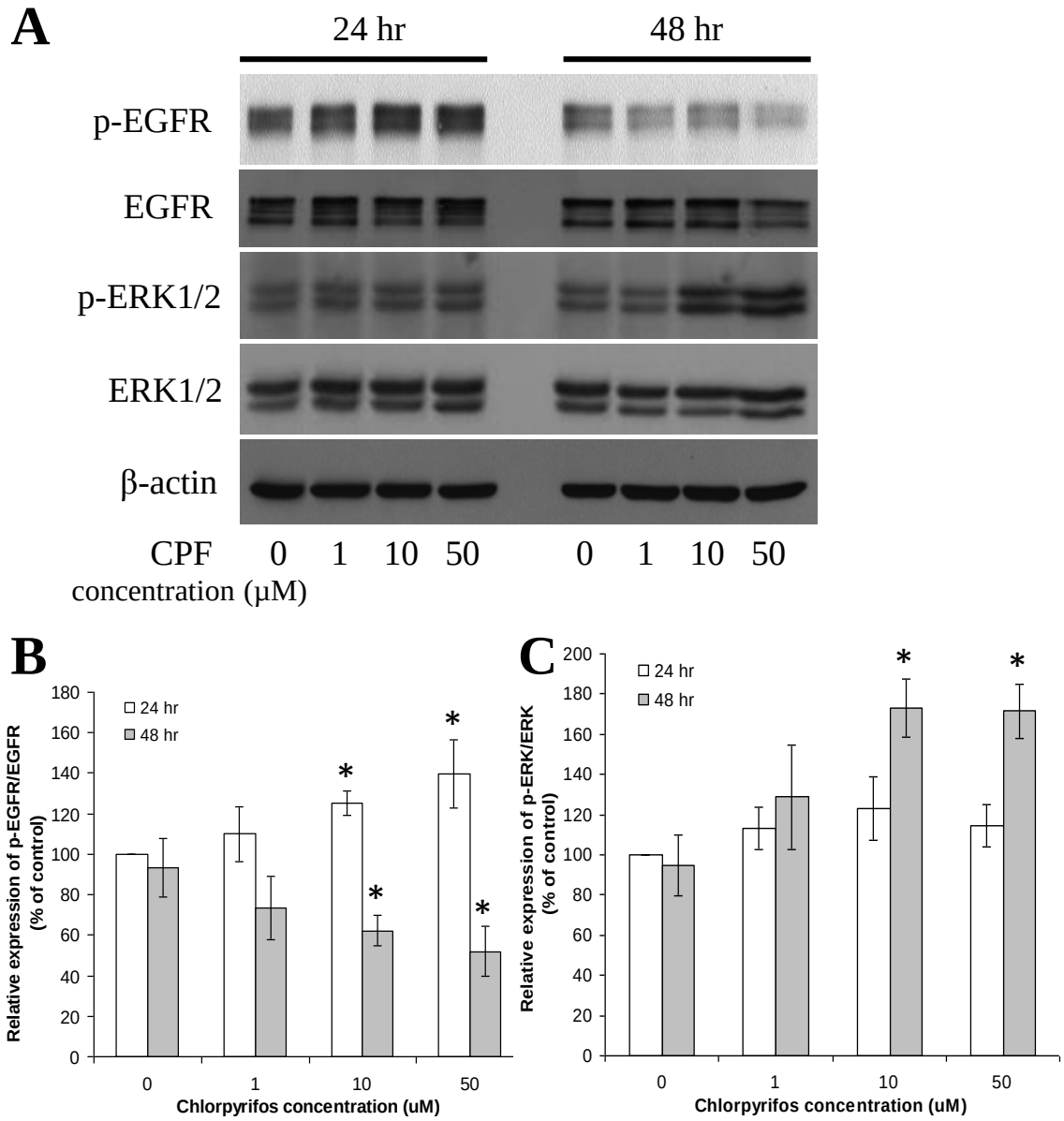

Figure 8: Effects of chlorpyrifos (CPF) on the activation of epidermal growth factor receptor (EGFR) and extracellular signal regulated kinase (ERK1/2). Colorectal adenocarcinoma H508 cells were starved in serum free condition for 24 hr and then further incubated with 1-50 μM of CPF in serum free medium for 24-48 hr. Expression of phosphorylated EGFR (p-EGFR), total EGFR, phosphorylated ERK1/2 (p-ERK1/2), and total ERK1/2 were measured by Western immunoblotting assay. (A) Representative immunoblots band of p-EGFR, total EGFR, p-ERK1/2, and total ERK1/2. The β-actin was used to ensure equal amount of loaded protein. (B) Ratio of relative expression of p-EGFR to total EGFR proteins. (C) Ratio of relative expression of p-ERK1/2 to total ERK1/2 proteins. Data are the mean±standard error of three

independent experiments and expressed as a relative to the control (0.1% ethanol) of 24 hr exposure. * represents statistically significant difference from the control of 24 hr exposure at $P < 0.05$.

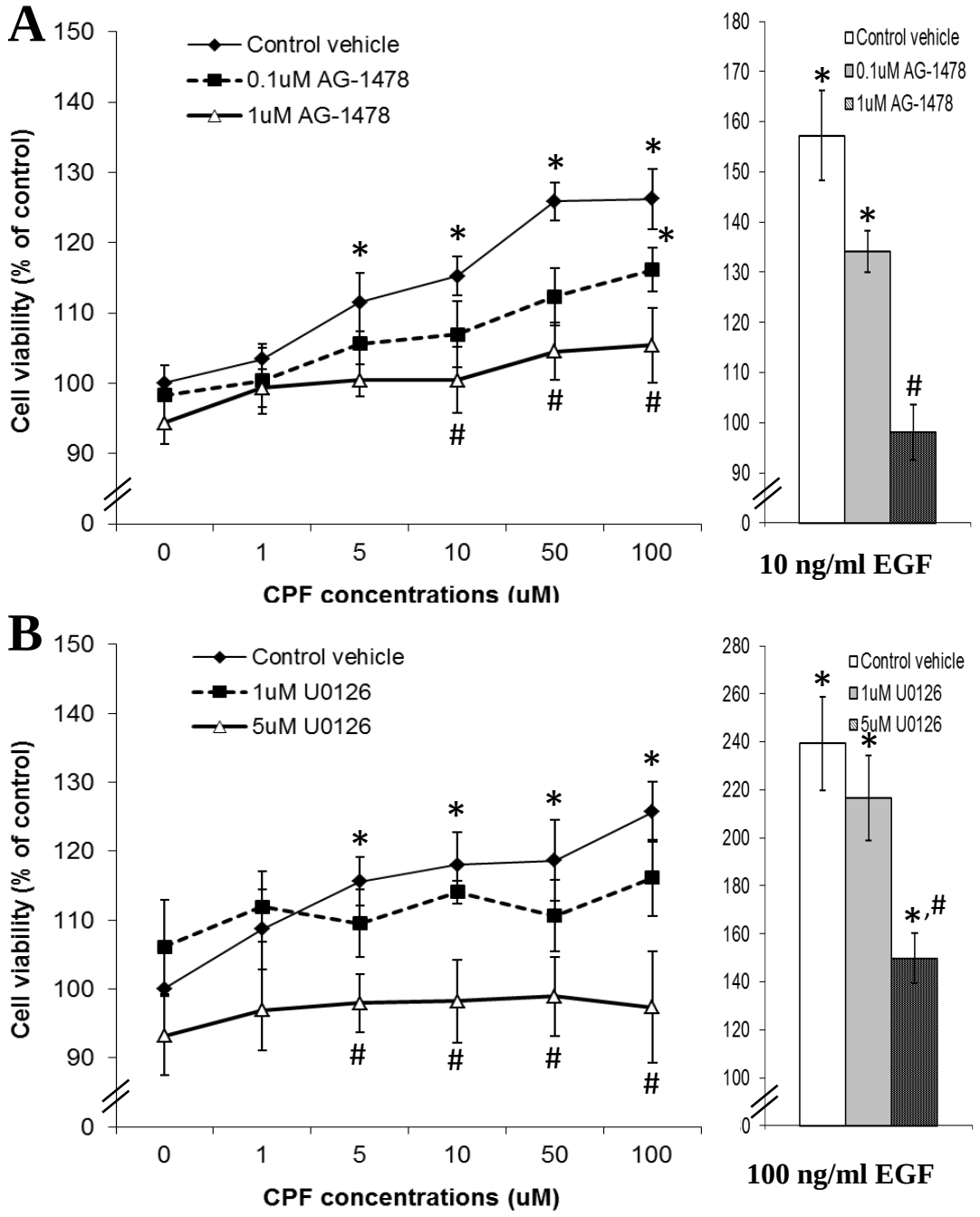

Figure 9: Effects of epidermal growth factor receptor (EGFR) and MEK inhibitors on the growth promoting effect of chlorpyrifos (CPF). Colorectal adenocarcinoma H508 cells were starved in serum free condition for 24 hr. Starved cells were pre-treated for 30 min with (A) 0.1- 1 μM of AG-1478 (a specific EGFR tyrosine kinase inhibitor) or (B) 1- 5 μM of U0126 (a specific MEK inhibitor) and then further co-incubated with 1-100 μM of CPF or 10-100 ng/ml of epidermal growth factor (EGF) (positive control) in serum free medium for 48 hr. Cell growth was

assessed by PrestoBlue™ cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to the control (0.1% ethanol + 0.1% DMSO). * represents statistically significant difference from the control group and # represents statistically significant difference from the control vehicle at the same concentration of CPF at $P< 0.05$.

5. Chlorpyrifos stimulates reactive oxygen species in colorectal adenocarcinoma H508 but this effect does not involved in the growth promoting effect of chlorpyrifos

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), have been shown to induce phosphorylation of EGFR, in part, due to its protein-tyrosine phosphatase inhibition (Kamata et al., 2000). Since, it has been shown that CPF generated oxidative stress by inducing the production of ROS (Ki et al., 2013), next we determined the effect of chlorpyrifos on the ROS level in H508 cells. The results showed that 24 and 48 hr exposure of CPF (10-100 μM) concentration dependently increased ROS in H508 cells (Fig 10A). Pretreatment the cells with 50 mM of H_2O_2 for 15 min (positive control) also significantly increased the level of ROS when compared to the control. Moreover, the involvement of oxidative stress in CPF-induced growth of H508 cells was also determined. The cells were pretreated with 2-4 mM of the antioxidant, NAC, for 30 min before co-treatment with CPF or EGF for 48 hr. The MTT viability assay revealed that pre- and co-treatment with NAC did not reduce the growth promoting effect of CPF. As expected, NAC also did not mitigate the growth promoting effect of EGF (Fig. 10B).

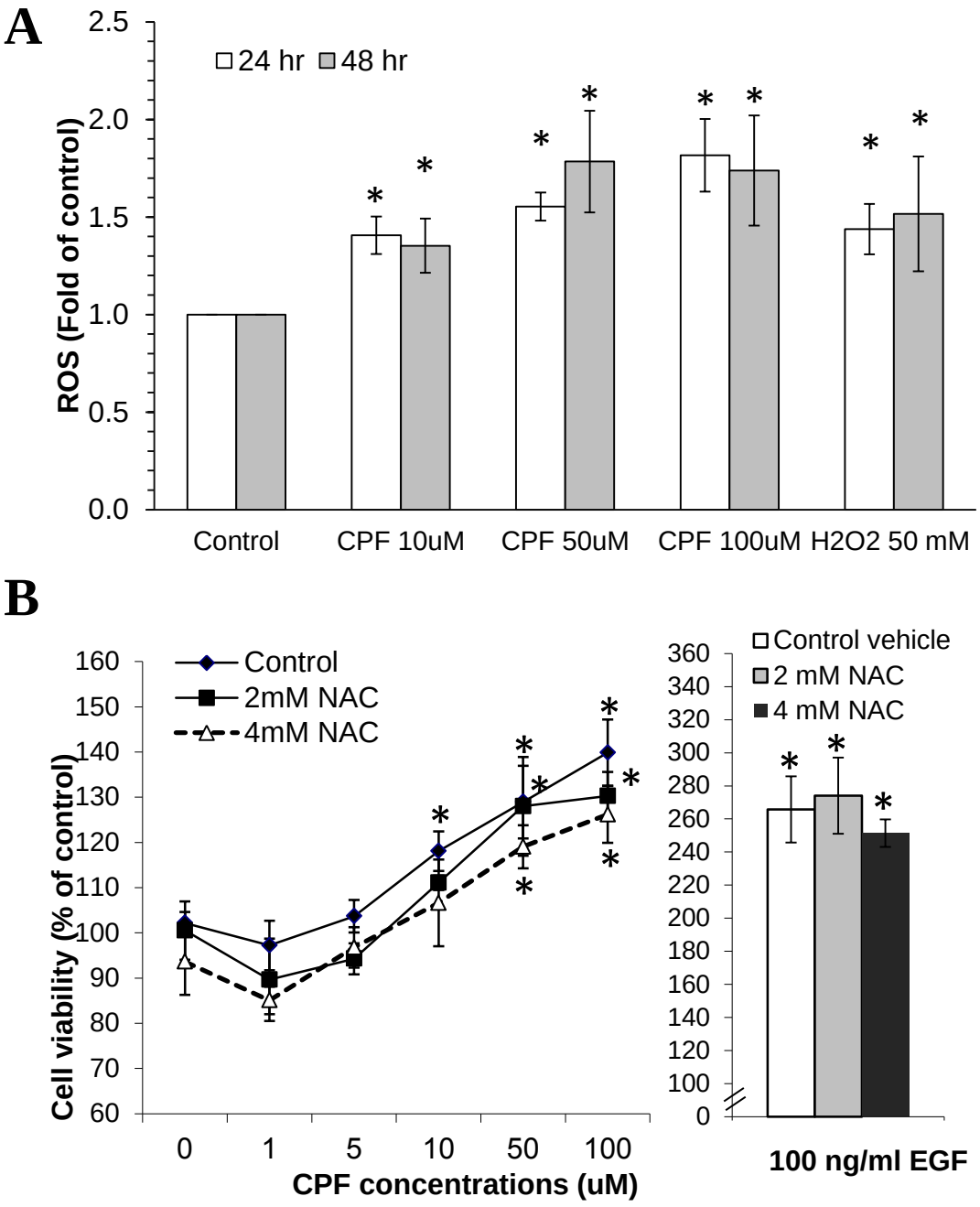

Figure 10: (A) Effect of chlorpyrifos (CPF) on the production of reactive oxygen species (ROS). Colorectal adenocarcinoma H508 cells were starved in serum free condition for 24 hr. Starved cells were treated with 10-100 µM of CPF in serum free medium for 24-48 hr or 50 mM of hydrogen peroxide (H_2O_2) for 15 min (positive control). ROS were stained with H_2DCF-DA dye and measured by flow cytometer. Each data point represents the mean±standard error of three independent experiments and expressed as a fold of the control. (B) Effects of antioxidant on the growth promoting effect of CPF. Serum starved cells were pre-treated for 30 min with 2- 4

mM of NAC and then further co-incubated with 1-100 μM of CPF or 100 ng/ml of epidermal growth factor (EGF) (positive control) in serum free medium for 48 hr. Cell growth was assessed by MTT cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to the control . * represents statistically significant difference from the control group at $P< 0.05$.

Discussion

The Agricultural Health Study (AHS) which is a largest perspective cohort study has identified an association between exposure to CPF and increased lung, brain, and colorectal cancer risk in pesticide applicators (Weichenthal et al., 2010). However, the carcinogenic mechanisms of CPF remain unclear. The present study provides the novel information on the carcinogenic effect of CPF in colorectal cancer. This study showed the growth promoting effect of CPF in colorectal adenocarcinoma H508 cells through the activation of EGFR/ERK1/2 signaling pathway.

The present study showed that CPF at the concentration up to 100 μM did not affect the viability of normal hepatocyte THLE-3 and hepatocellular carcinoma HepG2 cells. Previous study demonstrated that CPF at the concentration of 25-200 μM, concentration dependently reduced the viability of human neuroblastoma SH-SY5Y cells (Ki et al., 2013). Furthermore, it has been shown that CPF significantly increased cytotoxicity and the IC50 value (the concentration that inhibited 50% growth) at 24 hr for CPF was about 100 μM in human neuron PC12 (Lee et al., 2012) and SH-SY-5Y cells (Park et al., 2013). The difference in sensitivity among cell lines to CPF could be explained in part due to the difference in molecular characteristics of the different cancer cells especially anti-oxidant capacity, since it has been shown that oxidative stress is involved in CPF-induced apoptosis (Ki et al., 2013; Ventura et al., 2015). Note that, glutathione system including glutathione, glutathione reductase, glutathione peroxidases and glutathione *S*-transferases, which is one of an important anti-oxidant enzymatic system, is particularly at high level in hepatocytes (Hayes et al., 2005). The viability assay also showed that CPF-O at the concentration of 50-100 μM demonstrated more toxic

effect to reduce the cell viability than it parent compound. Since, CPF-O is about 28-180 orders of magnitude more potent than CPF in inhibition of brain AChE activity (Monnet-Tschudi et al., 2000), suggesting the involvement of an over activation of cholinergic signaling by the inhibition of AChE on the cytotoxic toxic effect of CPF-O. However, previous study suggested that CPF-O-induced apoptosis in rat cortical neurons may occur independently of AChE inhibition (Caughlan et al., 2004). It will be interesting to investigate if there is any correlation between AChE inhibition and the cytotoxic effect of CPF-O. Furthermore, the present study showed that PrestoBlue™ metabolic activity assay showed similar pattern of MTT metabolic activity assay with a higher sensitivity. However, opposite result was observed only in CPF-O-treated H508 cells. The cytotoxic effect of CPF-O in H508 cells had been evidenced by MTT assay and cell morphology under microscope observation. The fault positive result on the induction of cell viability in CPF-O-treated H508 cells had been found by PrestoBlue™ assay. Further study should be conducted to understand this specific fault positive result in this cell line.

Interestingly, the present study found that CPF at the concentration range 10-100 μM significantly increased the viability of normal colon epithelial CCD841 and colorectal adenocarcinoma H508 cells. The cell cycle analysis showed the induction of cell in the S phase, and the EdU incorporation assay revealed the induction of the DNA synthesis in CPF-treated H508 cells. These results demonstrated the growth promoting effect of CPF in H508 cells. Previous study also demonstrated that CPF at very low concentration (0.05 μM) promoted cell proliferation in the hormone-dependent breast cancer MCF-7 cells through estrogen receptor (ER) signaling pathway but higher concentrations of this insecticide caused cell death (Ventura et al., 2012). Furthermore, CPF showed a strong aryl hydrocarbon receptor

(AhR) agonistic activity compared to other pesticides, including methiocarb, chlorothalonil, tribenuron-methyl, paclobutrazol and tolchlofos-methyl (Long et al., 2003). In addition, Src-mediated cross-talk between AhR and EGFR signaling pathways strongly activate proliferation of H508 cells (Xie et al., 2012). However, we found that pretreatment either with a specific AhR antagonist, CH223191 (100 nM), or a high affinity ER antagonist, ICI182780 (10 μM), failed to attenuate the CPF-induced H508 cell growth (data not show). Even though, the inhibitory effect on the AChE activity was observed, atropine, which is a muscarinic receptor antagonist, or mecamylamine, which is a nicotinic receptor antagonist, did not antagonize the growth promoting effect of CPF. This may be partially due to the concentrations of ACh in the cells may not be high enough to activate cholinergic receptors and activate cellular responses. Altogether, these results suggested that AhR, ER, or cholinergic receptors did not involve in the growth promoting effect of CPF in H508 cells in the experimental condition of this study.

Furthermore, the results of this study showed that CPF caused an activation of EGFR by increasing the phosphorylation of EGFR at tyrosine 1173 residue after 24 h incubation period. However, the reduction of the phosphorylation of EGFR at tyrosine 1173 residue was observed after 48 h incubation period. This dynamic status of EGFR activation is possible in response to high activation of EGFR. This carboxy terminal tyrosine residue on EGFR is the major sites of autophosphorylation, which occurs as a result of ligand binding, and have been shown to play a critical role in the activation of the MAPK cascade following EGF stimulation (Schmidt-Ullrich et al., 1997; Sturla et al., 2005). In line with the activation of EGFR, CPF also increased the phosphorylation of ERK1/2 which is one of the most important downstream effectors of EGFR activation, after 48 h incubation period. Importantly, the present study provide a new evidence that CPF induces H508 cell growth via an activation of EGFR/ERK1/2

signaling pathway, since EGFR antagonist (AG1478) and MAK inhibitor (U0126) completely mitigated CPF-mediated H508 cell growth. Note that, there are no studies reported that CPF elicited ability to interact with EGFR or activate EGFR/ERK1/2 signaling pathway which lead to the proliferation of the cancer cells. Furthermore, many studies revealed that activation of MAPK signaling pathways are involved in regulating CPF-induced apoptosis. For example, CPF (100 μM) induced apoptosis involving the activation of MAPK pathways including JNK, ERK1/2, and p38 MAPK, through oxidative stress in human neuroblastoma SH-SY5Y cells (Ki et al., 2013). A recent study also demonstrated that CPF (50 μM) inhibited cell proliferation in breast cancer MCF-7 and MDA-MB-231 cells through an increment of phosphorylation of p-ERK1/2 levels mediated by oxidative stress (Ventura et al., 2015). Even though the increase in the production of ROS by CPF treatment was observed in this study, oxidative stress may not be involved in the growth promoting effect of CPF in H508 cells, since antioxidant NAC could not reverse CPF-induced H508 cell growth.

The growth promoting effect of CPF seems to be cancer and tissue specific in that colon cancer cells were relatively response to CPF than normal colon epithelial cells or other type of cancers. The difference in the response of CPF among cell lines could be explained in part due to the difference in molecular characteristics of the different cells especially the basal background level of phosphorylated/activated form of EGFR or the mutation of EGFR. It has been shown that the increase in basal phosphorylation of EGFR was evidenced when tyrosine 1173 was mutated (Sturla et al., 2005). Even though, the mutation in EGFR in H508 cells was not reported (Ahmed et al., 2013; Yeh et al., 2009), this study found that the basal background level of phosphorylated/activated form of EGFR at tyrosine 1173 residue was very high in H508 cells. This may explain the finding on the growth promoting effect of CPF likely specific to the

colon especially in H508 cells but not liver cells. However, this hypothesis need further study to clarify.

Although, CPF is highly metabolized and quickly detoxified in human, normal colon epithelial cells or colon cancer cells which line in the inner layer of the colon can directly contact with CPF. Following oral intake of CPF by rat, 90% was removed in the urine as metabolized forms and 10% was excreted in the feces as an unchanged form (Smith et al., 1967). Furthermore, the growth promoting concentration of CPF in this study was started at 5 μM which is not too high and may be possibly found in the gut.

In conclusion, the present study found that CPF promoted the growth of colorectal adenocarcinoma H508 cells via EGFR/ERK1/2 signaling pathway. Even though, the inhibition of AChE was evidenced, non-neuronal cholinergic system did not involve in the growth promoting effect of CPF in this cholinergic-responded cancer cells. It should be noted that this study is a preliminary *in vitro* study; further study regarding the colorectal cancer promotion of CPF should be conducted. For example, the growth promoting effect of CPF in the other type of colorectal cancer cell line should be investigated. Importantly, animal study should also be carried out to verify this carcinogenic effect of CPF.

References

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknaes, M., Hektoen, M., Lind, G.E., Lothe, R.A., 2013. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2, e71.

Belo, A., Cheng, K., Chahdi, A., Shant, J., Xie, G., Khurana, S., Raufman, J.P., 2011. Muscarinic receptor agonists stimulate human colon cancer cell migration and invasion. American Journal of Physiology-Gastrointestinal and Liver Physiology 300, G749-G760.

Budd, D.C., McDonald, J., Emsley, N., Cain, K., Tobin, A.B., 2003. The C-terminal tail of the M3-muscarinic receptor possesses anti-apoptotic properties. Journal of Biological Chemistry 278, 19565.

Caughlan, A., Newhouse, K., Namgung, U., Xia, Z., 2004. Chlorpyrifos induces apoptosis in rat cortical neurons that is regulated by a balance between p38 and ERK/JNK MAP kinases. Toxicol Sci 78, 125-134.

Cheng, K., Samimi, R., Xie, G., Shant, J., Drachenberg, C., Wade, M., Davis, R.J., Nomikos, G., Raufman, J.P., 2008a. Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. American Journal of Physiology-Gastrointestinal and Liver Physiology 295, G591-G597.

Cheng, K., Samimi, R., Xie, G., Shant, J., Drachenberg, C., Wade, M., Davis, R.J., Nomikos, G., Raufman, J.P., 2008b. Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 295, G591-597.

Colt, J.S., Lubin, J., Camann, D., Davis, S., Cerhan, J., Severson, R.K., Cozen, W., Hartge, P., 2004. Comparison of pesticide levels in carpet dust and self-reported pest treatment practices in four US sites. Journal of Exposure Science and Environmental Epidemiology 14, 74-83.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7, 88-95.

Espanol, A.J., de la Torre, E., Fiszman, G.L., Sales, M.E., 2007. Role of non-neuronal cholinergic system in breast cancer progression. Life Sci 80, 2281-2285.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. Annu Rev Pharmacol Toxicol 45, 51-88.

Health Canada, 2003. Proposed acceptability for continuing registration: Phase 2 of the Re-evaluation of chlorpyrifos. Pest Management Regulatory Agency, Ottawa, Ontorio, Canada.

Howard, M.D., Mirajkar, N., Karanth, S., Pope, C.N., 2007. Comparative effects of oral chlorpyrifos exposure on cholinesterase activity and muscarinic receptor binding in neonatal and adult rat heart. Toxicology 238, 157-165.

IARC, 2010. IARC Monographs on the evaluation of carcinogenic risks to humans., In: Cancer), I.A.f.R.o. (Ed.).

Kamata, H., Shibukawa, Y., Oka, S.I., Hirata, H., 2000. Epidermal growth factor receptor is modulated by redox through multiple mechanisms. Effects of reductants and H_2O_2. Eur J Biochem 267, 1933-1944.

Ki, Y.W., Park, J.H., Lee, J.E., Shin, I.C., Koh, H.C., 2013. JNK and p38 MAPK regulate oxidative stress and the inflammatory response in chlorpyrifos-induced apoptosis. Toxicol Lett 218, 235-245.

Lee, J.E., Park, J.H., Shin, I.C., Koh, H.C., 2012. Reactive oxygen species regulated mitochondria-mediated apoptosis in PC12 cells exposed to chlorpyrifos. Toxicol Appl Pharmacol 263, 148-162.

Lee, W.J., Alavanja, M.C., Hoppin, J.A., Rusiecki, J.A., Kamel, F., Blair, A., Sandler, D.P., 2007. Mortality among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. Environ Health Perspect 115, 528-534.

Lee, W.J., Blair, A., Hoppin, J.A., Lubin, J.H., Rusiecki, J.A., Sandler, D.P., Dosemeci, M., Alavanja, M.C., 2004. Cancer incidence among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. J Natl Cancer Inst 96, 1781-1789.

Long, M., Laier, P., Vinggaard, A.M., Andersen, H.R., Lynggaard, J., Bonefeld-Jorgensen, E.C., 2003. Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line. Toxicology 194, 77-93.

Mileson, B.E., Chambers, J.E., Chen, W.L., Dettbarn, W., Ehrich, M., Eldefrawi, A.T., Gaylor, D.W., Hamernik, K., Hodgson, E., Karczmar, A.G., Padilla, S., Pope, C.N., Richardson, R.J., Saunders, D.R., Sheets, L.P., Sultatos, L.G., Wallace, K.B., 1998. Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol Sci 41, 8-20.

Monnet-Tschudi, F., Zurich, M.G., Schilter, B., Costa, L.G., Honegger, P., 2000. Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetylcholinesterase and neuronal and glial markers in aggregating brain cell cultures. Toxicol Appl Pharmacol 165, 175-183.

Negroni, M.P., Fiszman, G.L., Azar, M.E., Morgado, C.C., Espanol, A.J., Pelegrina, L.T., de la Torre, E., Sales, M.E., 2010. Immunoglobulin G from breast cancer patients in stage I stimulates muscarinic acetylcholine receptors in MCF7 cells and induces proliferation: participation of nitric oxide synthase-derived nitric oxide. J Clin Immunol 30, 474-484.

Novotny, A., Ryberg, K., Heiman Ullmark, J., Nilsson, L., Khorram-Manesh, A., Nordgren, S., Delbro, D.S., Nylund, G., 2011. Is acetylcholine a signaling molecule for human colon cancer progression? Scand J Gastroenterol 46, 446-455.

Paleari, L., Grozio, A., Cesario, A., Russo, P., 2008. The cholinergic system and cancer. Semin Cancer Biol 18, 211-217.

Panuwet, P., Prapamontol, T., Chantara, S., Thavornyuthikarn, P., Montesano, M.A., Whitehead Jr, R.D., Barr, D.B., 2008. Concentrations of urinary pesticide metabolites in small-scale farmers in Chiang Mai Province, Thailand. Science of the total environment 407, 655-668.

Park, J.H., Lee, J.E., Shin, I.C., Koh, H.C., 2013. Autophagy regulates chlorpyrifos-induced apoptosis in SH-SY5Y cells. Toxicol Appl Pharmacol 268, 55-67.

Parnell, E.A., Calleja-Macias, I.E., Kalantari, M., Grando, S.A., Bernard, H.U., 2012. Muscarinic cholinergic signaling in cervical cancer cells affects cell motility via ERK1/2 signaling. Life Sci 91, 1093-1098.

Pettersson, A., Nilsson, L., Nylund, G., Khorram-Manesh, A., Nordgren, S., Delbro, D.S., 2009. Is acetylcholine an autocrine/paracrine growth factor via the nicotinic alpha7-receptor subtype in the human colon cancer cell line HT-29? Eur J Pharmacol 609, 27-33.

Rayford, W., Noble, M.J., Austenfeld, M.A., Weigel, J., Mebust, W.K., Shah, G.V., 1997. Muscarinic cholinergic receptors promote growth of human prostate cancer cells. Prostate 30, 160-166.

Sasaki, T., Hiroki, K., Yamashita, Y., 2013. The role of epidermal growth factor receptor in cancer metastasis and microenvironment. Biomed Res Int 2013, 546318.

Schmidt-Ullrich, R.K., Mikkelsen, R.B., Dent, P., Todd, D.G., Valerie, K., Kavanagh, B.D., Contessa, J.N., Rorrer, W.K., Chen, P.B., 1997. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. Oncogene 15, 1191-1197.

Schuller, H.M., 2009. Is cancer triggered by altered signalling of nicotinic acetylcholine receptors? Nat Rev Cancer 9, 195-205.

Shah, N., Khurana, S., Cheng, K., Raufman, J.P., 2009. Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296, C221-232.

Smegal, D.C., 2002. Human health risk assessment- Chlorpyrifos. U.S. Environmental Protection Agency, Washington DC.

Song, P., Spindel, E.R., 2008. Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in lung cancer provides a new target for cancer therapy. J Pharmacol Sci 106, 180-185.

Spano, J.P., Fagard, R., Soria, J.C., Rixe, O., Khayat, D., Milano, G., 2005. Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. Ann Oncol 16, 189-194.

Sturla, L.M., Amorino, G., Alexander, M.S., Mikkelsen, R.B., Valerie, K., Schmidt-Ullrichr, R.K., 2005. Requirement of Tyr-992 and Tyr-1173 in phosphorylation of the epidermal growth factor receptor by ionizing radiation and modulation by SHP2. J Biol Chem 280, 14597-14604.

Sultatos, L.G., Shao, M., Murphy, S.D., 1984. The role of hepatic biotransformation in mediating the acute toxicity of the phosphorothionate insecticide chlorpyrifos. Toxicol Appl Pharmacol 73, 60-68.

Ventura, C., Nunez, M., Miret, N., Martinel Lamas, D., Randi, A., Venturino, A., Rivera, E., Cocca, C., 2012. Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide. Toxicol Lett 213, 184-193.

Ventura, C., Venturino, A., Miret, N., Randi, A., Rivera, E., Nunez, M., Cocca, C., 2015. Chlorpyrifos inhibits cell proliferation through ERK1/2 phosphorylation in breast cancer cell lines. Chemosphere 120, 343-350.

Xie, G., Peng, Z., Raufman, J.P., 2012. Src-mediated aryl hydrocarbon and epidermal growth factor receptor cross talk stimulates colon cancer cell proliferation. Am J Physiol Gastrointest Liver Physiol 302, G1006-1015.

Yeh, J.J., Routh, E.D., Rubinas, T., Peacock, J., Martin, T.D., Shen, X.J., Sandler, R.S., Kim, H.J., Keku, T.O., Der, C.J., 2009. KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. Mol Cancer Ther 8, 834-843.

Zhao, Y., Wang, X., Wang, T., Hu, X., Hui, X., Yan, M., Gao, Q., Chen, T., Li, J., Yao, M., Wan, D., Gu, J., Fan, J., He, X., 2011. Acetylcholinesterase, a key prognostic predictor for hepatocellular carcinoma, suppresses cell growth and induces chemosensitization. Hepatology 53, 493-503.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า)
   - อยู่ในระหว่างดำเนินการจัดส่งบทความขอเพื่อเผยแพร่ ในชื่อ "Tawit Suriyo, Phum Tachachartvanich, Daranee Visitnonthachai, Piyajit Watcharasit, and Jutamaad Satayavivad. Chlorpyrifos promotes the growth of colorectal adenocarcinoma H508 cells through the activation of EGFR/ERK1/2 signaling pathway but not cholinergic pathway"
2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     - ยังไม่มี
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     - ยังไม่มี
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     - ยังไม่มี
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
     - จากผลการดำเนินการโครงการวิจัย "การศึกษาผลของยาฆ่าแมลงคลอร์ไพริฟอสต่อการเจริญเติบโต อยู่รอด และเคลื่อนแผ่กระจายของเซลล์มะเร็งลำไส้และตับ: บทบาทของระบบนอนนิวโรนอลคอลิเนอร์จิก" โดยสามารถพบว่ายาฆ่าแมลงคลอร์ไพริฟอสสามารถกระตุ้นการเจริญเติบโตของเซลล์มะเร็งลำไส้ใหญ่และทวารหนักได้ และสามารถอธิบายว่าผลในการกระตุ้นนี้เกี่ยวข้องกับการกระตุ้นการทำงานของตัวรับสัญญาณ EGF ซึ่งโครงการวิจัยนี้สามารถสร้างองค์ความรู้ใหม่ และผลิตบุคลากรนักวิจัยทางด้านพิษวิทยาสิ่งแวดล้อมของประเทศ โดยส่วนหนึ่งของผลการวิจัยของโครงการวิจัยนี้ได้รับรางวัล SOT/AstraZeneca/SOT Endowment/IUTOX Travel Award จากสมาคมพิษวิทยานานาชาติและสมาคมพิษวิทยาแห่งสหรัฐอเมริกา ให้ไปนำเสนอในการประชุมประจำปีของสมาคมพิษวิทยาแห่งสหรัฐอเมริกา ครั้งที่ 54 เมือง San Diego รัฐ California ประเทศสหรัฐอเมริกา ระหว่างวันที่ 21-27 มีนาคม 2558: Suriyo T, Tachachartvanuch P, Watcharasit P, Statyavivad J. 2015. Chlorpyrifos promotes the growth of colorectal adenocarcinoma H508 cells through the activation of

EGFR/ERK1/2 signaling pathway. *The Toxicologist*, Supplement to *Toxicological Sciences*, 144(1), Abstract #2155, 463.

3. อื่นๆ (เช่น หนังสือ การจดสิทธิบัตร)
   - ยังไม่มี

ภาคผนวก

บทความที่เตรียมสำหรับการขอเผยแพร่

(Manuscript)

Chlorpyrifos promotes the growth of colorectal adenocarcinoma H508 cells through the activation of EGFR/ERK1/2 signaling pathway but not cholinergic pathway

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Abstract

Aside from the effects on neuronal cholinergic system, epidemiological studies suggest an association between chlorpyrifos (CPF) exposure and cancer risk. This *in vitro* study examined the effects of CPF and its toxic metabolite, chlorpyrifos oxon (CPF-O), on the growth of human colorectal adenocarcinoma H508, normal colon epithelial CCD841, liver hepatocellular carcinoma HepG2, and normal liver hepatocyte THLE-3 cells. The results showed that CPF (0.1-100 µM) concentration-dependently increased viability of H508 and CCD841 cells in serum-free conditions. This increasing trend was not found in HepG2 and THLE-3 cells. In contrast, CPF-O (50-100µM) reduced the viability of all cell lines. Cell cycle analysis showed the induction of cells in the S phase, and EdU incorporation assay revealed the induction of DNA synthesis in CPF-treated H508 cells indicating that CPF promotes cell cycle progression. Despite the observation of acetylcholinesterase inhibition and reactive oxygen species (ROS) generation, atropine (a non-selective muscarinic acetylcholine receptor antagonist) and N-acetylcysteine (a potent antioxidant) failed to inhibit the growth-promoting effect of CPF. CPF increased the phosphorylation of epidermal growth factor receptor (EGFR) and its downstream effector, extracellular signal regulated kinase (ERK1/2), in H508 cells. AG-1478 (a specific EGFR tyrosine kinase inhibitor) and U0126 (a specific MEK inhibitor) completely mitigated the growth promoting effect of CPF. Altogether, these results suggest that CPF promotes the growth of colorectal adenocarcinoma H508 cells through the activation of EGFR/ERK1/2 signaling pathway.

Keywords: Chlorpyrifos, chlorpyrifos oxon, colon cancer, cancer cell growth, EGFR, H508 cells

Introduction

Chlorpyrifos [O,O-diethyl-O-(3,5,5-trichloro-2-pyridyl)-phos-phorothioate] (CPF) is the most extensively used broad-spectrum organophosphate insecticide and has been widely applied in agriculture throughout the world (Colt et al., 2004; Panuwet et al., 2008). Although the U.S. Environmental Protection Agency (EPA) banned CPF for residential pest control due to health effects, many other countries still heavily use CPF (Panuwet et al., 2008). The primary target of CPF toxicity is both the central and peripheral cholinergic neural systems, due to its ability to inhibit acetylcholinesterase (AChE) activity (Mileson et al., 1998). CPF is a weak anti-AChE compound. To inhibit AChE activity, CPF must undergo an oxidation desulfuration to become its oxygen (oxon) analogue, chlorpyrifos oxon (CPF-O). This biotransformation is executed by the cytochrome P450 monooxygenase system, which is found in the liver with a high level (Sultatos et al., 1984). It has been reported that CPF-O-induced inhibition of AChE activity is up to 28 and 180 orders of magnitude more potent than the parent compound, CPF, in immature and differentiated brain cells, respectively (Monnet-Tschudi et al., 2000). As a result of irreversible binding of CPF and CPF-O to the active site of AChE, the enzyme's ability to hydrolyze neurotransmitter acetylcholine (ACh) becomes defective, which causes an accumulation of ACh at the neuronal cholinergic synapses and over-activation of cholinergic signaling, resulting in cholinergic adverse effects (Howard et al., 2007).

The major function of non-neuronal cholinergic system in certain cancers is well documented, including lung (Song and Spindel, 2008), colon (Cheng et al., 2008b; Novotny et al., 2011; Pettersson et al., 2009), liver (Zhao et al., 2011), prostate (Rayford et al., 1997), cervical (Parnell et al., 2012) and breast cancers (Espanol et al., 2007; Negroni et al., 2010). The non-neuronal cholinergic system plays a key role in regulation of important cell functions, including proliferation, differentiation, migration, organization of the cytoskeleton, cell-to-cell communication, and other features critical for cancer progression (Paleari et al., 2008; Schuller, 2009; Shah et al., 2009). A recent study has shown that the expression of AChE is often down-regulated in hepatocellular carcinoma and functions as a tumor growth suppressor in regulating cell

proliferation and increasing drug sensitivity via its enzymatic activity (Zhao et al., 2011). Additionally, for example, human colon cancer cell can increase physiological responses, invasion, migration and proliferation via cholinergic muscarinic receptor activation (Belo et al., 2011; Cheng et al., 2008a). It has been demonstrated that the expression of anti-apoptotic protein, Bcl-2, can be induced by cholinergic muscarinic receptor signaling, resulting in elevating the cell viability and hindering cell death (Budd et al., 2003).

The evidence that CPF is involved in carcinogenesis is still scarce. Up to now, the International Agency for Research on Cancer (IARC) has not evaluated the carcinogenicity of CPF (IARC, 2010). Furthermore, the weight of biological evidence reviewed by the U.S. EPA and Canadian Pest Management Regulatory Agency (PMRA) do not suggest that CPF is a carcinogenic pesticide (Health Canada, 2003; Smegal, 2002). However, epidemiological studies related to occupational pesticide exposures and cancer incidences in the Agricultural Health Cohort Study (AHS) showed that pesticide applicators with the highest lifetime exposure-days for CPF had increased colorectal and lung cancer risk with a significant exposure-response relationship relative to non-exposed applicators (Lee et al., 2007; Lee et al., 2004). Furthermore, a recent study demonstrated action of CPF as an environmental breast cancer risk factor due to its effects on the mechanisms that modulate breast cancer cell proliferation (Ventura et al., 2012). However, it is difficult to conclude, based on limited evidence at this time, the causal nature of these associations. Therefore, further studies are strongly needed.

It has long been known that CPF causes ACh accumulation in the neuronal cholinergic synapses leading to overstimulation of cholinergic receptors (Howard et al., 2007). Together with cholinergic receptor activation, CPF causes non-neuronal cholinergic cancer cell proliferation, particularly colorectal and liver cancers (Cheng et al., 2008a; Paleari et al., 2008; Zhao et al., 2011). We hypothesize that as a result of AChE inhibiting action of CPF and CPF-O, an accumulation of ACh in cancer cell occurs and further causes cancer cell growth through activation of cholinergic signaling. This *in vitro* study examined the effects of CPF and CPF-O on the growth of human colorectal

adenocarcinoma H508, normal colon epithelial CCD841, liver hepatocellular carcinoma HepG2, and normal liver hepatocyte THLE-3 cells.

Materials and Methods

Chemicals and reagents

Chlorpyrifos (diethyl 3,5,6-trichloro-2-pyridyl phosphorothionate) (CPF; purity 99.9%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Chlorpyrifos oxon (CPF-O; purity 98.9%) was ordered from Chem Service (West Chester, PA, USA). Epidermal growth factor (EGF) was obtained from BD Biosciences (Bedford, MA, USA). Carbamoylcholine chloride (carbachol), atropine sulfate, mecamylamine hydrochloride, N-acetylcysteine (NAC) and tyrphostin AG-1478 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solutions of CPF and CPF-O were prepared in ethanol at the concentration of 100 mM (Sigma-Aldrich, St. Louis, MO, USA). EGF, carbachol, mecamylamine, and atropine were freshly prepared by dissolved in sterile water at the concentrations of 100 µg/ml, 1 M, 100 mM, and 10 mM, respectively. Tyrphostin AG-1478 was prepared as a stock solution in ethanol at the concentration of 30 mM.

Cell lines

Cell lines including Hep-G2 cell line (a human epithelial hepatocellular carcinoma), THLE-3 cell line (a human normal liver epithelial-immortalized with SV40 large T-antigen), NCI-H508 cell line (a human epithelial colorectal adenocarcinoma), and CCD-841-Con cell line (a human normal colon epithelial-immortalized with SV40 large T-antigen) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Hep-G2 cells were cultured in minimum essential medium (MEM) (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine (Gibco, Carlsbad, CA, USA), 1.0 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA), 100 unit/ml penicillin, 100 µg/ml streptomycin (Gibco, Carlsbad, CA, USA), and 10% (vol/vol) fetal bovine serum (FBS) (JR Scientific, Woodland, CA, USA). THLE-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS, 25 mM HEPES (Sigma-Aldrich, St. Louis, MO, USA), 100 unit/ml of penicillin and 100 µg/ml streptomycin. NCI-H508 cells were maintained

in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 1.0 mM sodium pyruvate, 4.5 g/l glucose (Sigma-Aldrich, St. Louis, MO, USA), 100 unit/ml of penicillin, 100 µg/ml streptomycin and 10% FBS. CCD-841-Con cells were cultured in DMEM supplemented with 10% FBS, 100 unit/ml of penicillin and 100 µg/ml streptomycin. All cell lines were maintained at 37ºC in a humidified atmosphere containing 95% air and 5% CO_2.

Cell viability assay

Cell viability was measured by PrestoBlue™ cell viability assay (Molecular Probes, Invitrogen, Carlsbad, CA, USA) or a quantitative colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO, USA) which both assays detecting metabolically active cells. Cells were seeded in 96-well plate (1×10^4 cells/well) and cultured overnight to allow attachment. After incubation, cells were starved by maintaining in serum free medium for 24 hr. The starving cells were treated with various concentrations of CPF or CPF-O (0.1-100 µM) for 48 hr. The final concentration of ethanol in all treatment conditions was 0.1%, which did not affect the cell viability in control plates. At the end of the respective incubation period, 10 µl of PrestoBlue™ cell viability reagent was added directly to each well, and cells were further incubated at 37ºC for 30 min. The fluorescence intensity was determined at 560 nm for excitation and 590 nm for emission using microplate-scanning spectrophotometer (SpectraMax® M3, Molecular Devices, Sunnyvale, CA, USA). For MTT assay, 100 µl of MTT (500 µg/ml MTT in serum free medium) was added to each well. Cells were incubated for 4 hr at 37^{o}C for formazan formation, MTT was removed and formazan was dissolved with dimethylsulfoxide (DMSO) (Sigma-Aldrich, ST. Louis, MO, USA). After formazan solubilization, the plate was measured at the absorbance of 570 nm with reference wavelength of 650 nm using microplate-scanning spectrophotometer.

Cell cycle assay

The cells (5×10^6 cells) were seeded in a 100 mm plate and processed as previously described in the cell viability assay. After 48 hr incubation with CPF, the cells were analyzed for the distribution of G1, S, and G2/M phases of cell cycle by flow cytometer with propidium iodide (PI) staining. Briefly, the medium was removed, and

cells were harvested with trypsinization (Gibco, Carlsbad, CA, USA). Cell suspension was centrifuged at 500 × g, 4°C for 5 min. Cell pellets were washed with phosphate buffer saline (PBS) (Gibco, Carlsbad, CA, USA) and subsequently fixed in 70% ethanol overnight at -20^{o}C. After fixing with 70% ethanol, the cells were washed with cold PBS and incubated with 50 µg/ml of PI (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 µg/ml of RNAse (Sigma-Aldrich, St. Louis, MO, USA) at ambient temperature for 15 min to stain nucleic acids and digest RNA, respectively. The cell cycle stages were measured by flow cytometer (BD FACSCanto, BD Biosciences, Franklin Lakes, NJ, USA) and the data was analyzed by Modfit LT software (Verity House Software, Topsham, ME, USA).

Cell proliferation assay

Cell proliferation was determined by the incorporation of 5-ethynil-2-deoxyuridine (EdU) into newly synthesis DNA stand using Click-iT® EdU microplate assay (Molecular probes, Invitrogen, Carlsbad, CA, USA). Briefly, cells were seeded in 96-well plate (1×10^4 cells/well) and processed as previously described in the cell viability assay. At the end of the respective incubation period, a working stock of 10X EdU in pre-warmed complete media was added to each well at final concentration of 10 µM and further incubated at 37°C for 3 hr. The incorporated EdU in DNA was coupled with Oregon Green-azide dye, and then subsequently incubated with horseradish peroxidase-labeled anti-Oregon Green antibody, Amplex® UltraRed, and N-acetyl-3, 7-dihydroxyphenoxazine. The fluorescence intensity was determined at 490 nm for excitation and 585 nm for emission using microplate-scanning spectrophotometer.

Enzymatic activity of acetylcholinesterase assay

Acetylcholinesterase (AChE) enzymatic activity in cells was determined by the Ellman method (Ellman et al., 1961) modified for use with microplate. Briefly, cells (5×10^6 cells) were seeded in a 100 mm plate processed as previously described in the cell viability assay. After 48 hr incubation with CPF, cells were washed with cold PBS and lysed in lysis buffer containing 10 mM Tris-HCl pH 7.4 (Sigma-Aldrich, St. Louis, MO, USA), 150 mM NaCl (Sigma-Aldrich, St. Louis, MO, USA), 1% triton X-100 (Bio-Rad, Hercules, CA, USA), 0.1

mM PMSF (Sigma-Aldrich, St. Louis, MO, USA), 1 mM Na_3VO_4 (Sigma-Aldrich, St. Louis, MO, USA), 20 mM NaF (Sigma-Aldrich, St. Louis, MO, USA), and protease cocktail inhibitor (Calbiochem, Germany). Cell lysates were sonicated and incubated at 4°C for 30 min then centrifuged at 16,000 · g for 15 min at 4°C. The concentration of protein was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Afterwards, 100 µl of protein sample containing 300 µg proteins was mixed with 50 µl of dithiobisnitrobenzoate (DTNB) solution (1.25 mM DTNB (Sigma-Aldrich, St. Louis, MO, USA), 0.1875 mg/ml $NaHCO_3$ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M PBS pH 8.0). The mixture was allowed to sit at room temperature for 5 min, and then 50 µl of acetylcholine iodine (ATCI) substrate solution (1.87 mM ATCI (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M PBS pH 8.0) was added. The absorbance was monitored every 2 min intervals for 12 min at 410 nm at 25°C using microplate-scanning spectrophotometer. The absorbance was corrected by subtracting the absorbance observed in a blank. AChE activity was extrapolated from standard curve of the purified AChE standard (Sigma-Aldrich, St. Louis, MO, USA).

Western immunoblotting assay

The cells (5×10^6 cells) were seeded in a 100 mm plate and processed as previously described in the cell viability assay. At the end of the respective incubation period, the total protein cell lysates were prepared as previously described in AChE enzymatic activity assay. The protein (50 µg) was mixed with Laemmli loading buffer (Bio-Rad, Hercules, CA, USA) and boiled at 95°C for 5 min. The proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis in a Mini-PROTEAN II system (Bio-Rad, Hercules, CA, USA). The separated protein bands were transferred onto a nitrocellulose membrane (GE Healthcare, United Kingdom). The membrane was incubated in blocking buffer containing 5% non-fat dry milk in TBST buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, and 0.05% Tween-20) for 1 h at room temperature followed by overnight incubation at 4°C with primary antibodies. The antibodies against AChE, phosphorylated EGFR at tyrosine 1173 residue, and total EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against phosphorylated ERK1/2, total ERK1/2, and β-actin were

obtained from Cell Signaling Technology (Cell Signaling, Beverly, MA, USA). After washing with TBST buffer, the membrane was incubated with horseradish-peroxidase conjugated secondary antibodies (GE Healthcare, United Kingdom) for 2 hr at room temperature. The protein bands stained by the antibody were visualized by using enhanced chemiluminescence (GE Healthcare, United Kingdom) followed by exposure to x-ray films (Pierce-Perbio, Brazil). Relative protein expressions were calculated from band intensities using computerized densitometry with ImageQuantTL software (GE Healthcare, United Kingdom).

Reactive Oxygen Species (ROS) Assay

The cells were seeded in a 6-well plate (7×10^5 cells/well) and processed as previously described in the cell viability assay. The cells were incubated with 25 µM of H_2DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min before the end of treatment. The treated cells were trypsinized and centrifuged at 2,300 $\times$ g for 5 min to remove excessed H_2DCF-DA dye. Then cells were re-suspended with cold PBS. The fluorescence was measured using BD LSRFortessaTM Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Data was expressed as the means ± standard error of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Fisher's Least Significant Difference (LSD) test. A two-tailed *p*-value less than 0.05 was considered as a statistically significant difference.

Results

1. Chlorpyrifos increases the viability of normal colon epithelial CCD841 cells and colorectal adenocarcinoma H508 cells

After treatment with CPF and CPF-O in a serum free condition for 48 hr, cell viability was assessed by PrestoBlue™ or MTT metabolic activity assays. The results from MTT assay showed that 5 and 10 µM of CPF significantly increased the viability of normal colon epithelial CCD841 cells but this increasing response slightly decreased at higher concentrations (Fig. 1A). CPF (0.1-100 µM) concentration-dependently increased the viability of colorectal adenocarcinoma H508 cells (Fig. 1B). The significant differences from the control occurred at 10, 50, and 100 µM of CPF treated groups. At the tested concentration range (0.1-100 µM), the viability of normal hepatocyte THLE3 cells and hepatocellular carcinoma HepG2 cells were not affected by CPF (Fig. 1C and 1D). Meanwhile, CPF-O at two highest tested concentrations (50 and 100 µM) dramatically reduced the cell viability of all tested cell lines, but not in H508 cell line while CPF-O at lower concentrations (0.1-10 µM) did not affect the cell viability. It should be noted that, the liver cell lines (THLE-3 and HepG2) are more likely to be sensitive to the toxic effect of CPF-O than the colon cell lines (CCD841 and H508).

For comparison, the results of PrestoBlue™ assay showed similar pattern with MTT assay, but higher sensitivity in all cell lines except H508 cells (Fig. 2). PrestoBlue™ cell viability assay showed that CPF-O (0.1-100 µM) concentration dependently increased the viability of H508 cells. In contrast, at the two highest tested concentrations of CPF (50 and 100 µM), the MTT assay showed the reduction of H508 cell viability. Observation under phase contrast microscopy revealed that cells treated with these high concentrations of CPF-O (50 and 100 µM) were detached from the plate and reduced in cell size (data not shown). These results suggested that CPF increased CCD841 and H508 cell viability in serum-free condition.

2. Chlorpyrifos stimulates growth of colorectal adenocarcinoma H508 cells

Further study was conducted to investigate the effect of CPF on cell cycle and DNA synthesis of H508 cells. Cell cycle was measured by flow cytometry with propidium iodide (PI) staining. The results indicated that CPF concentration dependently increased the percentage of cells in the S phase (Fig. 3). The percentage of H508 cells in the S phase increased from 8.44% for the control to 9.92-17.10% for cells treated with 1-100 µM of CPF. Significant differences from the control were observed at 50 and 100 µM of CPF treated groups in the S group. Furthermore, the increased percentage of cells in S phase was accompanied with the decreased percentage of cells in G0/G1 phase. The positive control (10 ng/ml of EGF) significantly increased the percentage of cells in the S and G2/M phases, while decreasing the percentage of cells in G0/G1 phase.

Next, the EdU incorporation assay was utilized to determine the proliferation by measuring the rate of DNA synthesis during S phase of cell cycle. CPF at concentrations of 10 and 50 µM induced proliferation by activation of DNA synthesis, significantly higher than the control about 108.7 and 164.7%, respectively (Fig. 4). Notably, the positive control (10 ng/ml of EGF) also dramatically activated cell proliferation by 372.4% compared to the control. These results suggested that CPF stimulated colorectal adenocarcinoma H508 cell growth.

3. Non-neuronal cholinergic system does not involve in chlorpyrifos-stimulated growth of colorectal adenocarcinoma H508 cells

It is well established that non-neuronal cholinergic system plays an important role in colon epithelial tumorigenesis (Novotny et al., 2011). We studied the role of non-neuronal cholinergic system in CPF-induced H508 cell growth. Initial experiment was performed to measure the effects of CPF on the protein expression and enzymatic activity of acetylcholinesterse (AChE). Cells were treated with 1-50 µM of CPF in a serum free medium for 24 and 48 hr, the protein expression of AChE was measured by Western immunobloting assay. The AChE enzymatic activity in cells was determined by the modified Ellman

method. The results showed that CPF did not alter the protein expression level of AChE in either 24 or 48 hr (Fig 5A); meanwhile, it decreased AChE enzymatic activity in a concentration-dependent manner (Fig. 5B). Note that the significant differences from the control were observed at 10 and 50 µM of CPF-treated groups.

To examine whether the growth stimulating effect of CPF is related to the non-neuronal cholinergic system, cells were pretreated for 30 min with specific cholinoceptor antagonists, including 10 µM of atropine (a non-selective muscarinic acetylcholine receptor antagonist) or 100 µM of mecamylamine (a non-selective nicotinic acetylcholine receptor antagonist), and then cells were further co-treated with 1-50 µM of CPF or 1 mM of carbachol (a cholinoceptor agonist) in serum free medium for 48 hr. The results of PrestoBlue™ assay showed that either mecamylamine or atropine failed to attenuate the growth promoting effect of CPF (Fig. 6). Notably, the positive control, carbachol, significantly stimulated growth of H508 cells and atropine, but not mecamylamine, which completely blocked this growth promoting effect.

4. Chlorpyrifos induces colorectal adenocarcinoma H508 cell growth via an activation of EGFR/ERK1/2 signaling pathway

The epidermal growth factor receptor (EGFR) is over-expressed in colorectal cancer patient populations and it is recognized as an important player in colon cancer initiation and progression (Sasaki et al., 2013; Spano et al., 2005). We determined the expression level of EGFR in colon and liver cell lines. The results showed that the background levels of the total and active/phospholylated forms of EGFR were varying among colon and liver cell lines. H508 and THLE-3 cells highly expressed the total form of EGFR when compared to HepG2 and CCD841 cells (Fig. 7). Interestingly, the basal background level of the active/phosphorylated form of EGFR at tyrosine 1178 in colon cells especially H508 cells were higher than the tested liver cells.

We studied the effects of CPF on EGFR signaling in H508 cells. Cells were treated with 1-50 µM of CPF in serum free medium for 24 and 48 hr. The activation of EGFR and its downstream signaling cascade were determined by Western immunobloting assay. The results of 24 hr incubation period showed

that CPF increased the expression level of active/phosphorelated EGFR at tyrosine 1173 residue in a concentration-dependent manner with a significant difference from the control at 10 and 50 μM of CPF (Fig. 8B). In contrast, the results of 48 hr incubation period showed that the expression level of active EGFR concentration dependently reduced by CPF treatment. Extracellular signal regulated kinase (ERK1/2), which is one of the most important downstream effectors of EGFR activation, was slightly (though, non-significantly) activated by CPF treatment at 24 hr exposure period (Fig. 8C). Furthermore, the activation of ERK1/2 was significantly increased by CPF treatment at 48 hr exposure period.

In order to determine the role of EGFR/ERK1/2 signaling in CPF-induced H508 cell growth, cells were pretreated with 0.1-1 μM of AG-1478 (a specific EGFR tyrosine kinase inhibitor) or 1-5 μM of U0126 (a specific MEK inhibitor) for 30 min before co-treatment with CPF for 48 hr. The PrestoBlue™ viability assay revealed that the growth promoting effect of CPF was significantly mitigated by pre- and co-treatment with 1 μM of AG-1478 (Fig. 9A). For MEK inhibitor, the growth stimulating effect of CPF was also significantly attenuated by pre- and co-treatment with 5 μM of U0126 (Fig. 9B). Though the 1 μM of AG-1478 and 5 μM of U0126 did not affect the viability of H508 cells, but they significantly attenuated the growth-stimulating effect of 10 and 100 ng/ml of EGF, respectively.

5. Chlorpyrifos stimulates reactive oxygen species in colorectal adenocarcinoma H508 but this effect does not involved in the growth promoting effect of chlorpyrifos

Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), have been shown to induce phosphorylation of EGFR, in part, due to its protein-tyrosine phosphatase inhibition (Kamata et al., 2000). Since, it has been reported that CPF can generate oxidative stress by inducing the production of ROS (Ki et al., 2013), we determined the effect of CPF on the level of ROS in H508 cells. The results showed that 24 and 48 hr exposure of CPF (10-100 μM) concentration dependently increased ROS in H508 cells (Fig 10A). Pretreatment the cells with 50 mM of H_2O_2 for 15 min (positive control) also significantly

increased the level of ROS when compared to the control. Moreover, the involvement of oxidative stress in CPF-induced growth of H508 cells was also determined. Cells were pretreated with 2-4 mM of the antioxidant N-acetylcysteine (NAC) for 30 min before co-treatment with CPF or EGF for 48 hr. The MTT viability assay revealed that pre- and co-treatment with NAC did not reduce the growth promoting effect of CPF. As expected, NAC also did not mitigate the growth promoting effect of EGF (Fig. 10B).

Discussion

The Agricultural Health Study (AHS) which is a largest perspective cohort study has identified an association between exposure to CPF and increased lung, brain, and colorectal cancer risk in pesticide applicators (Weichenthal et al., 2010). However, the carcinogenic mechanisms of CPF remain unclear. The present study provides the novel information on the carcinogenic effect of CPF in colorectal cancer. This study showed the growth promoting effect of CPF in colorectal adenocarcinoma H508 cells through the activation of EGFR/ERK1/2 signaling pathway.

The present study showed that CPF at the concentration up to 100 μM did not affect the viability of normal hepatocyte THLE-3 and hepatocellular carcinoma HepG2 cells. A previous study demonstrated that CPF at the concentration of 25-200 μM, concentration dependently reduced the viability of human neuroblastoma SH-SY5Y cells (Ki et al., 2013). Furthermore, it has been shown that CPF significantly increased cytotoxicity and the IC50 value (the concentration that inhibited 50% growth) at 24 hr for CPF was about 100 μM in human neuron PC12 (Lee et al., 2012) and SH-SY-5Y cells (Park et al., 2013). The difference in sensitivity among cell lines to CPF could be explained in part due to the differences in molecular characteristics of the different cancer cells, especially anti-oxidant capacity, since oxidative stress is involved in CPF-induced apoptosis (Ki et al., 2013; Ventura et al., 2015). The glutathione system including glutathione, glutathione reductase, glutathione peroxidases, and glutathione *S*-transferases are particularly at high level in hepatocytes (Hayes et al., 2005). The viability assay showed that CPF-O at concentrations of 50-100 μM demonstrated more toxic effects in reducing cell viability than its parent

compound. CPF-O is about 28-180 orders of magnitude more potent than CPF in inhibiting brain AChE activity (Monnet-Tschudi et al., 2000), suggesting over-activation of cholinergic signaling from CPF-O-induced AChE inhibition. However, a previous study suggested that CPF-O-induced apoptosis in rat cortical neurons may occur independently of AChE inhibition (Caughlan et al., 2004). Further research should investigate potential correlations between AChE inhibition and the cytotoxic effect of CPF-O. The present study showed that PrestoBlue™ metabolic activity assay showed similar patterns of MTT mitochondrial metabolic activity assay with higher sensitivity. Contrasting results were observed only in CPF-O-treated H508 cells. The cytotoxic effect of CPF-O at the high concentrations in H508 cells had been demonstrated by the MTT assay and cell morphology observations under phase contrast microscope. The fault positive result on the induction of cell viability in CPF-O-treated H508 cells had been found by PrestoBlue™ assay. Further study need to be further investigated to better understand this specific fault positive result.

The present study found that CPF at the concentration range 10-100 μM significantly increased the viability of normal colon epithelial CCD841 and colorectal adenocarcinoma H508 cells. The cell cycle analysis showed the induction of cells in the S phase, and the EdU incorporation assay revealed the induction of the DNA synthesis in CPF-treated H508 cells. These results demonstrated the growth-promoting effect of CPF in H508 cells. A previous study also demonstrated that low-levels of CPF (0.05 μM) promoted cell proliferation in the hormone-dependent breast cancer MCF-7 cells through estrogen receptor (ER) signaling pathway, but higher concentrations of this insecticide caused cell death (Ventura et al., 2012). Furthermore, CPF showed a strong aryl hydrocarbon receptor (AhR) agonistic activity compared to other pesticides, including methiocarb, chlorothalonil, tribenuron-methyl, paclobutrazol and tolchlofos-methyl (Long et al., 2003). In addition, Src-mediated cross-talk between AhR and EGFR signaling pathways strongly activate proliferation of H508 cells (Xie et al., 2012). However, we found that pretreatment either with a specific AhR antagonist, CH223191 (100 nM), or a high affinity ER antagonist, ICI182780 (10 μM), failed to attenuate the CPF-induced H508 cell growth (data not shown). Even though the inhibitory effect on the AChE activity was

observed, atropine (a muscarinic receptor antagonist) or mercamylamine (a nicotinic receptor antagonist) did not inhibit the growth-promoting effect of CPF. This may be partially due to insufficient concentrations of ACh in the cells to activate cholinergic receptors and cellular responses. Altogether, these results suggested that AhR, ER, or cholinergic receptors are not involved in the growth promoting effect of CPF in H508 cells in our experimental condition.

Furthermore, the results of this study showed that CPF caused an activation of EGFR by increasing the phosphorylation of EGFR at tyrosine 1173 residue after 24 h incubation period. However, the reduction of EGFR phosphorylation at tyrosine 1173 residue was observed after 48 h incubation period. This dynamic nature of EGFR activation is possibly due to the high activation of EGFR. The carboxy terminal tyrosine residue on EGFR is the major site of autophosphorylation, which occurs as a result of ligand binding. The autophosphorylation plays a critical role in the activation of the MAPK cascade following EGF stimulation (Schmidt-Ullrich et al., 1997; Sturla et al., 2005). In line with the activation of EGFR, CPF also increased the phosphorylation of ERK1/2, which is one of the most important downstream effectors of EGFR activation, after 48 h incubation period.

To the best of our knowledge, this is the first study to report new evidence showing that CPF induces H508 cell growth via activation of the EGFR/ERK1/2 signaling pathway, since EGFR antagonist (AG1478) and MAK inhibitor (U0126) completely mitigated CPF-mediated H508 cell growth. It is worth noting that, there are no studies reporting the CPF-elicited ability to interact with EGFR or activate EGFR/ERK1/2 signaling pathway, which leads to the proliferation of the cancer cells. Many studies revealed that activation of MAPK signaling pathways are involved in regulating CPF-induced apoptosis (CITATION?). For example, CPF (100 μM) induced apoptosis involving the activation of MAPK pathways including JNK, ERK1/2, and p38 MAPK, through oxidative stress in human neuroblastoma SH-SY5Y cells (Ki et al., 2013). A recent study also demonstrated that CPF (50 μM) inhibited cell proliferation in breast cancer MCF-7 and MDA-MB-231 cells through an incremental phosphorylation of p-ERK1/2 levels mediated by oxidative stress (Ventura et al., 2015). Even though the increase in the production of ROS by CPF treatment was observed in this study,

oxidative stress may not be involved in the growth-promoting effect of CPF in H508 cells, since antioxidant NAC did not reverse CPF-induced H508 cell growth.

The growth promoting effect of CPF seem to be cancer and tissue specific in that colon cancer cells were relatively response to CPF than normal colon epithelial cells or other type of cancers. The difference CPF responses among cell lines could be explained in part due to the molecular characteristics of the different cells, especially in the background level of phosphorylated/activated form of EGFR or the mutation of EGFR. It has been shown that the increase in basal phosphorylation of EGFR was evidenced when tyrosine 1173 was mutated (Sturla et al., 2005). Even though the mutation in EGFR in H508 cells was not reported (Ahmed et al., 2013; Yeh et al., 2009), this study found that the basal background level of phosphorylated/activated form of EGFR at tyrosine 1173 residue was very high in H508 cells. This may explain the finding on the growth-promoting effect of CPF is likely specific to the colon, especially in H508 cells but not liver cells. However, this hypothesis needs further study to clarify.

Although, CPF is highly metabolized and quickly detoxified in human, normal colon epithelial cells or colon cancer cells which line in the inner layer of the colon may directly contact with CPF. Following oral intake of CPF by rat, 90% was removed in the urine as metabolized forms and 10% was excreted in the feces as an unchanged form (Smith et al., 1967). Furthermore, the growth promoting concentration of CPF in this study was started at 5 µM which is not too high and may be possibly found in the gut.

In conclusion, the present study found that CPF promoted the growth of colorectal adenocarcinoma H508 cells via EGFR/ERK1/2 signaling pathway. Even through the inhibition of AChE was evidenced, non-neuronal cholinergic system was not involved in the growth-promoting effect of CPF in cholinergic-responded cancer cells.

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References

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknaes, M., Hektoen, M., Lind, G.E., Lothe, R.A., 2013. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2, e71.

Belo, A., Cheng, K., Chahdi, A., Shant, J., Xie, G., Khurana, S., Raufman, J.P., 2011. Muscarinic receptor agonists stimulate human colon cancer cell migration and invasion. Am J Physiol Gastrointest Liver Physiol 300, G749-G760.

Budd, D.C., McDonald, J., Emsley, N., Cain, K., Tobin, A.B., 2003. The C-terminal tail of the M3-muscarinic receptor possesses anti-apoptotic properties. J Biol Chem 278, 19565.

Caughlan, A., Newhouse, K., Namgung, U., Xia, Z., 2004. Chlorpyrifos induces apoptosis in rat cortical neurons that is regulated by a balance between p38 and ERK/JNK MAP kinases. Toxicol Sci 78, 125-134.

Cheng, K., Samimi, R., Xie, G., Shant, J., Drachenberg, C., Wade, M., Davis, R.J., Nomikos, G., Raufman, J.P., 2008. Acetylcholine release by human colon cancer cells mediates autocrine stimulation of cell proliferation. Am J Physiol Gastrointest Liver Physiol 295, G591-597.

Colt, J.S., Lubin, J., Camann, D., Davis, S., Cerhan, J., Severson, R.K., Cozen, W., Hartge, P., 2004. Comparison of pesticide levels in carpet dust and self-reported pest treatment practices in four US sites. J Expo Sci Environ Epidemiol 14, 74-83.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7, 88-95.

Espanol, A.J., de la Torre, E., Fiszman, G.L., Sales, M.E., 2007. Role of non-neuronal cholinergic system in breast cancer progression. Life Sci 80, 2281-2285.

Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. Annu Rev Pharmacol Toxicol 45, 51-88.

Health Canada, 2003. Proposed acceptability for continuing registration: Phase 2 of the Re-evaluation of chlorpyrifos. Pest Management Regulatory

Agency, Ottawa, Ontorio, Canada.

Howard, M.D., Mirajkar, N., Karanth, S., Pope, C.N., 2007. Comparative effects of oral chlorpyrifos exposure on cholinesterase activity and muscarinic receptor binding in neonatal and adult rat heart. Toxicology 238, 157-165.

IARC, 2010. IARC Monographs on the evaluation of carcinogenic risks to humans., In: Cancer), I.A.f.R.o. (Ed.).

Kamata, H., Shibukawa, Y., Oka, S.I., Hirata, H., 2000. Epidermal growth factor receptor is modulated by redox through multiple mechanisms. Effects of reductants and H2O2. Eur J Biochem 267, 1933-1944.

Ki, Y.W., Park, J.H., Lee, J.E., Shin, I.C., Koh, H.C., 2013. JNK and p38 MAPK regulate oxidative stress and the inflammatory response in chlorpyrifos-induced apoptosis. Toxicol Lett 218, 235-245.

Lee, J.E., Park, J.H., Shin, I.C., Koh, H.C., 2012. Reactive oxygen species regulated mitochondria-mediated apoptosis in PC12 cells exposed to chlorpyrifos. Toxicol Appl Pharmacol 263, 148-162.

Lee, W.J., Alavanja, M.C., Hoppin, J.A., Rusiecki, J.A., Kamel, F., Blair, A., Sandler, D.P., 2007. Mortality among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. Environ Health Perspect 115, 528-534.

Lee, W.J., Blair, A., Hoppin, J.A., Lubin, J.H., Rusiecki, J.A., Sandler, D.P., Dosemeci, M., Alavanja, M.C., 2004. Cancer incidence among pesticide applicators exposed to chlorpyrifos in the Agricultural Health Study. J Natl Cancer Inst 96, 1781-1789.

Long, M., Laier, P., Vinggaard, A.M., Andersen, H.R., Lynggaard, J., Bonefeld-Jorgensen, E.C., 2003. Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line. Toxicology 194, 77-93.

Mileson, B.E., Chambers, J.E., Chen, W.L., Dettbarn, W., Ehrich, M., Eldefrawi, A.T., Gaylor, D.W., Hamernik, K., Hodgson, E., Karczmar, A.G., Padilla, S., Pope, C.N., Richardson, R.J., Saunders, D.R., Sheets, L.P., Sultatos, L.G., Wallace, K.B., 1998. Common mechanism of toxicity: a case study of organophosphorus pesticides. Toxicol Sci 41, 8-20.

Monnet-Tschudi, F., Zurich, M.G., Schilter, B., Costa, L.G., Honegger, P., 2000. Maturation-dependent effects of chlorpyrifos and parathion and their oxygen analogs on acetylcholinesterase and neuronal and glial markers in aggregating brain cell cultures. Toxicol Appl Pharmacol 165, 175-183.

Negroni, M.P., Fiszman, G.L., Azar, M.E., Morgado, C.C., Espanol, A.J., Pelegrina, L.T., de la Torre, E., Sales, M.E., 2010. Immunoglobulin G from breast cancer patients in stage I stimulates muscarinic acetylcholine receptors in MCF7 cells and induces proliferation. Participation of nitric oxide synthase-derived nitric oxide. J Clin Immunol 30, 474-484.

Novotny, A., Ryberg, K., Heiman Ullmark, J., Nilsson, L., Khorram-Manesh, A., Nordgren, S., Delbro, D.S., Nylund, G., 2011. Is acetylcholine a signaling molecule for human colon cancer progression? Scand J Gastroenterol 46, 446-455.

Paleari, L., Grozio, A., Cesario, A., Russo, P., 2008. The cholinergic system and cancer. Semin Cancer Biol 18, 211-217.

Panuwet, P., Prapamontol, T., Chantara, S., Thavornyuthikarn, P., Montesano, M.A., Whitehead Jr, R.D., Barr, D.B., 2008. Concentrations of urinary pesticide metabolites in small-scale farmers in Chiang Mai Province, Thailand. Sci Total Environ 407, 655-668.

Park, J.H., Lee, J.E., Shin, I.C., Koh, H.C., 2013. Autophagy regulates chlorpyrifos-induced apoptosis in SH-SY5Y cells. Toxicol Appl Pharmacol 268, 55-67.

Parnell, E.A., Calleja-Macias, I.E., Kalantari, M., Grando, S.A., Bernard, H.U., 2012. Muscarinic cholinergic signaling in cervical cancer cells affects cell motility via ERK1/2 signaling. Life Sci 91, 1093-1098.

Pettersson, A., Nilsson, L., Nylund, G., Khorram-Manesh, A., Nordgren, S., Delbro, D.S., 2009. Is acetylcholine an autocrine/paracrine growth factor via the nicotinic alpha7-receptor subtype in the human colon cancer cell line HT-29? Eur J Pharmacol 609, 27-33.

Rayford, W., Noble, M.J., Austenfeld, M.A., Weigel, J., Mebust, W.K., Shah, G.V., 1997. Muscarinic cholinergic receptors promote growth of human prostate cancer cells. Prostate 30, 160-166.

Sasaki, T., Hiroki, K., Yamashita, Y., 2013. The role of epidermal growth factor receptor in cancer metastasis and microenvironment. Biomed Res Int 2013, 546318.

Schmidt-Ullrich, R.K., Mikkelsen, R.B., Dent, P., Todd, D.G., Valerie, K., Kavanagh, B.D., Contessa, J.N., Rorrer, W.K., Chen, P.B., 1997. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. Oncogene 15, 1191-1197.

Schuller, H.M., 2009. Is cancer triggered by altered signalling of nicotinic

acetylcholine receptors? Nat Rev Cancer 9, 195-205.

Shah, N., Khurana, S., Cheng, K., Raufman, J.P., 2009. Muscarinic receptors and ligands in cancer. Am J Physiol Cell Physiol 296, C221-232.

Smegal, D.C., 2002. Human health risk assessment- Chlorpyrifos. U.S. Environmental Protection Agency, Washington DC.

Smith, G.N., Watson, B.S., Fischer, F.S., 1967. Investigations on Dursban Insecticide. Metabolism of [^{36}Cl]*O*,*O*-Diethyl *O*-3,5,6-Trichloro-2-pyridyl Phosphorothioate in Rats. J Agric Food Chem 15, 132-138.

Song, P., Spindel, E.R., 2008. Basic and clinical aspects of non-neuronal acetylcholine: expression of non-neuronal acetylcholine in lung cancer provides a new target for cancer therapy. J Pharmacol Sci 106, 180-185.

Spano, J.P., Fagard, R., Soria, J.C., Rixe, O., Khayat, D., Milano, G., 2005. Epidermal growth factor receptor signaling in colorectal cancer: preclinical data and therapeutic perspectives. Ann Oncol 16, 189-194.

Sturla, L.M., Amorino, G., Alexander, M.S., Mikkelsen, R.B., Valerie, K., Schmidt-Ullrichr, R.K., 2005. Requirement of Tyr-992 and Tyr-1173 in phosphorylation of the epidermal growth factor receptor by ionizing radiation and modulation by SHP2. J Biol Chem 280, 14597-14604.

Sultatos, L.G., Shao, M., Murphy, S.D., 1984. The role of hepatic biotransformation in mediating the acute toxicity of the phosphorothionate insecticide chlorpyrifos. Toxicol Appl Pharmacol 73, 60-68.

Ventura, C., Nunez, M., Miret, N., Martinel Lamas, D., Randi, A., Venturino, A., Rivera, E., Cocca, C., 2012. Differential mechanisms of action are involved in chlorpyrifos effects in estrogen-dependent or -independent breast cancer cells exposed to low or high concentrations of the pesticide. Toxicol Lett 213, 184-193.

Ventura, C., Venturino, A., Miret, N., Randi, A., Rivera, E., Nunez, M., Cocca, C., 2015. Chlorpyrifos inhibits cell proliferation through ERK1/2 phosphorylation in breast cancer cell lines. Chemosphere 120, 343-350.

Weichenthal, S., Moase, C., Chan, P., 2010. A review of pesticide exposure and cancer incidence in the Agricultural Health Study cohort. Environ Health Perspect 118, 1117-1125.

Xie, G., Peng, Z., Raufman, J.P., 2012. Src-mediated aryl hydrocarbon and epidermal growth factor receptor cross talk stimulates colon cancer cell proliferation. Am J Physiol Gastrointest Liver Physiol 302, G1006-1015.

Yeh, J.J., Routh, E.D., Rubinas, T., Peacock, J., Martin, T.D., Shen, X.J., Sandler, R.S., Kim, H.J., Keku, T.O., Der, C.J., 2009. KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. Mol Cancer Ther 8, 834-843.

Zhao, Y., Wang, X., Wang, T., Hu, X., Hui, X., Yan, M., Gao, Q., Chen, T., Li, J., Yao, M., Wan, D., Gu, J., Fan, J., He, X., 2011. Acetylcholinesterase, a key prognostic predictor for hepatocellular carcinoma, suppresses cell growth and induces chemosensitization. Hepatology 53, 493-503.

Figure Caption

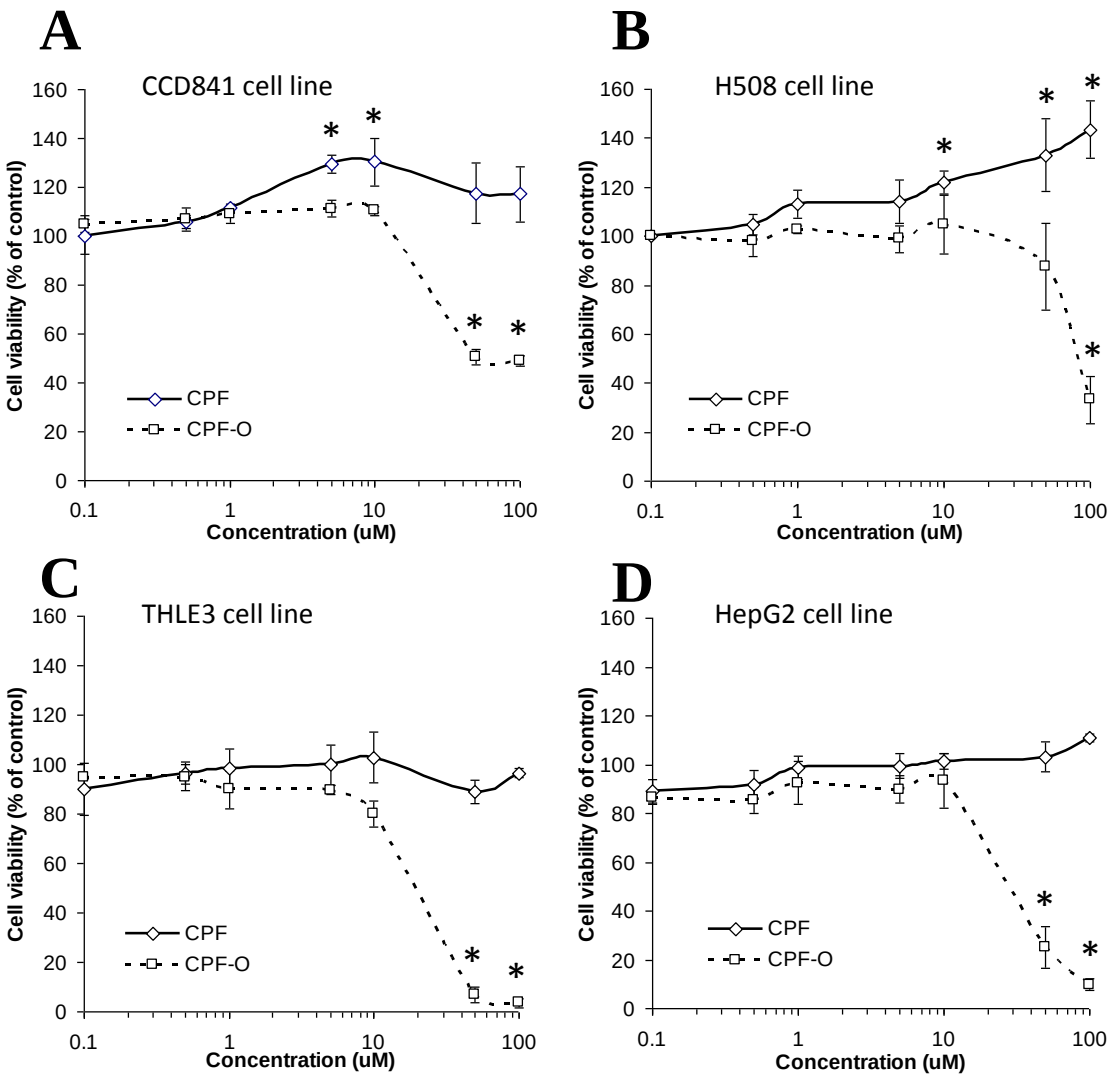

Figure 1: Effects of chlorpyrifos (CPF) and chlorpyrifos oxon (CPF-O) on the viability of (A) normal colon epithelial CCD841 cells, (B) colorectal adenocarcinoma H508 cells, (C) normal hepatocyte THLE3 cells, and (D) hepatocellular carcinoma HepG2 cells. Cells were starved in a serum free condition for 24 hr, and treated with 0.1- 100 µM of CPF or CPF-O in a serum free condition for 48 hr. Cell viability was assessed by MTT assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control at $p< 0.05$.

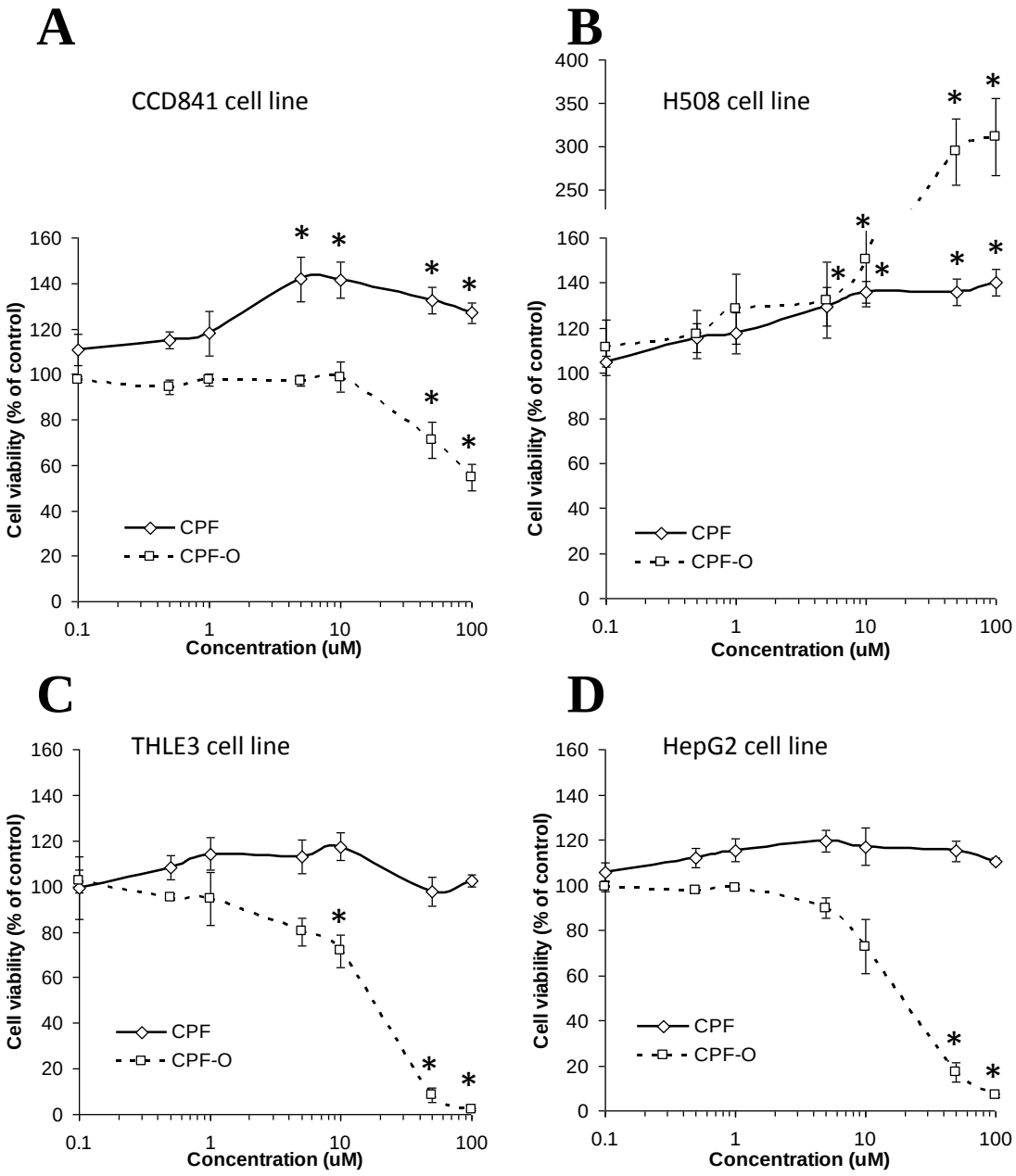

Figure 2: Effects of chlorpyrifos (CPF) and chlorpyrifos oxon (CPF-O) on the viability of (A) normal colon epithelial CCD841 cells, (B) colorectal adenocarcinoma H508 cells, (C) normal hepatocyte THLE3 cells, and (D) hepatocellular carcinoma HepG2 cells. Cells were starved in a serum free condition for 24 hr, and then treated with 0.1- 100 µM of CPF or CPF-O in a serum free condition for another 48 hr. Cell viability was assessed by PrestoBlue™ cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control at $P< 0.05$.

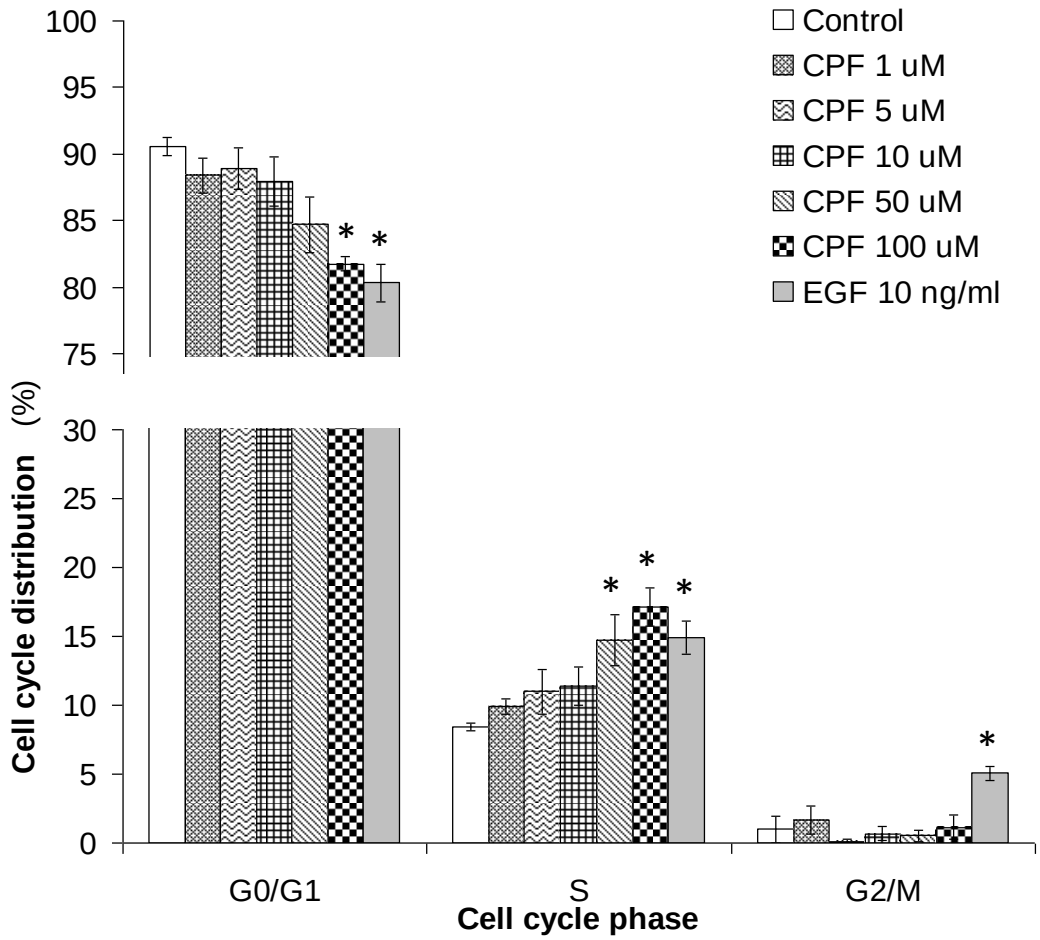

Figure 3: Effects of chlorpyrifos (CPF) on colorectal adenocarcinoma H508 cell cycle. Cells were starved in a serum free condition for 24 hr, and then treated with 1-100 µM of CPF or 10 ng/ml of epidermal growth factor (EGF) (positive control) in a serum free condition for 48 hr. Cell cycle phase distribution was measured by flow cytometry with propidium iodide staining. The data are the percentage mean of each cell cycle phase±standard error of three independent experiments. * represents statistically significant difference from the control at $P< 0.05$.

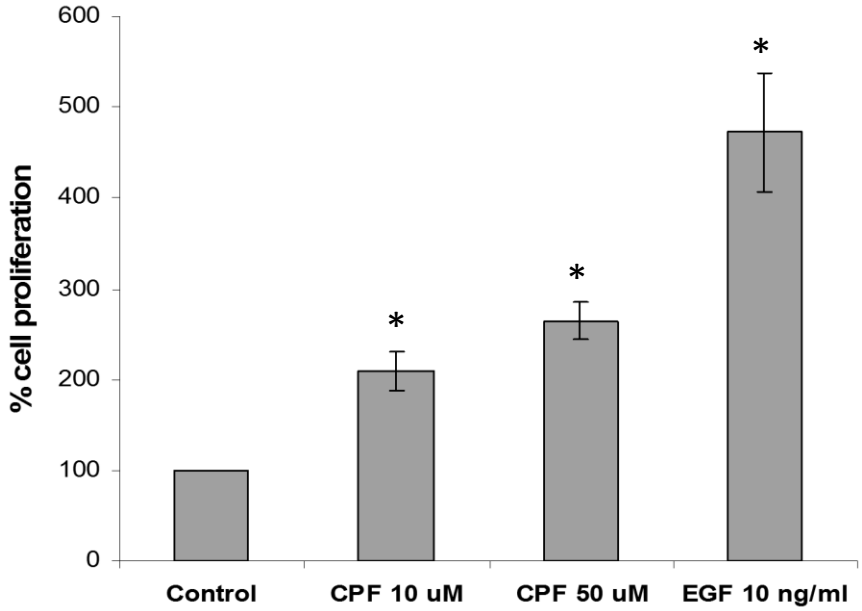

Figure 4: Effects of chlorpyrifos (CPF) on the proliferation of colorectal adenocarcinoma H508 cells. Cells were starved in serum free condition for 24 hr, and then treated with 10 or 50 µM of CPF or 10 ng/ml of epidermal growth factor (EGF) (positive control) in serum free condition for 48 hr. Cell proliferation was measured by Click-iT® EdU cell proliferation assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control at $P< 0.05$.

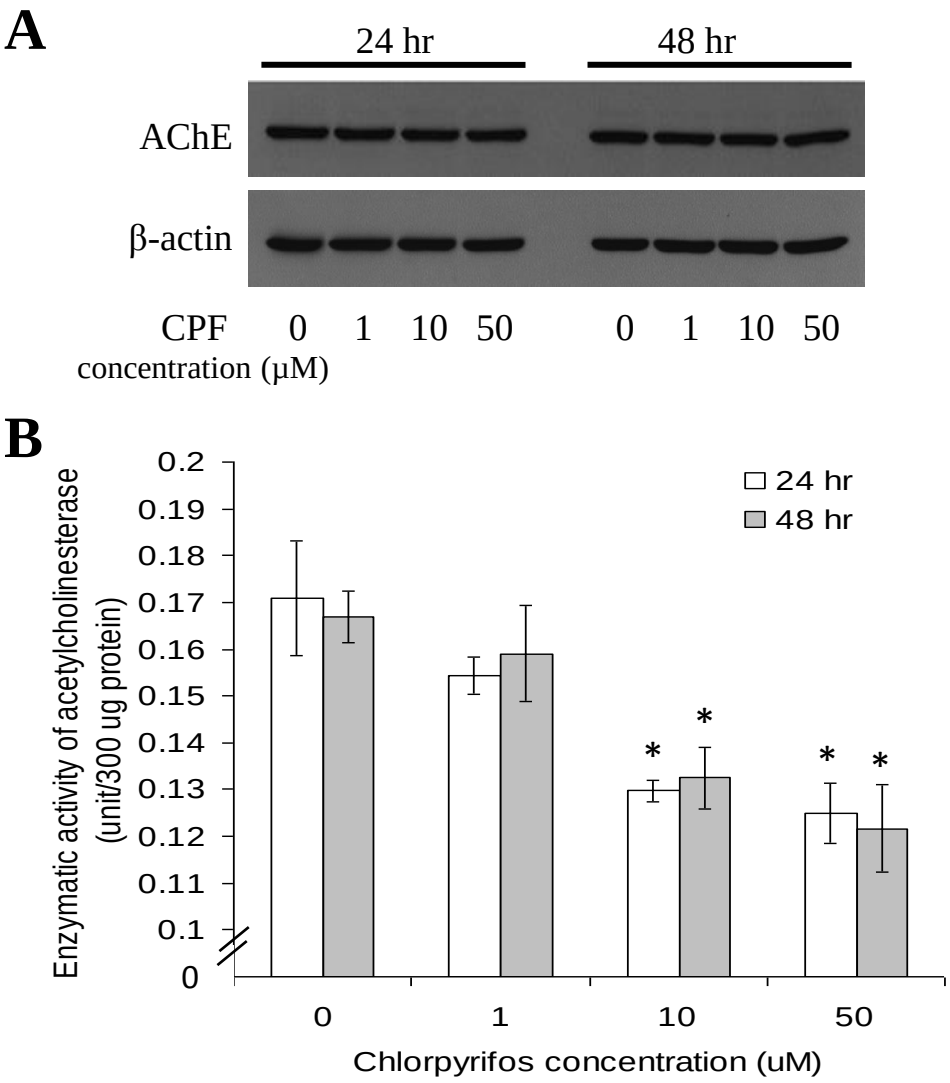

Figure 5: Effects of chlorpyrifos (CPF) on the expression and enzymatic activity of acetylcholinesterse (AChE). Cells were starved in a serum free condition for 24 hr, and then treated with 1, 10 or 50 μM of CPF in a serum free condition for another 24-48 hr. Protein expression of AChE was measured by Western immunobloting assay and AChE enzymatic activity was determined by the modified Ellman method. (A) Representative immunoblots band of AChE. The β-actin was used to ensure equal amount of loaded protein. (B) Enzymatic activity of AChE. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control at $P< 0.05$.

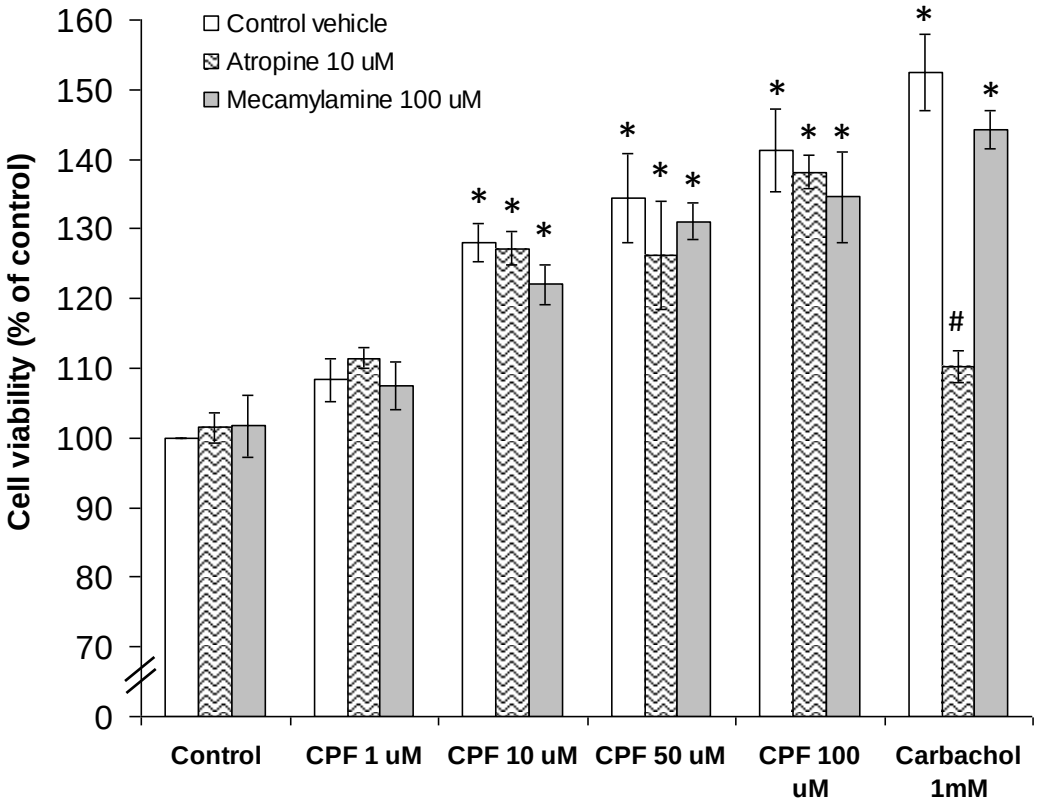

Figure 6: Effects of cholinoceptor antagonists on the growth promoting effect of chlorpyrifos (CPF). Colorectal adenocarcinoma H508 cells were starved in a serum free condition for 24 hr. Starved cells were pre-treated for 30 min with 10 µM of atropine or 100 µM of mecamylamine and then further co-treated with 1-50 µM of CPF or 1 mM of carbachol (positive control) in serum free medium for 48 hr. Cell growth was assessed by PrestoBlue™ cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to control. * represents statistically significant difference from the control and # represents statistically significant difference from the control vehicle at $P< 0.05$.

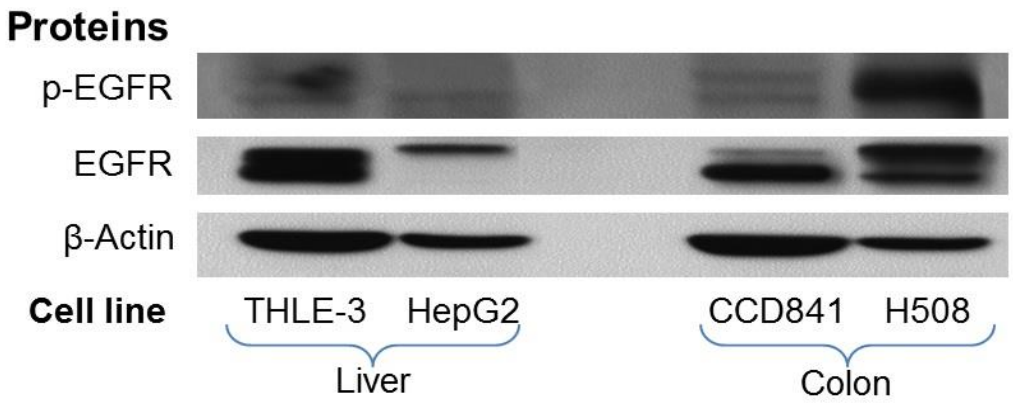

Figure 7: Background expression levels of the total and active/phosphorylated forms of epidermal growth factor receptor (EGFR) in liver (THLE-3 and HepG2) and colon (CCD841 and H508) cell lines. Expression of active/phosphorylated EGFR at tyrosine 1173 (p-EGFR), and total EGFR were measured by Western immunoblotting assay.

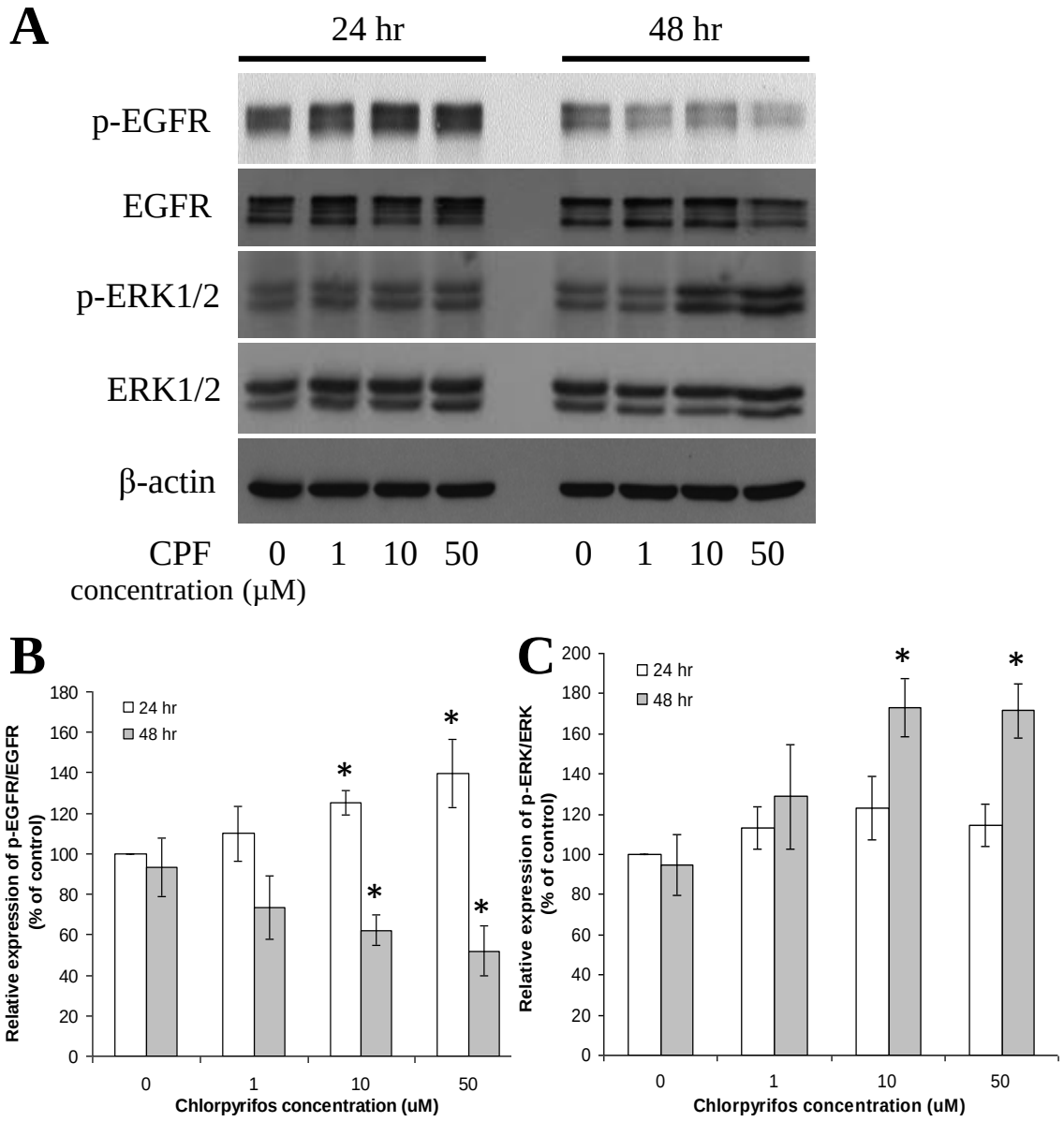

Figure 8: Effects of chlorpyrifos (CPF) on the activation of epidermal growth factor receptor (EGFR) and extracellular signal regulated kinase (ERK1/2). Colorectal adenocarcinoma H508 cells were starved in serum free condition for 24 hr and then further incubated with 1-50 μM of CPF in serum free medium for 24-48 hr. Expression of phosphorylated EGFR (p-EGFR), total EGFR, phosphorylated ERK1/2 (p-ERK1/2), and total ERK1/2 were measured by Western immunoblotting assay. (A) Representative immunoblots band of p-EGFR, total EGFR, p-ERK1/2, and total ERK1/2. The β-actin was used to ensure equal amount of loaded protein. (B) Ratio of relative expression of p-EGFR to total EGFR proteins. (C) Ratio of relative expression of p-ERK1/2 to total ERK1/2 proteins. Each data point represents mean±standard error of three independent experiments and expressed as a relative to the control of 24 hr exposure. * represents statistically significant difference from the control of 24 hr exposure at $P< 0.05$.

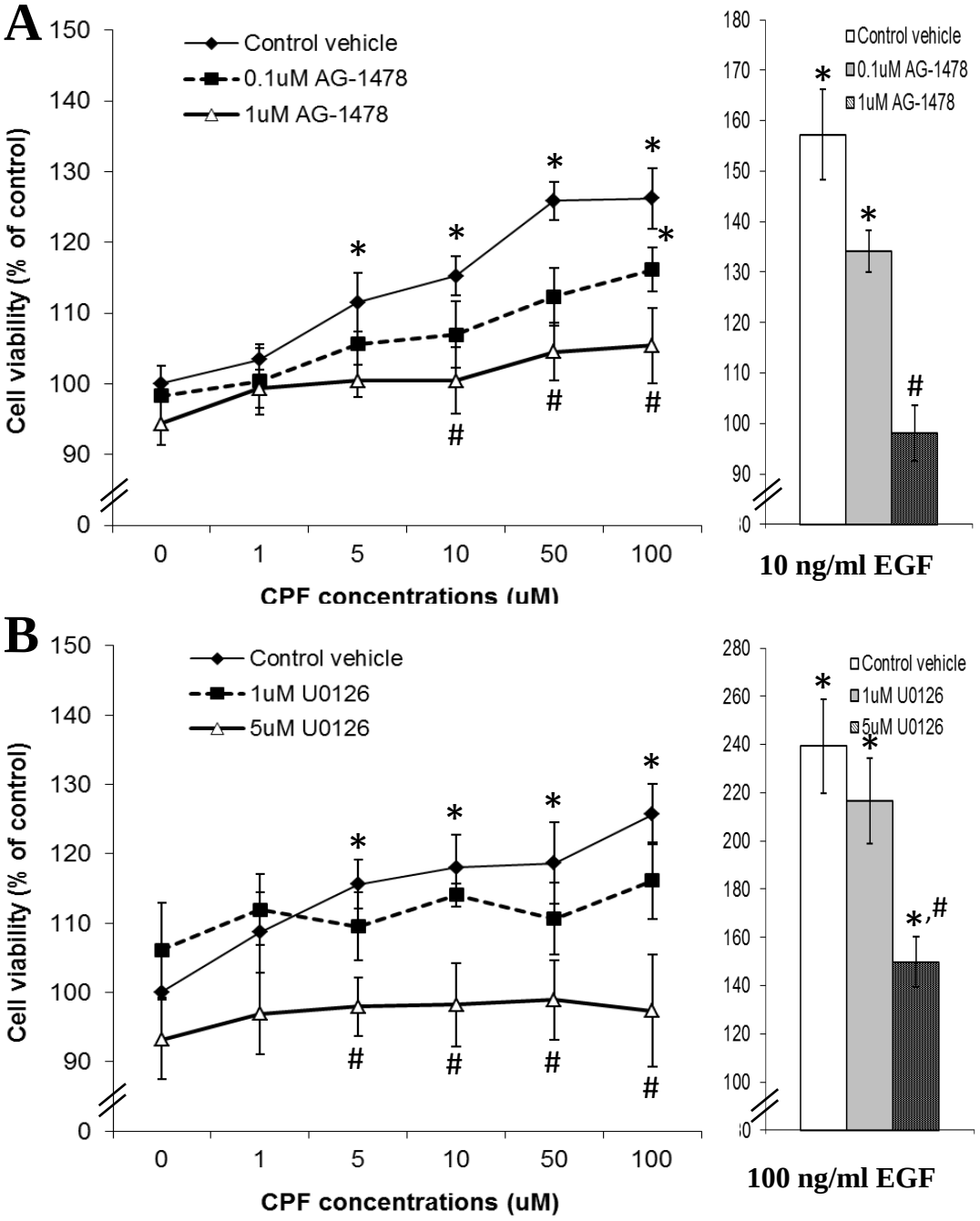

Figure 9: Effects of epidermal growth factor receptor (EGFR) and MEK inhibitors on the growth promoting effect of chlorpyrifos (CPF). Colorectal adenocarcinoma H508 cells were starved in a serum free condition for 24 hr. Starved cells were pre-treated for 30 min with (A) 0.1- 1 µM of AG-1478 (a specific EGFR tyrosine kinase inhibitor) or (B) 1- 5 µM of U0126 (a specific MEK inhibitor) and then further co-incubated with 1-100 µM of CPF or 10-100 ng/ml of epidermal growth factor (EGF) (positive control) in a serum free medium for 48 hr. Cell growth was assessed by PrestoBlue™ cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to the control. * represents statistically significant difference from the control group and # represents statistically significant difference from the control vehicle at the same concentration of CPF at $P< 0.05$.

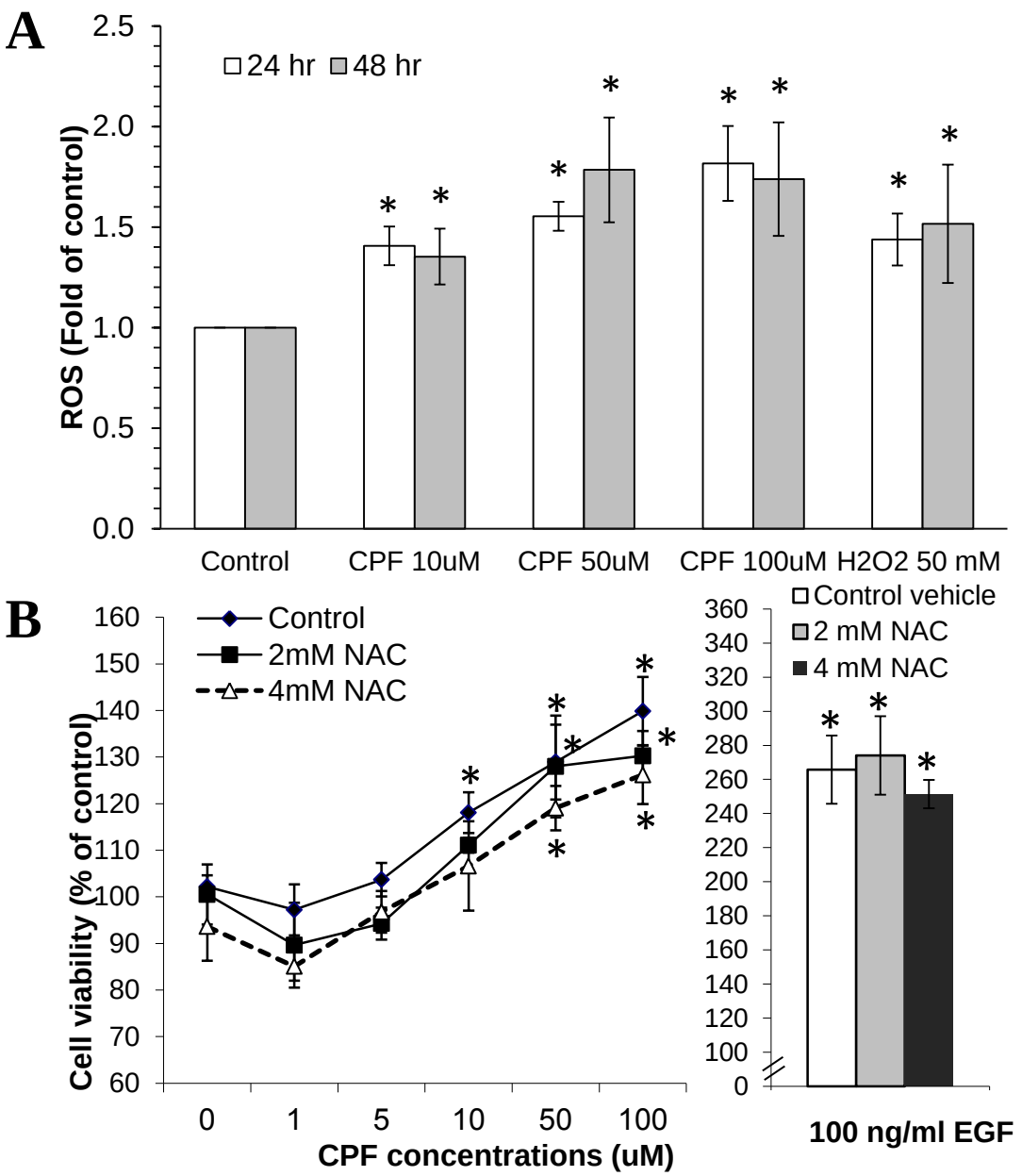

Figure 10: (A) Effect of chlorpyrifos (CPF) on the production of reactive oxygen species (ROS). Colorectal adenocarcinoma H508 cells were starved in serum free condition for 24 hr. Starved cells were treated with 10-100 µM of CPF in serum free medium for 24-48 hr or 50 mM of hydrogen peroxide (H_2O_2) for 15 min (positive control). ROS were stained with H_2DCF-DA dye and measured by flow cytometer. Each data point represents the mean±standard error of three independent experiments and expressed as a fold of the control. (B) Effects of antioxidant on the growth promoting effect of CPF. Serum starved cells were pre-treated for 30 min with 2- 4 mM of NAC and then further co-incubated with 1-100 µM of CPF or 100 ng/ml of epidermal growth factor (EGF) (positive control) in serum free medium for 48 hr. Cell growth was assessed by MTT cell viability assay. Each data point represents the mean±standard error of three independent experiments and expressed as a relative to the control. * represents statistically significant difference from the control group at $P< 0.05$.