The Cyclin-dependent kinase 1 Inhibitor CGP74514A inhibits cell proliferation, induces apoptosis and causes downregulation of Cyclin B1and accumulation of p53 in HepG2 cells

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Abstract: Cancer cells are characterized by dysregulation of proliferation, cell cycle and apoptosis. Cell cycle and apoptosis are linked through proteins that play a role in the regulation of both pathways such as cyclin-dependent kinase 1 (CDK1). Therefore, inhibition of CDK1 could be an attractive target in cancer therapy. Several pharmacologic inhibitors of CDKs have been developed and some are currently in clinical trials. The goal of the present work was to study the effect of the CDK1 inhibitor CGP74514A on proliferation, cell cycle and apoptosis using the HepG2 liver cancer cell line and to understand the molecular mechanisms underlying these effects. 5 µM CGP74514A markedly inhibited proliferation of HepG2 cells, induced an accumulation of cells in the G2/M phase of the cell cycle, inhibited colony formation and induced apoptosis. Studying the molecular changes in the protein expression of cell cycle and apoptosis regulatory proteins revealed that treatment of HepG2 cells with CGP74514A resulted in marked down regulation of cyclin B protein and the T161 phosphorylation site of CDK1 (both are positive regulators of CDK1 activity) but no changes in CDK1 protein levels were observed. Furthermore, CGP74514A down regulated Chk1, however, it stabilized the protein levels of p53 and reduced the levels of the antiapoptotic protein Bcl2. Taken together, the molecular mechanisms underlying the anitproliferative and apoptotic effects of CGP74514A involved down regulation of cyclin B, abrogated phosphorylation at the T161 of Cdk1, reduced levels of Chk1, p53 stabilization and down regulation of Bcl2. Therefore, CGP74514A may have therapeutic implications in liver cancer.

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1. Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths globally (**Shariff *et al.*, 2009**). It contributes to 14.8% of all cancer deaths in Egypt, with higher incidence in males (17.3%) than in females (11.5%) and it is the second most frequent cancer type in Egyptian males after bladder and the eighth in Egyptian females **(Anwar *et al.*, 2008**). For patients with advanced/metastatic disease or those refractory to transcatheter arterial chemoembolisation (TACE), no conventional cytotoxic or hormonal systemic therapy was able to improve patient survival during the last decades **(Lopez *et al.*, 2006; Zhu, 2006; Worns *et al.*, 2009**) leading to a 6 months prognosis in this stage of the disease (**Llovet *et al.*, 2008**). Therefore, new therapeutic agents are critically needed.

Cancer cells are characterized by dysregulation in proliferation, cell cycle and apoptosis. Pharmacologic compounds that arrest the cell cycle have been widely used for research purposes and recently also for therapeutic purposes. The cell cycle is regulated by cyclin-dependent kinases (Cdks). Cdks are activated by binding to cyclin regulatory subunits. These are expressed in different combinations during specific phases of the cell cycle (**Aleem *et al.*, 2004**).

There are several Cdk/cyclin complexes, the best characterized are Cdk1, Cdk2, Cdk4 and Cdk6. Targeting Cdks in mice demonstrated that Cdk1 is the only essential Cdk for the mammalian mitotic cell division **(Santamaria *et al.*, 2007**). Cdk1 binds classically to cyclin A and B to regulate G2 and M phase progression but it has been also shown in genetically targeted mice that Cdk1 has the ability to bind to cyclin E **(Aleem *et al.*, 2005**) and D **(Santamaria *et al.*, 2007**) to drive all phases of the cell cycle in the absence of other Cdks. CDKs have been an attractive target for pharmacologic drug development (**Senderowicz and Sausville, 2000)**. All CDK inhibitors developed to date can be subdivided into two main groups: broad-range inhibitors (flavopiridol, olomoucine, roscovitine) and specific inhibitors such as purvalanol A, BML-259, PD0332991 **(Cicenas and Valius, 2011**). Flavopiridol is currently in clinical trials **(Holkova *et al.*, 2011**). Other pharmacological CDK inhibitors in preclinical development include CGP74514A **(Senderowicz and Sausville, 2000; Dai *et al.*, 2002**).

CGP74514A is a purine derivative, structurally

related to olomoucine, which has been reported to function as a potent and selective inhibitor of CDK1 (**Imbach *et al.*, 1999**). Previous studies demonstrated that treatment with CGP74514A induced apoptosis caused by extensive mitochondrial damage and caspase activation in human leukemia cells **(Dai *et al.*, 2002**) and in prostate cancer cells (**Mohapatra *et al.*, 2005**). In a previous study (**Abdel Aleem, 2005**) showed that CGP74514A inhibits cell proliferation and induces apoptosis in A549 lung carcinoma, RKO colon cancer and in Saos-2 osteosarcoma cell lines. Since the effect of CGP74514A has not been studied before on human liver cancer cell lines the purpose of the present work was to investigate the anticancer effects of CGP74514A in HepG2 cells and the molecular mechanisms by which these effects are mediated.

2. Material and Methods

Cell culture

The human hepatocellular carcinoma cell line HepG2 (ATCC, USA) was grown in RPMI 1640 (Gibco, UK) supplemented with penicillin (100 units/ml), streptomycin (50 µg/ml) and 10% Fetal Bovine Serum (FBS) (Gibco, UK). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and treated with 5 µM CGP74514A (Merck, Germany). The stock solution of CGP74514A (20 mM) was prepared in dimethyl sulfoxide (DMSO) and stored in -20ºC. In all experiments, the final DMSO concentration was <0.1%.

Viability assay

Cell viability was measured using alamar blue® viability assay (**Ahmed *et al.*, 1994**) according to manufacturer's protocol. Briefly, cells were seeded at a density of 5000 cells per one well of 96-well plates in triplicates and allowed to grow for 24 hrs. 5 µM CGP74514A were then added and cells incubated for the required time period. Four hours before analysis 10% of alamar blue®solution diluted in culture medium was added and cells were incubated at 37ºC. Absorbance was measured using a microplate reader.

Cell Cycle and flow cytometry

Cells were seeded in 10 cm dishes and treated with 5 µM CGP74514A for different time points. For the determination of the percentage of cells in S phase cells were pulse-labelled with 100 µM bromo deoxyuridine (BrdU; Sigma-aldrich, Germany) for 1 h in the dark then harvested. Cells were washed with PBS and fixed with 70% ethanol overnight. Cells were then treated with RNase (0.1 mg/ml) for 10 min at 37 ^{o}C, permeabilized with 0.05% pepsin and 2N HCl followed by staining with mouse anti-BrdU antibody (Becton Dickinson, San Jose, CA) (1:100) for 1h at room temperature (RT). After washing, cells were stained with FITC-conjugated secondary antibody (DAKO) for 45 min at RT, followed by propidium iodide (PI) staining (25 µg/ml) for 1h at RT. The samples were analyzed with a FACS Calibur (Becton Dickinson), and the data were processed with the Cell Quest software (Becton Dickinson).

Annexin V/PI assay

Cells were treated with 5 µM CGP74514A for 48 hrs. Early apoptosis was studied using Annexin V/PI method (Annexin V-FLUOS staining kit, Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly $1x10^{6}$ cells were washed with ice-cold PBS without Ca^{+2} or Mg^{+2} (Gibco, UK). The cells were then resuspended in 100 µl of labelling solution and incubated with 0.1µg/ml PI and 2 µl Annexin V-fluorescein for 15 min in the dark at RT. Flow cytometric analysis was immediately performed using FACS Calibur (Becton Dickinson).

Focus formation assay

Cells were seeded in 10 cm dishes at low densities (10,000 cells per plate) and left to grow for 24 hrs then treated with 5 µM of CGP74514A and left to grow for 2-3 weeks. The plates were then washed with PBS, fixed with cold methanol for 10 min then stained with 0.5% of crystal violet dye (Sigma, St Louis, MO) for 10 min at RT. The dishes were then rinsed with tap water.

Preparation of lysates

Cells were lysed in modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate). Proteases inhibitor cocktail (Roche, Germany) and phosphatase inhibitors (Sigma-aldrich, Germany) were freshly added. Lysates were centrifuged for 30 min at 16,100 x *g* at 4ºC, and supernatants were frozen at -80ºC until use. Protein concentrations were determined using the BCA protein assay (Pierce Biotechnology, Rockford, USA) according to manufacturer's protocol and measured in a microplate reader.

Antibodies and Western blot

Lysates (15 µg total protein) were resolved on 12 % Bis-Tris SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes (Hybond, Amersham, UK), blocked with either 5% milk or bovine serum albumin (BSA) in a Tris-Buffered Saline and Tween 20 buffer (TBST) [19.97 mM Tris base, 135 mM NaCl, 0.1% Tween 20] and blotted with the primary antibodies below. Membranes were

routinely washed using TBST and incubated with either anti-rabbit-, anti-mouse-, or anti-goat IgG horseshoe peroxidase (HRP)-conjugated secondary antibody (Amersham, UK) and bands detected using chemiluminescence (ECL, Amersham, UK). The following primary antibodies from (Cell Signaling Technology, Beverly, MA, USA) were used for Western blot: mouse anti-Bcl-2, rabbit anti-phospho-T161 Cdk1, rabbit anti-phospho-S345 Chk1, mouse anti-Chk1, and mouse anti-cyclin B. Other antibodies used in the present study were rabbit anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-p53 (Becton Dickenson) and rabbit anti-Cdk1 (Calbiochem).

Statistical analysis

Data were analyzed for mean and standard deviation and significance was determined using student's t test. A p value ≤ 0.05 was considered significant.

3. Results

Effect of CGP74514A on viability of HepG2 cells

In the present study, HepG2 cells were treated with 5 µM of CGP74514A and their viability was measured daily over a period of five days (Fig.1). CGP74514A significantly reduced HepG2 cells' viability (p = 0.03). After 72 hrs of treatment the surviving fraction of HepG2 cells was only 8.2% of the untreated cells.

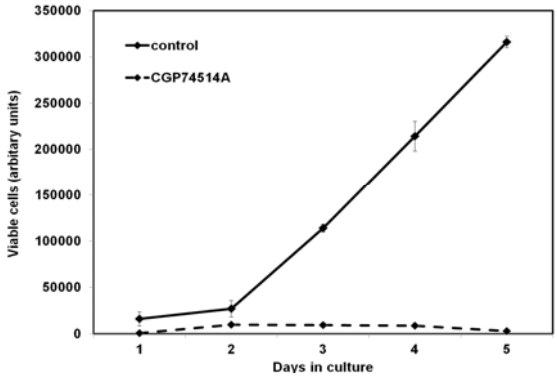

Figure 1. Effect of CGP74514A on the viability of HepG2 cells

CGP74514A induces apoptosis

Treatment of HepG2 cells for 48 hrs with 5 µM CGP74514A resulted in an average of 8-fold increase in apoptotic cells in comparison to cells treated with vehicle (DMSO) as detected by Annexin V/PI method which detects both early apoptosis (in which cells stain positively for annexin) and late apoptosis (in which cells stain positively for both annexin and PI) (Fig. 2A, B). This increase was statistically significant and the values in Fig. 2C represent the total of early and late apoptotic cells expressed as fold change relative to control.

CGP74514A inhibits colony formation

In the present study treatment of HepG2 cells seeded at low densities with 5 µM CGP74514A inhibited the formation of colonies (Fig. 3).

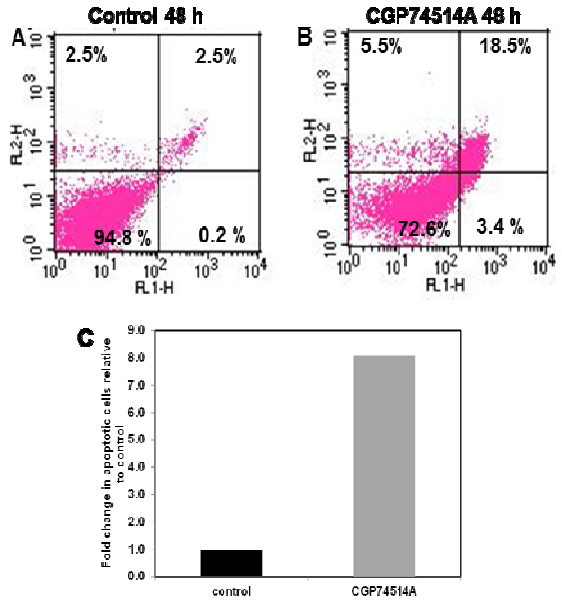

Figure 2. CGP74514A induces apoptosis in HepG2 cells. (A) control cells receiving vehicle (DMSO), (B) cells treated with 5 µM CGP74514A. The four quadrants in (A) or (B) represent viable cells (lower left), early apoptotic (lower right, annexin+ve), late apoptotic (upper right, annexin+ve/ PI +v) and necrotic cells (upper left). The values plotted in the histogram (C) represent the total percentage of apoptotic cells (early and late) as fold change relative to control cells.

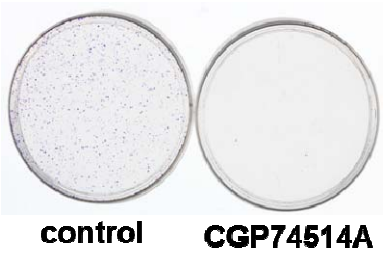

Figure 3. **CGP74514A inhibits colony formation in HepG2 cells.**

CGP74514A induces G2/M cell cycle phase arrest

In the present study treatment of cells with 5 µM CGP74514A induced a modest accumulation of

cells in G2/M phase of the cell cycle. I harvested the cells after 8 and 24 hrs of treatment with CGP74514A and analysed their cell cycle profiles using flow cytometry (Fig. 4). The selection of these two time points was based on my previous findings that CDK1 kinase activity inhibition is detected as early as 8 hours in HepG2 and in other cancer cell lines (unpublished). After 8 hrs of treatment the percentages of HepG2 cells in G1 and in S phases were similar in both treated and control cells, however, CGP74514A induced a slight increase in the percentage of cells in G2/M phase of the cell cycle (1.54 fold increase relative to control) (Fig. 4A, B, C). The accumulation of cells in G2/M persisted also at the 24 hrs time point, at which the percentage of cells in G2/M was higher than that in control cells by 1.67 (Fig. 4 D, E, F).

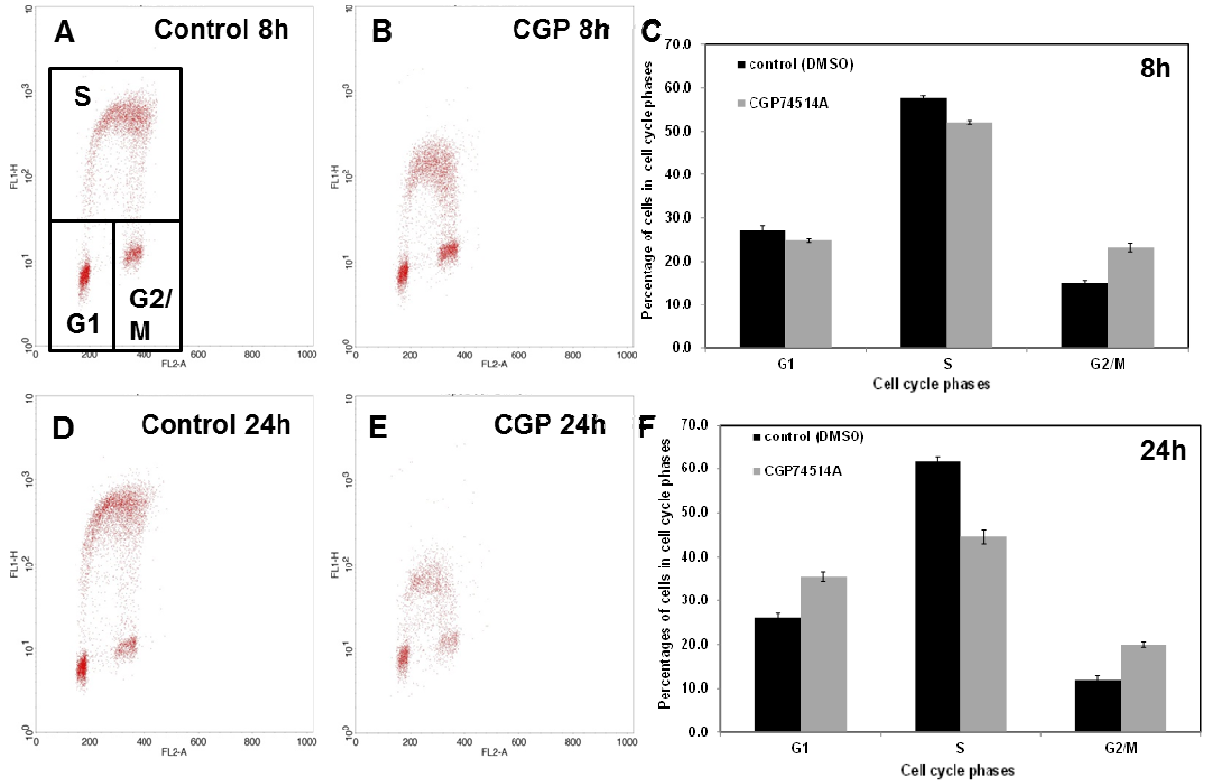

Figure 4. **Effect of CGP74514A on cell cycle kinetics.** (A) the cell cycle profile of control cells (treated with DMSO) after 8hrs. Cells in G1, S and G2/M are shown in labeled quadrants. (B) the cell cycle profile of cells treated with 5 µM CGP74514A after 8h. (C) Histogram showing the percentages of cells in the different cell cycle phases (y axis) after 8 h. (D) the cell cycle profile of control cells (treated with DMSO) after 24hrs. (E) the cell cycle profile of cells treated with 5 µM CGP74514A after 24hrs. (F) Histogram showing the percentages of cells in the different cell cycle phases (y axis) after 24 hrs.

CGP74514A causes downregulation of cyclin B, T161 phospho-Cdk1 and an accumulation of p53

In order to understand the molecular mechanism through which CGP74514A induces the accumulation of cells in G2/M phase of the cell cycle, and apoptosis, cells were treated with 5 µM CGP74514A for 24 hrs and the protein expression of cell cycle and apoptosis regulatory proteins was studied by Western blot (Fig. 5). CGP74514A induced dramatic and significant downregulation of cyclin B (Fig. 5A, top panel), an essential activator of Cdk1 kinase activity, and of the T161 phospho site of Cdk1 (Fig. 5A, second panel from top). This site is essential for activation of CDK1 kinase activity by the CDK activating kinase (CAK). However, there was a negligible effect of CGP74514A on CDK1 total protein levels (Fig. 5A, panel 3 from top). Furthermore, CGP74514A caused downregulation of Chk1 and its S345 phospho site (Fig. 5A, panels 4 and 5 from top). On the other hand CGP74514A was found in the present study to stabilize p53 (a negative regulator of CDK1 activity) (Fig. 5B, top panel) and to decrease the levels of Bcl2, one of the major antiapoptotic proteins (Fig. 5B, second panel from top).

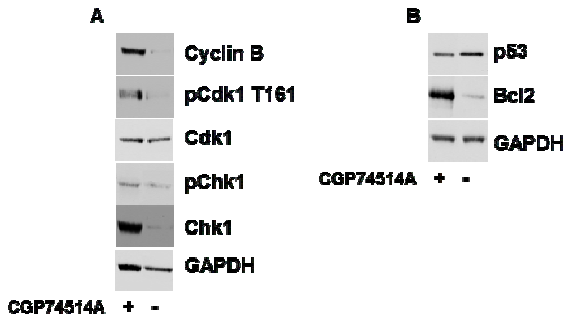

Figure 5. Effect of CGP74514A on cell cycle and apoptosis regulatory proteins. Western blot of lysates from HepG2 cells after treatment with 5 µM CGP74514A for 24 hrs (15 µg protein/lane). The membranes were probed with antibodies directed against Cyclin B, the T161phospho site of Cdk1, Cdk1, the S345 phospho site of Chk1, Chk1 (A) and against p53 and Bcl2 (B). Membranes were subsequently stripped and reprobed with antibodies to GAPDH.

4. Discussion

In the present study the potential molecular mechanisms through which CGP74514A; a potent CDK1 inhibitor inhibits cell proliferation, induces accumulation of cells in G2/M phase of the cell cycle and induces apoptosis in HepG2 cells were studied. It was found that 5 µM CGP74514A inhibited cell proliferation, induced accumulation of cells in G2/M arrest and apoptosis. This is the first study to show the antiproliferative and apoptotic effects of CGP74514A on a liver cancer cell line. Previous studies have demonstrated similar results in prostate cancer cell lines (**Mohapatra *et al.*, 2005**), as well as in human leukaemia cells **(Dai *et al.*, 2002**). There is accumulating evidence that both apoptosis and dysregulation of cell cycle progression are linked (**Evan and Vousden, 2001)**. For example CDK1 while it can solely drive all phases of the cell cycle it also regulates the inhibitor of apoptosis protein BIRC5 (survivin). Inhibition of CDK1 rapidly downregulates survivin expression and induces MYC-dependent apoptosis **(Goga *et al.*, 2007**). Furthermoe, we have previously shown that Cdk2 is required for Myc-induced-, and for cisplatin-induced apoptosis **(Deb-Basu *et al.*, 2006; Price *et al.*, 2006**).

In the current study the percentage of cells accumulated in the G2/M phase of the cell cycle was a relatively small percentage relative to control. In contrast a higher percentage of CGP74514A-treated HepG2 cells were either in early or late apoptosis in comparison to the untreated cells. This result is in agreement with the study by **Dai *et al.* (2002)** in which they reported that while a low dose of CGP74514A (1 µM) induced G2/M arrest, a higher dose (5 µM) induced apoptosis in leukaemia cells. In the present study CGP74514A completely inhibited cell proliferation and this may explain the total inhibition of colony formation as well.

CDK1activity is regulated by several mechanisms. Binding of CDK1 to cyclin B is essential for activation of the kinase. CDK1 bound to cyclin B is phosphorylated on residue T161 by CDK activating kinase (CAK) to stabilize the cyclin B-CDK1 interaction and to induce the conformational rearrangements needed for kinase activity **(Russo *et al.*, 1996; Larochelle *et al.*, 2007**). In the present study it was forund that CGP74514A reduced the levels of both Cyclin B and the T161 phosphorylation significantly. In the absence of Cyclin B and T161 phosphorylation CDK1 remains inactive. In agreement with my results **(Yuan *et al.*, 2004**) demonstrated that treatment of an array of cancer cell lines with small interfering RNA (siRNA) targeting Cyclin B1 resulted in cell cycle arrest in the G2/M phase in all tumor cell lines tested. Proliferation of tumor cells from different origins was suppressed by 50-80% 48 hrs after transfection with Cyclin B1 siRNA and apoptosis was increased from 5 to 40-50%. Furthermore, tumor cells showed less colony-forming ability after Cyclin B1 siRNA treatment **(Yuan *et al.*, 2004**) .

Checkpoint kinase 1 (Chk1) is one of the major players activated in response to DNA damage and it activates different cell cycle checkpoints including the G_1/S, the intra-S, G_2-M and the mitotic spindle checkpoint, contributing to the maintenance of genomic stability (**Carrassa and Damia, 2011**). Activation of Chk1 after DNA damage is known to require the phosphorylation of several C-terminal residues, including the highly conserved S345 site by ATR (**Zhao and Piwnica-Worms, 2001**). In the present study 5 µM CGP74514A markedly downregulated the protein levels of Chk1, however, it was still phosphorylated on (S345), indicating that Chk1 is still active. In agreement, transient depletion of Chk1 by RNAi in synchronized cells results in a block at the subsequent metaphase (**Tang *et al.*, 2006**). It has been proposed that Chk1's major mechanism is through phosphorylation of Cdc25C on S216, thereby marking Cdc25C for nuclear export and binding to 14-3-3 proteins in the cytoplasm. Sequestered outside the nucleus, Cdc25C cannot dephosphorylate and activate intranuclear CDK1**(Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997; Zeng *et al.*, 1998; Dalal *et al.*, 1999; Lopez-Girona *et al.*, 1999; Sanchez *et al.*, 1999**). Therefore, Chk1's phosphorylation of Cdc25C promotes cell cycle arrest in G2 phase. However, more studies are required to further investigate the

role of Chk1 in the accumulation of cells in G2 observed in the present study.

P53 is a central protein in the regulation of G2 arrest as well as apoptosis. In the present work CGP74514A induced stabilization of p53 in HepG2 cells which express wild-type p53. These results are supported by previous findings by (**Mohapatra *et al.*, 2005**) reporting that CGP74514A induced apoptosis in prostate cancer cells by a dual mechanism involving p53 accumulation and XIAP depletion. Furthermore, in the present study CGP74514A treatment resulted in marked downregulation of the antiapoptotic protein Bcl2. This is in agreement with previous results by **Wada *et al.*(1998)** demonstrating a predominant downregulation of Bcl2 and induction of apoptosis induced by the CDK inhibitor butyrolactone-I. Furthermore, it has been reported that Bcl2 is downregulated by p53 in breast cancer cells (**Haldar *et al.*, 1994**)

In conclusion, the present study demonstrated that the CDK1 inhibitor CGP74514A blocked cell proliferation, induced accumulation of HepG2 cells in G2/M phase of the cell cycle and induced apoptosis. The molecular mechanisms underlying these processes involved downregulation of cyclin B, abrogated phosphorylation at the T161 of Cdk1, reduced levels of Chk1, p53 stabilization and downregulation of Bcl2. Therefore, CGP74514A may have therapeutic implications in liver cancer.

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