

Anticancer pyridines induce G2/M arrest and apoptosis via p53 and JNK upregulation in liver and breast cancer cells

VASILIS P. ANDROUTSOPOULOS and DEMETRIOS A. SPANDIDOS

Laboratory of Clinical Virology, Medical School, University of Crete, Voutes, Heraklion 71003, Crete, Greece

Received October 27, 2017; Accepted November 28, 2017

DOI: 10.3892/or.2017.6116

Abstract. In the present study, the synthesis and biological evaluation of one novel pyridine and one novel pyridone anticancer compound is reported. The compounds 6-(2,4-dimethoxyphenyl)-4-(3,4-methylenedioxyphenyl)-1H-pyridin-2-one (1) and 2-(2,4-dimethoxyphenyl)-4-(3,4-methylenedioxyphenyl)pyridine (2) were synthesized from a chalcone precursor. 1 was more active than 2 in inhibiting the proliferation of MCF-7 and HepG2 cells, whereas HepG2 cells were more sensitive to the antiproliferative activity of these compounds compared with MCF-7 cells. The lowest IC₅₀ value was noted for compound 1 in HepG2 cells (IC₅₀=4.5±0.3 μM). The mechanism of action involved induction of G2/M arrest and apoptosis. Both 1 and 2 further induced downregulation of the cell cycle-associated protein cyclin D1 and upregulation of the cell cycle inhibitors p53 and p21 and the apoptosis-associated protein JNK in HepG2 cells. Compound 1 was further shown to induce phosphorylation of JNK in HepG2 cells. These results demonstrate promising cytostatic effects for the two novel anticancer compounds in human cancer cells.

Introduction

Liver cancer is the third most common cancer in the world and breast cancer the most frequently encountered gynecological malignancy. The one-year survival rate for liver cancer is particularly low, whereas breast cancer exhibits a favorable survival rate when diagnosed early (1,2). Current chemotherapeutic options for liver cancer include the alkylating agent cisplatin and the topoisomerase inhibitor doxorubicin, while the antimetabolite 5-fluorouracil and the taxane docetaxel are the chemotherapeutic drugs used frequently to treat breast cancer patients. Although the use of these chemotherapeutic regimens is well established, the major drawback encountered is their limited specificity for

the tumor site. Hence, in the past two decades considerable effort has been made to develop strategies that will target malignant cells more specifically. Notably compounds that inhibit the cell cycle of cancer cells and act in a cytostatic fashion, such as the EGFR inhibitor gefitinib have been investigated (3).

Natural products have played an essential role in the quest for developing chemotherapeutic agents with enhanced specificity and potency. Chalcones, which constitute an important class of natural products that belongs to the flavonoid family, display a wide range of biological activities, such as cytotoxic, anticancer, anti-inflammatory and anti-microbial activities (4). Specifically, chalcones that contain methoxy group substitutions have shown cytotoxic activities against the cancer cell lines ACHN, H460, HCT116, Panc1 and Calu1 (5). 2-Hydroxy-2,3,4,6-tetramethoxychalcone was found to demonstrate *in vitro* antiproliferative activity via upregulation of p53 and p21 and downregulation of p-Cdc2 and p-Rb in A549 cells and *in vivo* antitumor activity in A549 xenografts (6). However, various chalcones are known to be phototransformed from trans into cis isomers, in solution such as 3-hydroxy-3'-methylchalcone, and it is believed that the phototransformed cis analogues show more antitumorigenic activity than the original transform (7,8). This presents difficulties in evaluating the efficacy of these compounds as more than one isomers are present in solution.

To overcome the non-specific isomerization of chalcones, we synthesized one pyridone, namely 6-(2,4-dimethoxyphenyl)-4-(3,4-methylenedioxyphenyl)-1H-pyridin-2-one (compound 1) and one pyridine, 2-(2,4-dimethoxyphenyl)-4-(3,4-methylenedioxyphenyl)pyridine (compound 2) starting from a chalcone precursor (Fig. 1B). We further evaluated the anticancer properties of compounds 1 and 2 in MCF-7 human breast adenocarcinoma and HepG2 liver hepatoblastoma cells.

Materials and methods

Reagents. The reagents for chemical synthesis were from Merck KGa (Darmstadt, Germany) and Thermo Fisher Scientific, Inc., (Waltham, MA, USA). Media and cell culture reagents were from Merck KGa. Western blotting reagents were obtained from Bio-Rad Laboratories Ltd., (Watford, Hertfordshire, UK). Primary antibodies for cyclin D1, p53, p21 and NF-κB were from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA), whereas primary antibodies for JNK and p-JNK^{Thr183/Tyr185} were from Upstate Biotechnology, Inc. (Lake

Correspondence to: Dr Vasilis Androutsopoulos, Department of Clinical Virology, Medical School, University of Crete, Voutes, Heraklion 71003, Crete, Greece
E-mail: androuts@uoc.gr

Key words: breast cancer, liver cancer, pyridines, apoptosis, JNK, cell cycle arrest

Placid, NY, USA) and for β -actin from Merck KGa. Secondary antibodies used were from Santa Cruz Biotechnology, Inc.

Synthesis. The general procedure involved the synthesis of a chalcone precursor by a classical Claisen-Schmidt condensation reaction. This compound was used to prepare a pyridone compound that was in turn converted into a pyridine via hydrogenolysis from a chlorinated pyridine precursor. The exact synthetic strategy of these two compounds will be published in a separate article.

Cell culture. MCF-7 and HepG2 cells were maintained in RPMI-1640 with phenol red and DMEM respectively, containing 10% fetal calf serum and 2 mM glutamine. The cells were passaged every 2 to 3 days using trypsin EDTA (0.25%).

MTT assay. HepG2 and/or MCF-7 cells were seeded at a density of 10^4 cells/ml in 96-well flat-bottomed plates. After 24 h, the medium was carefully removed, and 1 and/or 2 were added to 200 μ l medium to obtain a final concentration of not more than 0.1% (v/v) DMSO. The cells were then allowed to grow for 96 h at 37°C. The medium was removed, and fresh medium containing MTT (0.4 mg/ml) was added to each well for 3 h. The formazan product generated by the viable cells was solubilised with 150 μ l of DMSO. The plates were vortexed, and the absorbance at 540 nm was determined using a Spectra Max M5/M5^e microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The results were expressed as the percentage of proliferation compared with the control wells, and the IC₅₀ was calculated using GraphPad Prism software. The dose range was in serial dilutions, namely 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078 μ M.

Flow cytometry. HepG2 and/or MCF-7 cells were pretreated with 1, 2 and/or 0.1% DMSO (negative control) for 24 h. The medium was aspirated and the cells were washed with cold PBS, fixed in 70% ethanol and stored at least for 2 h at -20°C. The cells were then resuspended in PI solution (70 μ g/ml in PBS) containing 13 Kunitz units of RNase and incubated at 37°C for 30 min. Fluorescence was measured with a FACS Beckman Coulter, Inc., (Oakley Court, Buckinghamshire, UK) flow cytometer. The instrument was calibrated, and the laser was aligned using fluorescent beads and a flow check protocol prior to each sample run. The doublets were separated from single cells in the G2/M phase by gating. At least 10,000 events were acquired. The induction of apoptosis was measured by the proportion of the cells in the subG1 phase.

Western blot analysis. HepG2 and/or MCF-7 cells were trypsinized and resuspended in 200 μ l lysis solution (Merck KGa) containing protease inhibitor cocktail (Merck KGa) and DTT (1 mM). A total of 20 μ l of each sample was mixed with loading buffer (containing 5% mercaptoethanol) at a 1:1 ratio. The samples were heated at 100°C for 4 to 5 min and each sample was loaded on an acrylamide gel (10% acrylamide for resolving gel and 5% acrylamide for stacking gel) at the required volume to obtain 15 μ g of protein for protein detection. Wet blotting was used to transfer the proteins from the gel to a nitrocellulose membrane. The membrane was incubated with 10% milk in 0.1% PBST at room temperature for 1 h on a shaker in order

to block non-specific binding. The primary antibodies were diluted in 1% milk in 0.1% PBST and incubated at 4°C overnight with the membrane. The membrane was then washed three times for 10 min each time with 0.1% PBST in order to remove the excess primary antibody. The membrane was incubated with secondary antibodies diluted in 1% milk in 0.1% TBST at room temperature for 1 h. The excess secondary antibody was washed three times with 0.1% PBST as described above, and the membrane was exposed to ECL reagents (Thermo Fisher Scientific, Inc.). Finally, the signal was transferred to a film, which was developed in the dark.

The primary antibody dilutions that used were as follows: Cyclin D1 at 1:300, p53 at 1:500, p21^{WAF/CIP1} at 1:200, NF- κ B at 1:500, JNK at 1:500 and p-JNK^{Thr183/Tyr185} at 1:500. The secondary antibody dilutions used were at 1:2,000 for anti-mouse and/or anti-rabbit antibodies.

Statistical analysis. The results are expressed as mean \pm standard deviation (SD) for at least three independent experiments.

Results

Synthesis and cytotoxicity of the novel pyridone and pyridine compounds. The precursor chalcone 4',6', dimethoxy, 3,4 methylene dioxy chalcone was synthesized by a Claisen-Schmidt condensation (Fig. 1A). This compound was used to prepare the pyridone 1 (Fig. 1A). Compound 1 was further used to yield an intermediate chloropyridine that was converted to the pyridine 2 via hydrogenolysis (Fig. 1B). The exact synthetic route that was used for the synthesis of 1 and 2 will be published in a separate article. In an initial approach to evaluate the antiproliferative effects of the two compounds, MTT assays were carried out in MCF-7 breast adenocarcinoma and HepG2 liver hepatoblastoma cells. The cells were treated with various concentrations of compounds covering a range of 0.08–20 μ M for 96 h, and cell viability was assessed spectrophotometrically. Compound 1 was more active in inhibiting proliferation of cancer cells than compound 2 (Fig. 2). Generally, HepG2 cells appeared more sensitive to compound treatment than MCF-7 cells, in terms of the IC₂₅ values (concentrations required for 25% inhibition of cell proliferation) (Fig. 2). The calculated IC₅₀s were 4.5 \pm 0.3 and 7.5 \pm 0.1 μ M for 1 and 2, respectively, in the HepG2 cell line and 6.3 \pm 0.4 μ M and 16 \pm 1.7 μ M in the MCF-7 cell line, respectively.

Compounds 1 and 2 induce G2/M phase arrest in MCF-7 and HepG2 cells. In order to provide more information on the mechanism of antiproliferative action of compounds 1 and 2, the cells were treated with the compounds for 24 h and further subjected to cell cycle analysis. Initial investigations using phase contrast microscopy revealed doublets of cells floating following treatment of 15 μ M with either of the compounds for 24 h (data not shown). Cell cycle analysis using propidium iodide (PI) staining demonstrated that both compounds 1 and 2 (15 μ M) induced G2/M arrest in MCF-7 and HepG2 cells following 24 h of treatment, while it was evident that a small percentage of cells underwent apoptosis as demonstrated by the subG1 peak (Figs. 3 and 4). Treatment of HepG2 cells with compound 1 and/or 2 caused an increase from 24 \pm 2.2% (control) to 56 \pm 3.2% and

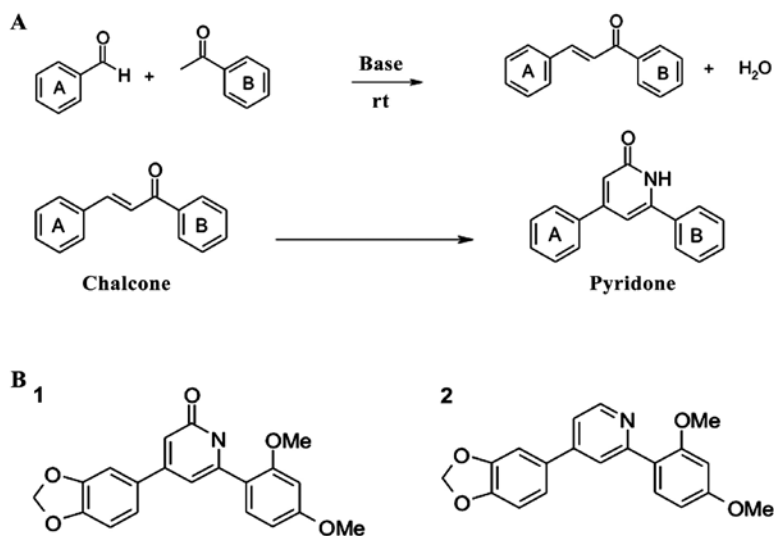


Figure 1. Chemical structures and synthetic routes of the compounds 1 and 2.

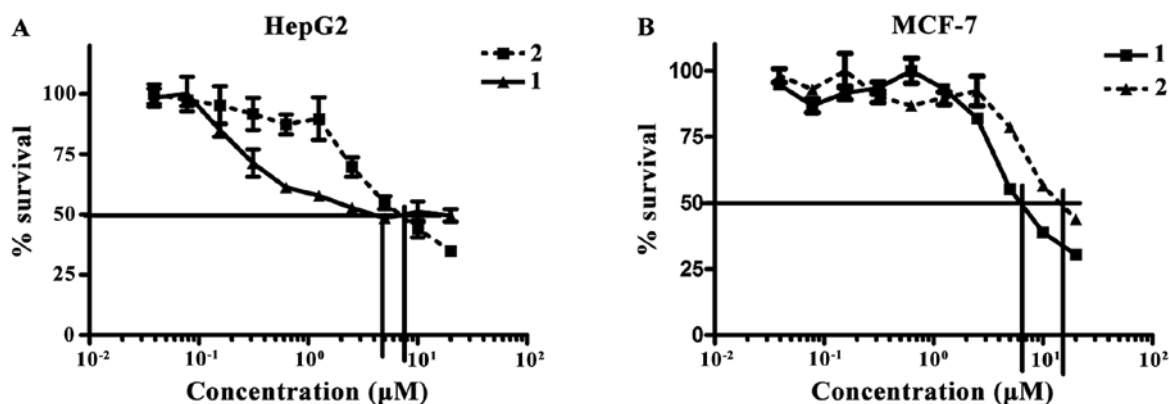


Figure 2. Cytotoxic effects of compounds 1 and 2 in (A) HepG2 and (B) MCF-7 cells. The compounds were incubated for 96 h at a concentration range of 0.078-20 μM , and cell viability was measured using the MTT assay. Error bars represent mean \pm SD for at least n=3 determinations. SD, standard deviation.

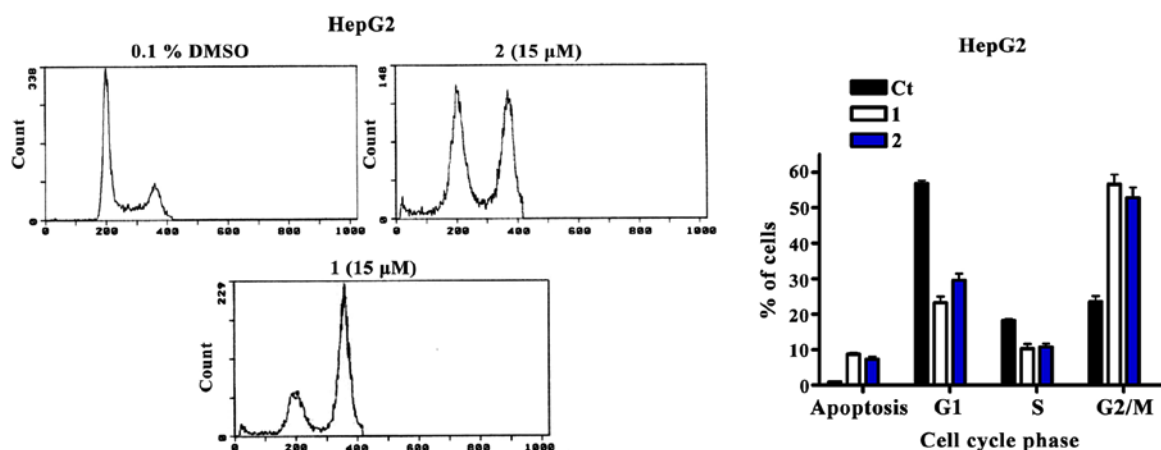


Figure 3. Cell cycle analysis of HepG2 cells treated with 15 μM compound 1 and/or 2 for 24 h. The cells were fixed in 70% ethanol and incubated with PI solution (70 $\mu\text{g}/\text{ml}$) containing RNase for 30 min. Fluorescence was measured using a Beckman-Coulter flow cytometer. Error bars represent mean \pm SD for n=3 determinations. SD, standard deviation.

51 \pm 3.8%, respectively, of the cell population at the G2/M phase, whereas a decrease was noted in the percentage of the cells at the G1 phase from 58 \pm 2.9% (control) to 23 \pm 2.2%

and 28 \pm 3.1% for compound 1 and 2 respectively, compared with the control samples (Fig. 3). The percentage of cells undergoing apoptosis was estimated to 8 \pm 0.2% and 6.7 \pm 0.3%

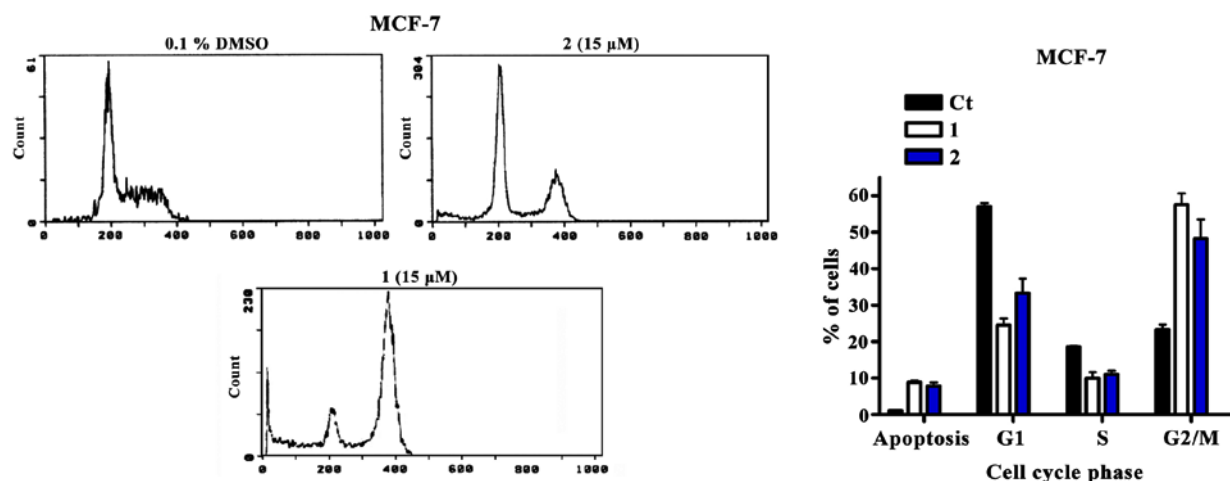


Figure 4. Cell cycle analysis of MCF-7 cells treated with 15 μM compound 1 and/or 2 for 24 h. The cells were fixed in 70% ethanol and incubated with PI solution (70 $\mu\text{g}/\text{ml}$) containing RNase for 30 min. Fluorescence was measured using a Beckman-Coulter flow cytometer. Error bars represent mean \pm SD for $n=3$ determinations. SD, standard deviation.

for compounds 1 and 2, respectively (Fig. 3). Compound 2 was somewhat less potent than compound 1 in inducing apoptosis and the G2/M block and, as a lower increase in the cell cycle distribution of the liver cancer cells was noted (Fig. 3). Similar results were noted for the MCF-7 cell line, although 2 appeared less potent in those cells compared with HepG2 cells (23) (Fig. 4, G2/M arrest: Control, $23 \pm 1.3\%$, 1, $58 \pm 3.5\%$; 2, $47 \pm 4.7\%$). The data derived from the cell cycle analysis were in agreement with the results obtained from the MTT cell viability assays.

Compounds 1 and 2 induce upregulation of p53 and JNK in HepG2 cells. In order to add insight to the mechanism of 1- and 2-mediated cell cycle inhibitory action, the protein expression of the cell cycle inhibitors and cell cycle-associated proteins following treatment of HepG2 liver cancer cells with the compounds was investigated. The investigation was focused on HepG2 cells, since they were more profoundly affected by the compounds compared with MCF-7 cells. The effects of compounds 1 and 2 on the proteins cyclin D1, p53 and p21 were evaluated. The data presented in this report indicated that both compounds 1 and 2 induced p53 expression in a concentration-dependent manner in HepG2 cells. Furthermore, the induction of p21^{WAF/CIP1} was lower than that noted for p53. Compound 2 induced a weak increase in p21^{WAF/CIP1} protein expression in HepG2 cells. Moreover, the expression of the protein cyclin D1 was profoundly decreased in the presence of compounds 1 and 2 (Fig. 5A), which is in concordance with the decrease in the percentage of HepG2 cells at the G1 phase, since this protein is involved in the G1/S phase transition.

In order to elucidate the molecular mechanism underlying the induction of apoptosis in the presence of compounds 1 and 2, we examined the effects of these two compounds on the expression of the cell signaling proteins JNK and NF- κB . Treatment of HepG2 cells with compound 1 and/or 2 (20–50 μM) for 24 h resulted in an upregulation of total JNK protein and total p-JNK^{T183/185} (Fig. 5). In contrast to compound 1, compound 2 caused only an upregulation of total JNK, while the levels of total p-JNK^{T183/185} remained relatively constant (Fig. 5A). In order to investigate the expression pattern

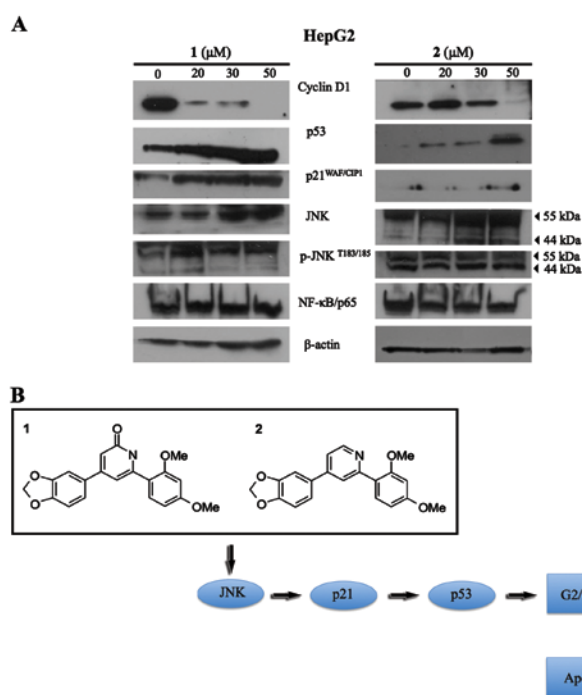


Figure 5. Induction of cell cycle associated proteins by compounds 1 and 2. (A) Western blot analysis of cell cycle-associated and apoptosis-associated proteins. HepG2 cells were treated with compounds 1 and/or 2 at a concentration range of 20–50 μM for 24 h, and expression of the proteins was determined using immunoblotting. (B) Schematic pathway depicting the cell signaling events of compounds 1 and 2 in HepG2 cells.

of upstream mediators of JNK, we incubated HepG2 cells with compounds 2 and 1 and examined the expression of NF- κB using western blot analysis. NF- κB is a transcription factor that is activated in response to stress and cytokines. This protein is overexpressed in certain cancer types and can lead to apoptosis via cell signaling with JNK proteins. The results indicated that the NF- κB protein levels did not change following treatment with either compounds 1 and/or 2 (Fig. 5A), thus implying that induction of apoptosis in HepG2 cells does not require NF- κB signaling (Fig. 5B).

Discussion

Inhibition of cellular proliferation and induction of apoptosis is a commonly encountered phenomenon for the majority of phytochemicals and synthetic anticancer compounds. Particular attention has been given to natural and synthetic agents that induce the apoptotic cascade and/or arrest the cell cycle of cancer cells at specific checkpoints (6,9,10). Chalcones have shown promising antitumor activity by 3 distinct modes of action: Antioxidant activity, cytotoxicity and induction of apoptosis. Recently, the exploration of structure-activity relationships has contributed towards the improvement of the anticancer properties of chalcones by substituting aryl rings and introducing heterocyclic moieties (11). Despite their promising antitumor activity, chalcones are frequently susceptible to photoisomerization in solution (12). Consequently, the present study explored the possibility of synthesizing novel pyridine and pyridone compounds from a chalcone precursor in order to overcome the non-specific photoisomerization. Compounds 1 and 2 were further evaluated in human cancer cells and it was demonstrated that they were more active in HepG2 liver cancer cells with IC_{50} s near the μ M range, whereas their mechanism of action involved induction of G2/M arrest and apoptosis via upregulation of the cell cycle inhibitors p21 and p53 and the cell signaling protein JNK (Fig. 5B).

Previous studies have reported the anticancer activity of pyridine derivatives against several types of cancer cells. A series of 6-amino-5-cyano-1-7(3-ethylphenyl)-2-oxo-4-substituted-1,2-dihydropyridine-3-carbo-nitriles 4a-p were synthesized and tested for *in vitro* anticancer activity against the Ehrlich Ascites Carcinoma (EAC) cell line and the liver human tumor cell line (HepG2) (13). These compounds demonstrated considerably low potency in inhibiting proliferation of HepG2 cells in terms of IC_{50} values (20-75 μ M) compared with the compounds investigated in the present study (3). In addition, 2-pyridones that contain aromatic and heteroaromatic rings with 4H-pyrans were considerably more active in a range of human cancer cell lines, namely MCF-7, HepG2 and A549 (IC_{50} of 8, 11.9 and 15.8 μ M, respectively) (14).

With regard to the potential mechanism of action of compounds that are structurally similar to 1 and 2, various modes of anticancer activity have been proposed, such as inhibition of histone deacetylases, inhibition of Cdc7 and inhibition of VEGFR (15-17). However, the mechanism of action that is more likely to be responsible for the antiproliferative activity of compound 1 and 2 is inhibition of the process of mitosis. This hypothesis is based partially on the data reported in the present study and on previous studies that have demonstrated antimetabolic activity of compounds that resemble the structure of compounds 1 and 2. The bidentate iron chelator CP20 (deferiprone) resembles the structure of compounds 1 and 2 and has been shown to increase the number of rat and human hepatoma cells in the G2/M phase of the cell cycle (18). Similarly, the anticancer compound ABT-751 is a novel orally active antimetabolic agent in phase II clinical development that exerts antivasculature effects and its structure contains a pyridine ring and a phenol group (19). It is interesting to note the structural similarity of compounds 1 and 2 with colchicine that contains 2 six-membered carbon benzene rings and one 7-membered carbon ring. Based on this

hypothesis, a recent study has demonstrated the antimetabolic activity of a series of new pyridine derivatives structurally related to ABT-751 that were initially tested for their cytotoxic activity *in vitro* against the HCT-116 and HepG-2 cancer cell lines. The results indicated that one of the pyridine compounds exhibited higher cytotoxic activity than the reference antimetabolic agent colchicine, against both tested cell lines, with IC_{50} of 0.52 and 1.40 μ M, respectively, while *in vitro* inhibition of tubulin polymerization showed comparable efficacy with that of colchicine (20). The present study is in agreement with these findings, although additional *in vitro* assays are required to elucidate the exact mechanism of action of compounds 1 and 2.

In addition to the inhibition of the mitotic process compounds 1 and 2 profoundly affected the expression of cell cycle and cell signaling proteins. The reduction in the levels of cyclin D1 and the increase in p21 and p53 that were caused by compounds 1 and 2 in HepG2 cells is expected due to the accumulation of the cells in the G2/M phase. Cyclin D1 is the dimerization partner of CDK4 that controls the phosphorylation of Rb and the G1/S phase transition. p53 is a transcription factor that is induced in response to DNA damage and/or cellular stress. p53 controls the G2/M checkpoint by allowing sufficient repairs to occur before the cell enters mitosis. p53 can also cause induction of apoptosis when irreversible damage to DNA or the cell cycle machinery occurs (21). The data presented in this report indicate that both compounds 1 and 2 induced p53 expression in a concentration-dependent manner in HepG2 cells. The p53-mediated cell cycle arrest was promoted by induction of the cell cycle inhibitor p21^{WAF/CIP1} (Fig. 5).

Furthermore, compounds 1 and 2 increased the expression of JNK in HepG2 cells and 1 further induced the phosphorylation of this protein. C-jun N terminal kinases belong to the family of MAPK kinases and are activated in response to stress and inflammatory signals. The activation of the kinase domain of JNK requires its phosphorylation at specific residues Thr183 and Tyr185. Activated JNK translocates to the nucleus where it regulates the phosphorylation of several transcription factors such as p53, STAT3 and ATF-2 (22). Consequently, the phosphorylation of JNK that was caused by compound 1 may be associated with the induction of p53 that in turn arrests the cells at the G2/M phase.

It is important at this point to note that 1 and 2 displayed antiproliferative activities with dissimilar potencies, as determined by MTT assays, cell cycle analysis and western blot analysis. This leads to the conclusion that the keto group on the middle ring of the pyridine structure plays a prominent role in enhancing the antiproliferative activity of the compound. Specifically, this functional group led to an approximately 3-fold increase in IC_{50} values in MCF-7 cells and a 2-fold increase in HepG2 cells, respectively. In the present study, we did not determine the exact nature of this structure-activity relationship with regard to the mechanism of action of the two compounds. However, this is an issue that warrants further investigation in future studies.

In conclusion, the present study described for the first time the synthesis and biological evaluation of one novel pyridine and one novel pyridone-based anticancer agent. The data demonstrated that both compounds inhibited the prolifera-

tion of human breast and liver cancer cells by inducing G2/M phase arrest via a p53-p21-mediated pathway and apoptosis via JNK upregulation. These initial *in vitro* results show promise for the further development of these compounds as anticancer agents and their biological evaluation in an *in vivo* study.

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