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ORIGINAL ARTICLE

Synergistic antiproliferative effects of curcumin and celecoxib in hepatocellular carcinoma HepG2 cells

Fatma M. Abdallah[1] · Maged W. Helmy[1] · Mohamed A. Katary[1] · Asser I. Ghoneim[1]

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Abstract

Hepatocellular carcinoma (HCC) is still a leading cancer killer in the community. Molecular targeted therapy with celecoxib (CXB) has shown promising antitumor effects; however, its use may be limited due to serious side effects. Curcumin (CUR) has also shown beneficial effects against HCC. Then, it was aimed to investigate the effects of adding CUR to CXB on HCC HepG2 cells. HepG2 cells were treated with CXB and/or CUR at increasing concentrations to investigate synergistic drug interactions, as calculated combination index (CI). Combination treatment effects on cell viability and caspase-3 activation were assessed. The levels of Akt, nuclear factor-kappa B (NF-κB), prostaglandin E_2 (PGE_2), malondialdehyde (MDA), cyclin D1 (CD1), and vascular endothelial growth factor (VEGF) were also evaluated. CXB (3.13–100 μM) and/or CUR (1.25–40 μM) reduced HepG2 cell viability dose-dependently. Nevertheless, lower combined concentrations showed higher synergism (CI < 1) and higher CXB dose reduction index (DRI > 1). Also, the addition of CUR to CXB resulted in increased cytotoxicity and caspase-3 activation, as compared to CXB alone. In addition, the selected combination significantly reduced the levels of Akt, NF-κB, PGE_2, MDA, CD1, and VEGF, as compared to either agent alone. In conclusion, CUR augmented the CXB-mediated antitumor effects in HepG2 cells through, at least in part, antiproliferative, antioxidant, and pro-apoptotic mechanisms. This may allow the further use of CXB at lower concentrations, combined with CUR, as a promising safer targeted strategy for HCC management.

Keywords Curcumin · Celecoxib · HepG2 · Proliferation · Apoptosis

Abbreviations

CD1	Cyclin D1
CI	Combination index
CUR	Curcumin
CXB	Celecoxib
DRI	Dose reduction index
HCC	Hepatocellular carcinoma
HepG2	Human liver-derived hepatoma G2
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
IC_{50}	Median inhibitory concentration
MDA	Malondialdehyde
MTT	3-[4, 5-Dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
p-Akt	Phospho-Akt
PGE_2	Prostaglandin E_2
SEM	Standard error of the mean
VEGF	Vascular endothelial growth factor

Introduction

Hepatocellular carcinoma (HCC), as the most common form of liver cancer, represents the third main cause of worldwide cancer deaths annually. In Egypt, there is a high incidence of HCC where it also represents the principal cause of death from all cancers. HCC prognosis is still extremely poor and advanced HCC is highly aggressive with a low response to traditional therapies (El–Serag and Rudolph 2007; Shaker et al. 2013). In this context, the significance relies on the massive worldwide burden that HCC represents, and particularly to the Egyptian community. The recent success of the Egyptian model experience in the treatment of HCV in collaboration with the WHO's Global Strategy has highlighted the importance of managing remaining complications including HCC (Elgharably et al.

✉ Asser I. Ghoneim
asser@damanhour.edu.eg; aighoneim40@gmail.com

[1] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Damanhour University, Damanhour 22514, Egypt

2017; Waked et al. 2016). Therefore, new strategies are urgently needed, including chemotherapy that remains a principal treatment method, especially the targeted one.

Targeted strategies include selective COX-2 inhibitors (COXIBs) that have been evaluated as potential anticancer agents for various malignancies. Specifically, celecoxib (CXB) has demonstrated anticancer effects in tumors, particularly, colon carcinoma and HCC both in vitro and in vivo (Cui et al. 2005; Harris 2009). The antiproliferative and pro-apoptotic effects of COXIBs on human HCC cell lines are likely to be multifactorial as they may use both cyclooxygenase (COX)-2 and non-COX-2 biochemical targets to mediate their antitumor activities (Cui et al. 2005; Grösch et al. 2006). It is reported that CXB may inhibit Akt phosphorylation through the COX-2-PGE_2-PI_3K pathway (Kulp et al. 2004). Also, the nuclear transcription factor-kappa B (NF-κB) and reactive oxygen species (ROS), among others, are targets for COXIBs (Cervello et al. 2013; Maeda and Omata 2008; Wyrebska et al. 2014). In addition, CXB induces cytochrome c release, activates caspase-9 and caspase-3, and eventually stimulates apoptosis in HCC (Grösch et al. 2006). Moreover, most COX-independent effects of CXB in vitro were only observed at supra-therapeutic concentrations that were 10–100 times higher than plasma concentrations measured clinically (Grösch et al. 2006). Nevertheless, the use of COXIBs and their dose have been associated with an increased incidence of cardiovascular toxicity (Bertagnolli et al. 2009). Several pharmacogenetics factors that may contribute to the toxicity profile of CXB were reviewed (Domiati and Ghoneim 2015).

A novel concept in anticancer pharmacology is the use of combination therapy. A combination consisting of CXB with agents that specifically affect relevant molecular targets may take advantage of increased antitumor effects and could reduce the toxicity associated with CXB (Cervello et al. 2013; Du et al. 2013; El-Awady et al. 2011; Lev-Ari et al. 2005a; Lev-Ari et al. 2005b; Morisaki et al. 2013; Narayanan et al. 2005). Then, aiming to keep the anticancer effect of CXB with minimal toxicity profile, we combined CXB at low concentration with curcumin (CUR), keeping in mind that CUR has a good safety profile (Cheng et al. 2001).

The pharmacological terms *combination index* (CI) and coefficient of drug interaction are used to analyze effects of drug combinations to indicate whether the combined drugs are synergistic (CI < 1), additive (CI = 1), or antagonistic (CI > 1) (Cervello et al. 2013; Chou 2006). Synergistic drug combination is able to reduce the dose of the drug used, thereby reducing toxicity while maintaining efficacy. The concept of *the dose-reduction index* (DRI) is a measure of how many folds the dose of a drug in combination may be reduced at a given effect compared with the dose of drug alone. Thus, DRI is important in clinical situations, in which dose reduction may reduce toxicity in patient while retaining therapeutic efficacy. The greater DRI > 1 value indicates a greater dose reduction for a given therapeutic effect (Chou 2006). In order to calculate CI, computerized algorithms were based on general theories for biological applications of mass-action law and receptor occupancy. These have established a dose-response *theory* of basic equations in biomedical sciences guided by Henderson, Michaelis, Hill, and Scatchard (Chou 2006; Chou and Martin 2007).

The HepG2 liver cancer cell line was selected as a HCC model due to several reasons. Firstly, it is of wild apoptotic p53 gene (Müller et al. 1997), and highly expresses the COX-2 enzyme needed to investigate both COX-2-dependent and COX-2-independent antiproliferative pathways in human liver cancer cells (Bae et al. 2001). Secondly, HepG2 represents the most widely used human liver cancer cell line in pharmacological research aiming to develop new potential drugs and combinations. It is phenotypically more hepatocytic than others and expresses many differentiated essential hepatic functions including drug-metabolizing enzymes (Donato et al. 2015; Duan et al. 2014; Fan et al. 2014; Feo et al. 2007; Khalil et al. 2015; Qiu et al. 2015).

CUR is the most important of three main curcuminoids present in turmeric and has a potential role in liver cancer (S Darvesh et al. 2012). CUR is a potent antioxidant having neuroprotective, hepatoprotective, anti-inflammatory, and antitumor activities in normal and cancer cells, as previously demonstrated by our teams and by others (Ghoneim 2012; Ghoneim 2009; Ghoneim et al. 2002; Ghoneim and Eldahshan 2012; Teiten et al. 2010). CUR also targets negatively NF-κB and positively caspase-3 (Ghoneim 2009; Shishodia et al. 2005). Furthermore, CUR has been shown to inhibit several signaling proteins such as COX-2, cyclin D1, vascular endothelial growth factor receptor (VEGF), and phosphoinositol-3 $(PI)_3$/Akt/mammalian target of rapamycin (mTOR) pathway (Aggarwal et al. 2003; Beevers et al. 2009).

Both CUR and CXB inhibit COX-2 via diverse mechanisms. CUR downregulates COX-2 mRNA and decreases its protein levels (Goel et al. 2001), whereas, CXB inhibits COX-2 directly by occupying its binding location (Hood et al. 2003). Furthermore, the combination of CUR and CXB was found to demonstrate a synergistic anticancer effect in colon cancer (Lev-Ari et al. 2005a) and in pancreatic cancer (Lev-Ari et al. 2005b). However, the combined effect of CUR and CXB against HCC cells remains unknown. Hence, the present study aimed to evaluate the anticancer effect of CUR combined with CXB in HCC HepG2 cells. The ultimate goal was to test the validity of the hypothesis stipulating the attainment of a better anticancer efficacy of this combination using a lower and safer dose of CXB. To better understand the molecular mechanisms underlying the interactive cytotoxic effects of this combination, several biomarkers were investigated along the proliferative signaling pathway of the Akt/NF-κB/PGE2/ROS axis, such as, proliferation (CD1), angiogenesis (VEGF), and apoptosis (caspase-3).

Materials and methods

Chemicals

Celecoxib (CXB) was obtained as a gift from Amriya Pharmaceutical Industries, PHARCO Co. Alexandria, Egypt). Curcumin (CUR), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (St. Louis, USA). Trypsin, Dulbecco's modified eagle medium (DMEM), phosphate buffer saline, and penicillin/streptomycin antibiotic mixtures were purchased from Maadi medical supplies (Cairo, Egypt). Ethanol was bought from El-Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt).

Cell lines and cell culture

HepG2 cell line was procured from the American Type Culture Collection (ATCC; USA). The cells (5 passages after thawing) were cultured in 75-cm^2 flasks in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin antibiotic mixture and kept in a humidified incubator with 95% air and 5% CO_2 at 37 °C (Thermo Electron Co., Waltham, Massachusetts, USA). The media were continuously changed after 3–4 days, and the cells passaged after reaching 80–90% confluence.

Cell viability assay

The effects of CUR and CXB and their combination on cell proliferation of Hep G2 were assessed by the MTT assay (van Meerloo et al. 2011). Cells were seeded at 5000 cell/well in 96-well plates containing 100 μL of DMEM and allowed to adhere to the plate overnight. DMEM was replaced by fresh medium comprising serially diluted concentrations of CUR (1.25–40 μM) and/or CXB (3.13–100 μM) dissolved in vehicle DMSO at a final concentration of 0.1%. Cells were protected from light for the duration of CUR treatment. The plates were incubated for 72 h at 37 °C and the medium was then aspirated; the cells were rinsed with PBS, and 200 μL of the MTT (0.5 mg/ml in DMEM) was incubated with cells for further 4 h in the dark. After removal of supernatant, 100 μL of DMSO was added to dissolve the crystals formed by maintaining agitation for 15 min. Absorbance was detected at 570 nm using a microplate reader (Bio-Rad, USA). Values were means of two separate experiments, each done in triplicate. Cell viability was expressed as percentage of the untreated control wells. The median inhibitory concentrations (IC_{50}) and the 30% inhibitory concentration (IC_{30}) values were calculated using the CompuSyn software (ComboSyn, Inc.) (Chou 2006).

Synergism experiments

Drug-drug interactions between CXB and CUR were evaluated in HepG2 cells by MTT assay. Cells were incubated with each drug independently and in combination for 72 h before assessment of cytotoxicity. The combination index (CI) was produced by Compusyn software (version 1.0.1), where CI < 1 indicate synergism, CI = 1 designate additive effect, and CI > 1 means antagonism as developed by Chou and Talalay (Chou 2006; Chou and Martin 2007).

Cells were then cultured in 75-cm^2 flasks to adhere for 24 h under routine conditions. After this period, each three of 75-cm^2 flasks containing cells were incubated with CXB (IC_{30}), CUR (IC_{50}), or a combination of both (Lev-Ari et al. 2005a; Lev-Ari et al. 2005b; Morisaki et al. 2013) for 72 h. Then, the cells were harvested, resuspended in 500 μl PBS, and kept frozen till measuring the following biomarkers.

Morphological changes in HepG2 cells

HepG2 cells normal appearance was observed under light microscope, and after being treated with CXB, CUR, or a combination of CXB and CUR. Cells were investigated for typical polygonal and intact cellular appearance. Otherwise, observation took place for any morphological changes characteristic of cell death, either by apoptosis and/or necrosis, particularly rounding, detachment from the surface of the plates and/or shrinkage and condensation.

Assay of caspase-3 activity

HepG2 cell pellets were lysed with lysis buffer for 30 min at 4 °C and the supernatants were collected and used to detect caspase-3 levels. A colorimetric test kit (Sigma-Aldrich, St. Louis, USA) was used. The hydrolysis of a peptide substrate (Ac-DEVD-pNA) by the enzyme released a p-nitroaniline part. Concentration of the cleaved moiety was calculated from a calibration curve with absorbance at 405 nm using a microtiter plate reader as previously described (Ghoneim 2009; Nicholson et al. 1995). Data were expressed as means ± SEM of three separate experiments, each done in triplicate.

Measurement of the levels of Akt, NF-κB, CD1, and VEGF

The Sandwich ELISA technique was used as a reliable quantitative method having high sensitivity and specificity (Gan and Patel 2013). Several ELISA kits were used according to the manufacturers' instructions for the detection of the following biomarkers in HepG2 cells. Cell pellet lysates were used for analysis of phospho-Akt (Akt [pS473] kit, DRG International, Inc., Massachusetts, USA), phospho-NF-κB (p-NF-κB p65 (S536) kit, Ray Biotech, Georgia, USA),

CD1 (MBS724349 kit, MyBioSource, CA, USA), and VEGF (CSB-E11718h kit, CUSABIO, Maryland, USA). The Sandwich ELISA kit used monoclonal antibody specific for each of the following biomarkers coated onto the wells provided. Firstly, the marker antigen binds to the monoclonal antibody. After washing, an antigen-specific antibody acted as a detector by binding to the captured marker. Finally, a horseradish peroxidase (HRP) antirabbit IgG was added, then a substrate solution was added to produce color after being acted upon by the peroxidase enzyme (Morisaki et al. 2013; Sasaki et al. 2013).

Assessment of prostaglandin-E2 (PGE2) and malondialdehyde (MDA) levels

Both PGE2 and MDA production levels were assessed based on the competitive binding of enzyme immunoassay using two specific ELISA kits from MyBioSource, CA, USA. A color change was detected at 450 nm. The concentration of target antigen was then determined using a standard curve (Bassiouny et al. 2010; Lev-Ari et al. 2005a; Morisaki et al. 2013).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons using GraphPad Prism 3.0 (Graph Pad Software Inc., CA, USA) as appropriate. Statistical difference significance was set at $p < 0.05$. Compusyn software (version 1.0.1) (Chou and Martin 2007) was used to estimate the synergistic effects of different drug combinations by generating the combination index (CI), where CI < 1, CI = 1, and CI > 1 designated synergism, additive effect, and antagonism, respectively (Chou 2006; Chou and Martin 2007).

Results

Effects of CXB and/or CUR on HepG2 cell growth

To evaluate the effect of CXB and/or CUR on the growth of the human HCC cell lines, HepG2 cell growth inhibition assay was done by MTT. Cells were treated with increasing concentrations of CXB (3.25–100 μmol/l) or CUR (1.25–40 μmol/l) (Fig. 1). The IC_{50} value, at which growth was half of that in the vehicle-treated control, was calculated using Compusyn software (version 1.0.1). CXB reduced cell viability dose-dependently with an IC_{50} of 89.9 μM and an IC_{30} of 42.5 μM in HepG2 cells. CUR also inhibited cell proliferation dose-dependently (IC_{50} 13 μM). It was clear that increasing concentrations of CXB began to significantly inhibit growth of cells only from 50 μmol/l, whereas, increasing concentrations of CUR began to significantly inhibit growth of cells from 10 μmol/l (Fig. 1). Interestingly, the IC_{30} of CXB (42.5 μM) is between its minimum effective concentration (50 μM) and its no-observed effect level concentration (25 μM), as illustrated by the line chart in Fig. 1.

Also, the addition of increasing concentrations of CUR (1.25–40 μmol/l) to increasing concentrations of CXB (3.125–100 μmol/l) consistently increased growth inhibition in HepG2 cells compared to the corresponding CXB-alone group (Fig. 2). Moreover, the combination treatment was found to be synergistic as calculated using Compusyn software (version 1.0.1) (Chou and Martin 2007).

Synergistic antiproliferative effects of celecoxib and curcumin in HepG2 cells

To examine the combined effects of CXB and CUR on HepG2 cells, these were treated with CXB, CUR, or both agents in an

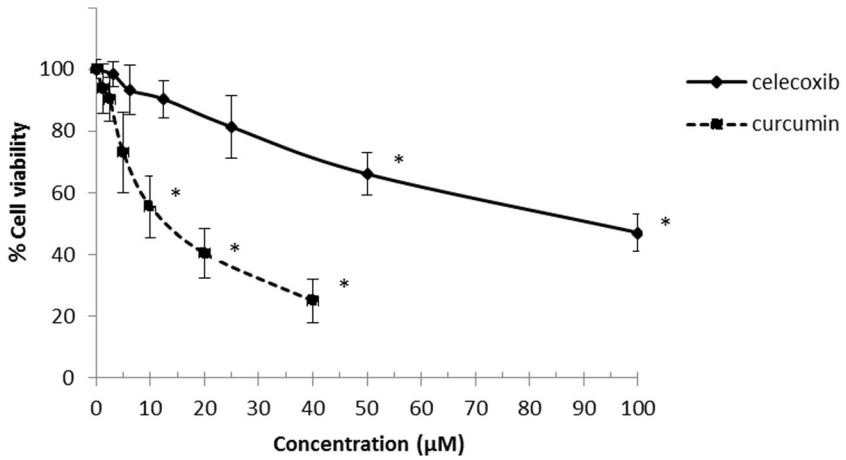

Fig. 1 Effects of CXB or CUR on viability of HepG2 cells. Cells were cultured in the presence of increasing concentrations of celecoxib (CXB) (3.125–100 μmol/l) or curcumin (CUR) (1.25–40 μmol/l) for 72 h in DMEM containing 10% FBS. Cell viability was assessed by MTT assay. Data points represent the means ± SEM of two separate experiments, each done in triplicate. *$p < 0.05$ indicated significant difference for CXB or CUR versus corresponding control group. Statistical analysis was determined by one-way ANOVA followed by Tukey post hoc test

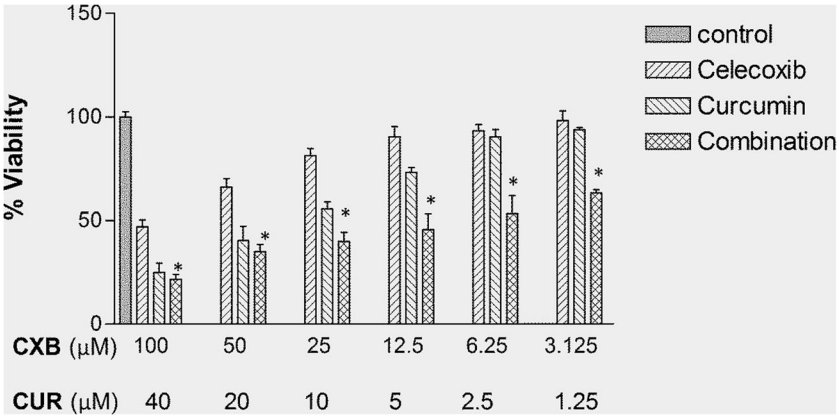

Fig. 2 Effects of adding CUR to CXB in combination on the viability of HepG2 cells. Cells were incubated for 72 h in the presence of the indicated increasing concentrations of celecoxib (CXB) (3.125–100 μmol/l) and curcumin (CUR) (1.25–40 μmol/l) alone or in combination. Data are expressed as the percentage of control cells and are the means ± SEM of three separate experiments, each of which was performed in triplicate.*$p < 0.05$ indicated significant difference for the combination versus corresponding CXB-alone group. Statistical analysis was determined by one-way ANOVA followed by the Tukey post hoc test

arbitrary ratio of 1 CUR: 2.5 CXB. Concerning synergy experiments, HepG2 cells were treated with the combination of CXB and CUR at doses indicated.

CompuSyn software was used to determine the type of drug interaction between the agents. Table 1 presents the combination indices (CIs) detected after treatment of HepG2 cells with different combinations of the two agents and indicated their interaction pattern. The CI values were estimated according to the method of Chou using CompuSyn software, where CI < 1, CI = 1, and CI > 1 designated synergism, additive effect, and antagonism, respectively (Chou 2006; Chou and Martin 2007; Cusimano et al. 2007; Morisaki et al. 2013). The results in Table 1 showed the addition of 100 μM CXB to 40 μM CUR significantly inhibited cell growth by 79% in HepG2 cells with CI = 1.15 which showed slight antagonistic effect with dose reduction index (DRI) of CXB = 2.8, while 50 μM CXB plus 20 μM CUR significantly inhibited cell growth by 70% in HepG2 cells with CI = 0.9; which began to show slight synergism that increased with lower concentrations, and with higher DRI of CXB = 3.8. The CI value of 25 μM CXB plus 10 μM CUR was 0.7 which meant better synergism, with much higher DRI of CXB = 5.1 (Table 1). Based on these experiments and observations, the CUR IC_{50} (13 μm) added to the CXB IC_{30} (42.5 μM) was selected as a novel combination to be investigated mechanistically for its underlying pharmacologic effects on the subsequent molecular biomarkers of HepG2 cytotoxicity. This combination is complying more with our approach to reduce CXB dose to decrease its side effects as much as possible.

Table 1 Combination index (CI) values of adding CUR to CXB in combination in HepG2 cells

CXB (μM)	CUR (μM)	Fa	CI	DRI CXB
100	40	0.79	1.15	2.8
50	20	0.7	0.9	3.8
25	10	0.6	0.7	5.1
12.5	5	0.54	0.44	8.4
6.25	2.5	0.47	0.30	12.7

HepG2 cells were treated with the combination of celecoxib and curcumin at doses indicated. CompuSyn software was used to analyze the data and calculate the CI value, where CI < 1, CI = 1, and CI > 1 indicated synergism, additive effect, and antagonism, respectively. The CI = (dA/DA) + (dB/DB), where dA and dB are the concentrations of CXB and CUR in combination, whereas, DA and DB are the concentrations of CXB or CUR, respectively, which produce the same effect alone. For example, for an IC_{50}, the CI = 0.38

CI combination index, *CUR* curcumin, *CXB* celecoxib, *DRI CXB* dose reduction index of celecoxib, *Fa* fraction affected

Effects of CXB and/or CUR on apoptosis of HepG2 cells

Firstly, the morphological effects of CXB and/or CUR in the HepG2 cells were determined by preliminary morphological assay. The HepG2 cells were treated with CXB (IC_{30}: 42.5 μM) and/or CUR (IC_{50}: 13 μM) for 72 h, and observed under a light microscope. The control normal HepG2 cells showed a typical polygonal and intact appearance. Treatment of cells with CXB and/or CUR induced morphological changes characteristic of cell death, particularly when exposed to combination of CXB and CUR, where the cells appeared spares, and detached from the surface of the plates with progressive cytoplasmic shrinkage and condensation (the typical morphologic signs of apoptosis under light microscope) (Fig. 3).

Secondly, to confirm apoptosis biochemically, the treatment of cells with combination of CXB and CUR led to a significant increase in caspase-3 activity as shown in Fig. 5c. Caspase-3 is a key biochemical apoptosis marker. The results

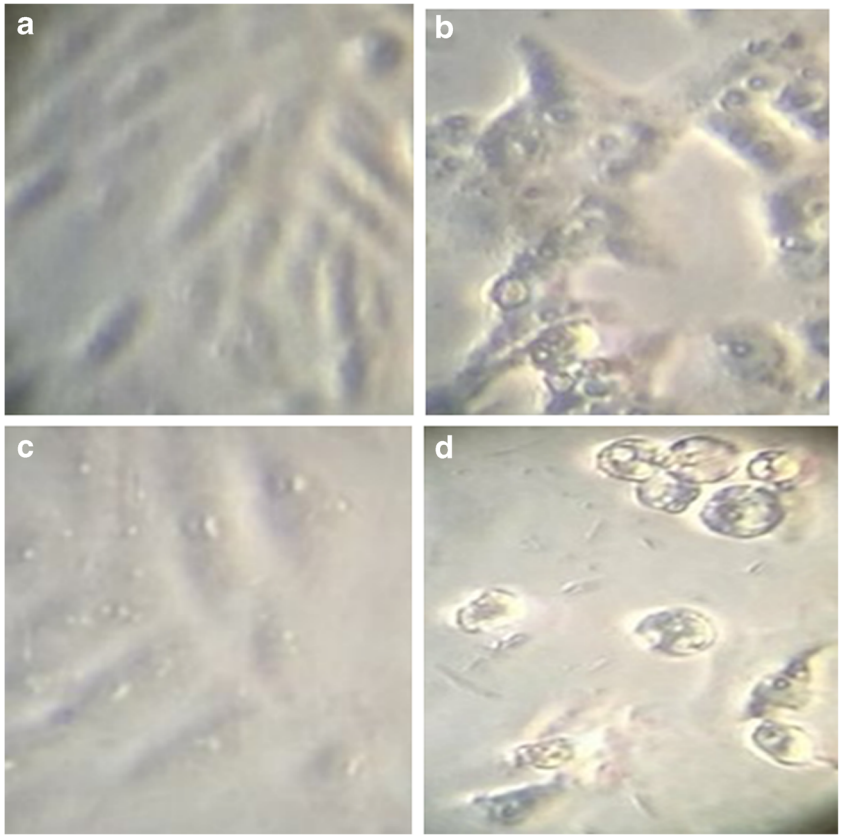

Fig. 3 Effects of CXB and/or CUR on the morphological changes in HepG2 cells. **a** Represents the control normal HepG2 cells which showed a typical polygonal and intact appearance under light microscope. Treatment of cells with CXB IC_{30} (**b**), CUR IC_{50} (**c**) or a combination of CXB and CUR (**d**) induced morphological changes characteristic of cell death, particularly in (**d**) where the cells appeared spares, and detached from the surface of the plates. The cells lost their usual morphology and looked smaller in size, rounded, and shrunken (characteristic of apoptosis)

showed that the combination of IC_{30} of CXB with IC_{50} of CUR led to increased caspase-3 activation in HepG2 cells by 42% compared to CXB-alone group.

Effects of CXB and/or CUR on the levels of Akt, NF-κB, PGE2, and MDA

The effects of combined drugs on the tumor proliferation signaling pathway of the Akt/NF-κB/PGE2/ROS axis were investigated. The levels of phospho-Akt, phosphorylated NF-κB, PGE2, and MDA were all reduced by CUR or CXB compared to the control group. It was found that treatment with the combination of IC_{30} of CXB and IC_{50} of CUR resulted in a greater reduction in Akt, NF-κB, PGE2, and MDA than what was observed by either agent alone (Fig. 4a–d). The addition of CUR to CXB decreased the levels of p-Akt, NF-κBp65, PGE2, and MDA by about 40, 29, 37, and 47%, respectively, compared to CXB-alone group, in HepG2 cells.

Effects of CXB and/or CUR on CD1, VEGF levels, and caspase-3 activity

The effects of combined drugs on the levels of effector tumor markers of proliferation (CD1), angiogenesis (VEGF), or apoptosis (active caspase-3) were assessed. As shown in Fig. 5, the results showed a significant decrease in the levels of CD1 and VEGF by CUR or CXB compared to the control group. Also, the addition of CUR to CXB decreased CD1 and VEGF levels by 38 and 43%, respectively, in HepG2 cells, as compared to CXB-alone group. Moreover, the results showed that the combination of CXB with of CUR led to increased caspase-3 activation in HepG2 cells by 42% compared to CXB-alone group.

Discussion

Combination therapy may take advantage of synergistic inhibitory effects against cancer, as well as, reduced toxicity associated with CXB. In this context, several combinations of CXB with several chemotherapeutic compounds have been investigated (Cervello et al. 2013; Cusimano et al. 2007; Narayanan et al. 2003). Similarly, CUR has been demonstrated to possess antiproliferative, pro-apoptotic, and antioxidative properties against a diversity of organ cancers including the liver (Cao et al. 2007). For that reason, the development of a treatment that comprises a safe phytochemical in combination with a safer low dose of CXB seems highly promising. Mechanistically, both CUR and CXB inhibit COX-2 and PGE2 (Lev-Ari et al. 2005a; Yoysungnoen et al. 2006). Interestingly, numerous non-COX-2 pathways are also targeted by both drugs, such as Akt (Kulp et al. 2004), NF-κB

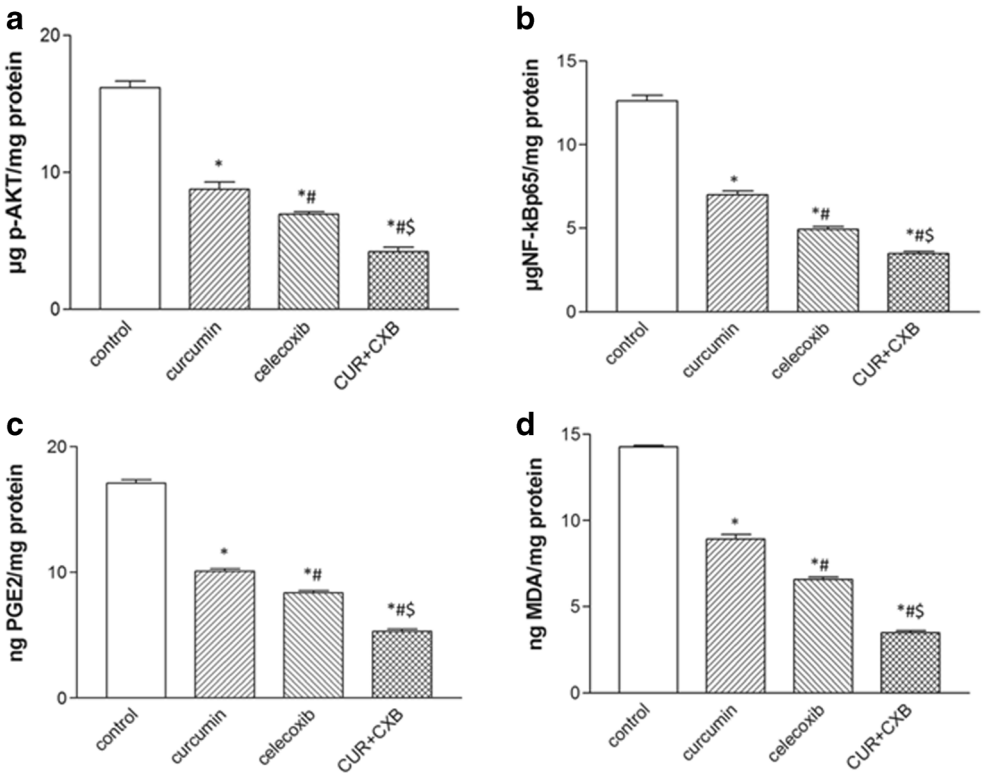

Fig. 4 Effects of CXB and/or CUR on Akt/NF-κB/PGE2/ROS axis in HepG2 cells. Cells were treated with CXB IC_{30} and/or CUR IC_{50} for 72 h. The levels of p-Akt (**a**), NF-κBp65 (**b**), PGE2 (**c**), and MDA (**d**) were measured using different ELISA techniques as described in "Materials and Methods" section. Columns represent the means ± standard error of mean (SEM) of three samples each performed in triplicate. Statistically significant differences between groups are designated as $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. curcumin (CUR) and $^{\$}p < 0.05$ vs. celecoxib (CXB) group. Statistical analysis was performed using ANOVA followed by the Tukey post hoc test. HepG2 human liver-derived hepatoma G2, NF-κB nuclear factor kappa B, PGE2 prostaglandin E2, ROS reactive oxygen species measured as malondialdehyde (MDA)

(Shishodia et al. 2003), oxidative stress (Lev-Ari et al. 2005a), CD1 (Liu et al. 2011), VEGF (Liu et al. 2011; Morisaki et al. 2013; Zhang et al. 2018), and apoptosis (Maeda and Omata 2008; Park and Hong 2016). Consequently, the present study aimed to investigate the anticancer effects and underlying mechanisms of the promising combination of CUR and CXB on HepG2 cells. Aiming to keep the anticancer effect of CXB with minimal toxicity profile, a minimal effective

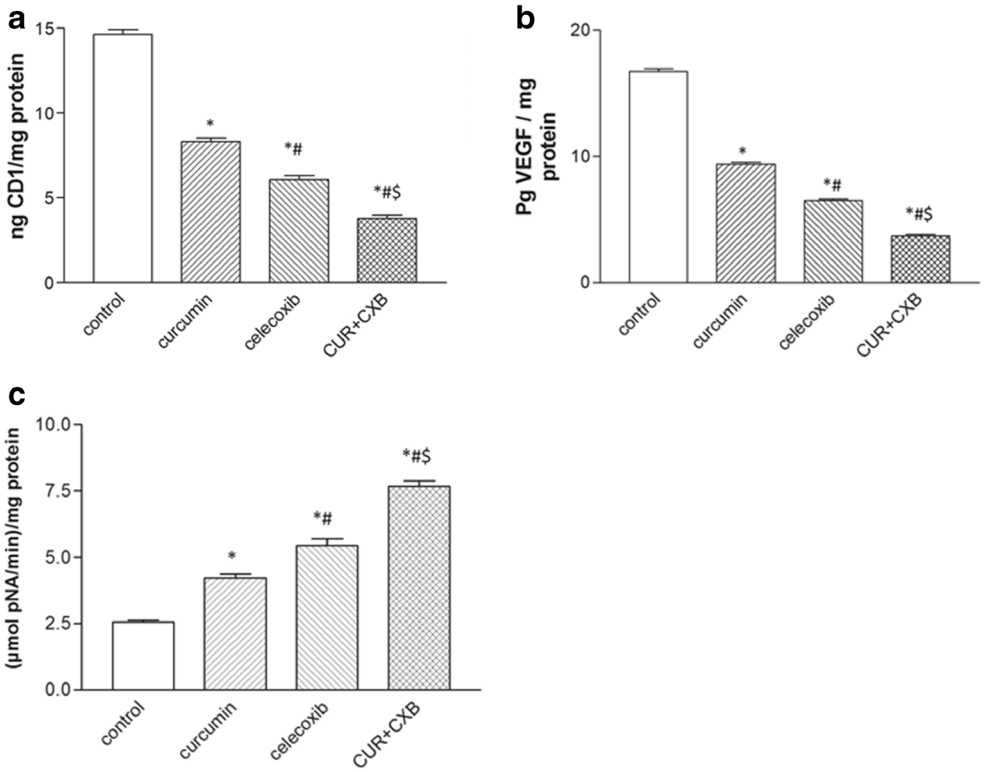

Fig. 5 Effects of CXB and/or CUR on markers of proliferation, angiogenesis, or apoptosis in HepG2 cells. The levels of effector tumor markers of proliferation (cyclin D1; CD1) (**a**), angiogenesis (vascular endothelial growth factor; VEGF) (**b**), or apoptosis (active caspase-3) (**c**) were measured using ELISA technique or colorimetrically as described in "Materials and Methods" section. Columns represent the means ± standard error of mean (SEM) of three samples each performed in triplicate. Statistically significant differences between groups are designated as $^{*}p < 0.05$ vs. control, $\#p < 0.05$ vs. curcumin (CUR) and $\$p < 0.05$ vs. celecoxib (CXB) group. Statistical analysis was performed using ANOVA followed by the Tukey post hoc test

concentration (IC_{30}) was combined with CUR at its half maximal inhibitory concentration (IC_{50}), keeping in mind that CUR showed a good safety profile (Cheng et al. 2001; Cheng et al. 2010; Wang 2012).

The present results showed that CUR inhibited HepG2 cells proliferation dose-dependently (IC_{50} 13 μM), in parallel with previous reports (Fan et al. 2014). CXB also reduced HepG2 cells viability in a dose-dependent manner (IC_{50} 89.9 μM; IC_{30} 42.5 μM), in line with previous reports (Cervello et al. 2013; Cui et al. 2005; Cusimano et al. 2007; Naruse et al. 2002). In addition, an increase of growth inhibition was observed for the combination of CUR and CXB, as previously documented for other combinations in different cancer cell lines (Banerjee et al. 2007; Gong et al. 2004; Narayanan et al. 2003; Somers-Edgar et al. 2008). When the IC_{30} of CXB was combined with the IC_{50} of CUR, the results showed enhanced cytotoxicity and apoptosis in HepG2 cells (Figs. 1, 2, 3, and 5C). Also, the molecular signaling pathways in HepG2 cells were significantly affected by the addition of CUR to CXB. The results showed greater reduction in p-Akt, NF-κB, PGE2, MDA, CD1, and VEGF, than with either agent alone (Figs. 4 and 5).

The present research was actually based on the previous studies of the combination of CXB and CUR in colorectal and pancreatic cancer cells, so as to perform more detailed pharmacologic, drug interaction, and mechanistic insights into HepG2 HCC cell lines. Previous studies focused only on COX-2-dependent pathway (Lev-Ari et al. 2005a; Lev-Ari et al. 2005b). They proved that the combination of CXB (5–25 μM) and CUR (10–15 μM) resulted in several-fold reduction in cell number. When they studied the colorectal HT-29 and pancreatic P-34 cell lines, the celecoxib IC_{30} was 25 and 10 μM in cancer cells of the pancreas and colon, respectively. When combined with CUR, it resulted in about 80% growth inhibitory effect in both cancer cells (Lev-Ari et al. 2005a; Lev-Ari et al. 2005b). In the present study, the IC_{30} of CXB was found to be 42.5 μM. These differences in the results may be due to the difference in the cell lines used. We studied HCC cell lines (HepG2) while Levi-Ari et al. studied the pancreatic P-34 cell lines with high COX-2 expression (Lev-Ari et al. 2005b) and the colorectal cancer cells (HT29) which are more sensitive for CXB effect as they were showing higher COX-2 expression than HepG2 cells when previously compared together (Bae et al. 2001). This reason is clear when Levi-Ari et al. themselves studied the effect of different concentrations of CXB and CUR combinations on different cell lines showing different sensitivities towards concentrations of either single or combined drugs (Lev-Ari et al. 2005a; Lev-Ari et al. 2005b). Moreover, the present research was based on the recommendations of Levi-Ari et al. for additional experiments needed for the identification of the precise synergistic (CI) and underlying non-COX-2 mechanisms that may be targeted by CUR and CXB, such as, Akt, NF-κB, CD1, VEGF, caspase-3, and ROS.

In the present results, the CI values were < 1 for most of the concentrations tested (Table 1), indicating a synergistic effect between CXB and CUR in HepG2 cells, particularly at low concentrations < IC_{50}, while in high concentrations, CI values showed additive to slight antagonistic effect between CUR and CXB in HepG2 cells. Synergistic drug combination is able to reduce the dose of the drug used, thereby maintaining safety with efficacy. In our synergy experiments, the DRI values showed the great benefits of adding CUR to CXB, particularly at low doses which allow us to avoid the toxicity of CXB to great extent (Table 1). Based on these results, the CXB IC_{30} (42.5 μM) and CUR IC_{50} (13 μM) were selected to study the subsequent biomarkers as it was found a higher synergism using the (IC_{30} 42.5 μM) of CXB than combining with its higher (IC_{50} 89.9 μM). In addition, there is a great advantage of reducing the CXB dose to prevent the serious side effects of large doses of CXB in contrast to CUR which is known to be safe even at large doses (IC_{50} 13 μM). These concentrations showed higher synergism with better CXB DRI lying between 3.8 and 5.1 (Table 1), and this is complying more with our approach to reduce CXB dose to decrease its side effects as much as possible. These findings agreed with the previous studies proving that CUR synergistically potentiated the inhibitory effect of CXB on cell growth in P-34 cells (Lev-Ari et al. 2005b). On the other hand, CXB showed antagonistic antiproliferative effects when combined with cisplatin in ovarian and colon cancer cell lines (Bijman et al. 2008). This difference may be due to the difference in the cell lines studied. El-Awady et al. reported an interaction of CXB with different anticancer agents as antagonistic in breast cell lines but not in cells of other cancers (El-Awady et al. 2011). The effect of the used cells on the studied drugs was proved by Lim et al. who showed that CXB attenuated the cytotoxic effect of 5-FU in human colon cancer cells proving that CXB may act through blocking of cell cycle advancement, and stopping apoptosis induced by the drug (Lim et al. 2007). Conversely, and while studying the effect of the same drugs by Bassiouny et al., a synergism of CXB on drug-induced apoptosis was observed in HCC patients (Bassiouny et al. 2010).

Non-COX hypotheses are supported by the fact that antitumor effectiveness has almost been attributed to concentrations of COXIBs usually exceeding clinical margin (5 μM) (Davies et al. 2000; Gong et al. 2012). Moreover, previous reports demonstrate that COX-2-deficient cells underwent apoptosis when exposed to CXB at concentrations of 50–100 μM (Hawk et al. 2002). Based on that, the selected CXB dose was decreased to its IC_{30} (42.5 μM) to be combined with the IC_{50} of CUR (Figs. 1 and 2) to investigate the combination effects on HepG2 cell death signaling pathways. HepG2 cells treated with this combination showed an increase in apoptosis, in which the cells lost their usual morphology, and looked smaller in size, rounded and shrunken, compared to use of either agent alone (Fig. 3), as was observed in similar situations (Cheng et al. 2010; Du et al. 2013; Ghoneim and Eldahshan 2012; Khalil

et al. 2015). These progressive cytoplasmic shrinkage and nuclear condensation as typical morphologic signs of apoptosis were previously established by Kerr et al. (Kerr et al. 1972). Biochemically, caspase-3 is a key apoptosis marker (Cui et al. 2005; Du et al. 2013; Ghoneim 2009; Khalil et al. 2015; Sato et al. 2011). It was found a rise in caspase-3 activity in HepG2 cells treated with CUR and CXB combination rather than either CUR or CXB alone (Fig. 5c). This indicated that CUR potentiated the apoptotic effect of CXB leading to an increase in apoptosis in line with studies in colorectal cancer cell lines (Lev-Ari et al. 2005a).

Concerning the signaling pathway of the Akt/NF-κB/PGE2/ROS axis, it is known that Akt activation is documented to be involved in COX-2-mediated HCC growth (Leng et al. 2003). The present results (Fig. 4a) agreed with studies of CXB effects on Akt phosphorylation in HCC cells alone and in combination with sorafenib (Morisaki et al. 2013; Sui et al. 2014). CXB, in turn, showed anticancer activity in different cancer cells by impeding the NF-κB pathway (Huang et al. 2010; Vaish et al. 2010), as well as, CUR (De Porras et al. 2016; Sato et al. 2011). In the present study, the levels of NF-κB p65 and MDA revealed (Fig. 4b, d) a greater reduction by CUR and CXB combination. CUR, in turn, scavenges oxygen radicals that represent a key player in carcinogenesis (FUJISAWA et al. 2004; Ghoneim 2009; Patial et al. 2015). Studies have also proven the significant inhibition of lipid peroxidation (LPO) produced by CXB (Ekor et al. 2013). These findings suggested that CUR potentiated the antioxidant effect of CXB. Nevertheless, this seemed to be different from a previous study on CXB and CUR (Lev-Ari et al. 2005a), which showed that synergism may be due to that MDA is a by-product of prostaglandin breakdown (VanderVeen et al. 2003). Regarding COX-dependent HepG2 cell death signaling pathways, the PGE2 levels showed (Fig. 4c) a significant decrease by combined drugs. That is comparable to the CXB and CUR combination effect in colorectal cancer cells (Lev-Ari et al. 2005a) and in pancreatic adenocarcinoma cells (Lev-Ari et al. 2005b).

Moreover, the present study investigated the effects of CXB and CUR on HepG2 cell markers of proliferation (CD1) and angiogenesis (VEGF). The levels of CD1 (Fig. 5a) indicated that CUR increased the antiproliferative effect of CXB in HepG2 cells. This was thought to be due to the enhanced effect of the combination on PI3K/Akt pathway, as Akt promotes G1-S phase progression by preventing the phosphorylation and degradation of cyclin D1 (Alao 2007). Furthermore, COX inhibition has been demonstrated to be a potential anti-angiogenic approach in various HCC tumors by inhibiting p-Akt and VEGF (Sui et al. 2014). Evaluating the levels of VEGF (Fig. 5b) gave evidence that CUR increased the anti-angiogenic effect of CXB. In connection with these results, both CUR and CXB have previously been shown to inhibit VEGF in HCC cells including the HepG2 ones (Liu et al. 2011; Morisaki et al. 2013; Zhang et al. 2018).

Previous signaling studies have generally established that Akt pathway is actively involved in the regulation of NFκB essential for oncogenic transformation (Kane et al. 1999). Indeed, inhibitors of Akt interfere with overexpression of NFκB, as previously proven (Bai et al. 2009). Crosstalk and direct relation between p-Akt and NF-κB is already proven in several cancer cells (Oeckinghaus et al. 2011), including HepG2 cells (Hu et al. 2017; Shan et al. 2014). Actually, it is already proven that CXB significantly inhibits the phosphorylation of Akt using Akt inhibitors in HepG2 cells (Leng et al. 2003). Many references built on that and further assessed the direct effects of CXB on p-Akt (Cui et al. 2014; Morisaki et al. 2013; Sui et al. 2014). The same complies also with CUR which is already proven to inhibit the Akt/NF-κB pathway, especially in liver cancer and HepG2 cells (S Darvesh et al. 2012; Shen and Tergaonkar 2009). That is why, many studies also assessed the direct effects of CXB or CUR on Akt/NF-κB pathway in HepG2 cells, particularly (Cusimano et al. 2007; Morisaki et al. 2013; Zhang et al. 2018).

In turn, NF-κB is one of the most important molecular targets in cancer therapy (Shen and Tergaonkar 2009) that is especially overexpressed in HCC (Tai et al. 2000). Additionally, NF-κB activation directly affects molecular hallmarks of cancer through modulating cell proliferation (CD1), angiogenesis (VEGF), inflammation (COX-2/PGE2), apoptosis (caspase-3), and oxidative stress (MDA) (Maeda and Omata 2008; Park and Hong 2016; Xia et al. 2014). Many studies assessed the anticancer effects of different drugs acting on the NF-κB signaling pathways (Oeckinghaus et al. 2011), particularly, in HepG2 cells (Ozaki et al. 2007; Xia et al. 2013; Zhang et al. 2018). In fact, it is already proven that both CXB and CUR have already been proven to be NF-κB inhibitors, especially in liver cancer and HepG2 cells (Cervello et al. 2011; Reuter et al. 2008; S Darvesh et al. 2012; Shen and Tergaonkar 2009; Wyrebska et al. 2014). Based on the above-mentioned facts, and similarly to the present research, many other studies directly assessed the effects of CXB or CUR on the NF-κB, CD1, VEGF, PGE2, and ROS in HepG2 cells, particularly (Liu et al. 2011; Morisaki et al. 2013; Ramyaa and Padma 2014; Zhang et al. 2018).

To our knowledge, evidence was provided for the first time that the combination of CXB (a COXIB) and CUR (a phytochemical) led to synergistic cytotoxic effect in the HepG2 cell line as demonstrated by increased apoptosis and inhibition of proliferation. It appeared also in this context that this combination acted via the signaling pathway of the Akt/NF-κB/PGE2/ROS axis that affects proliferation (CD1) and angiogenesis (VEGF) by induction, and apoptosis (caspase-3) by inhibition. Both CUR and CXB acted at different points in this signaling pathway that affected proliferation, angiogenesis, and apoptosis. This may, at least in part, underlie the synergistic cytotoxic effects of these interacting drugs on the viability of HepG2 cells (Fig. 6; graphical abstract).

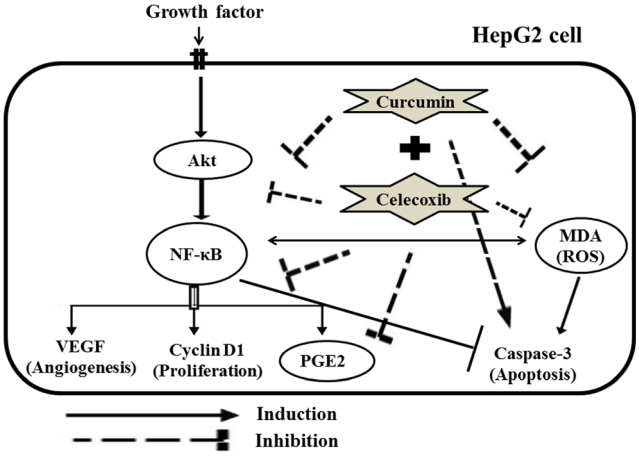

Fig. 6 Proposed underlying mechanisms of the synergistic antiproliferative effects of curcumin and celecoxib on HepG2 cells. Both curcumin and celecoxib act at different points in the signaling pathway of the Akt/NF-κB/PGE2/ROS axis that affects proliferation (CD1) and angiogenesis (VEGF) by induction and apoptosis (caspase-3) by inhibition. This may underlie the synergistic effect of the combined drugs on the viability of HepG2 cells when exposed to this combination. CD1 cyclin D1, HepG2 human liver-derived hepatoma G2, NF-κB nuclear factor kappa B, PGE2 prostaglandin E2, ROS reactive oxygen species measured as malondialdehyde (MDA), VEGF vascular endothelial growth factor

Conclusions

CUR showed synergistic antiproliferative interactions with CXB in HepG2 cells. This might further allow for the use of lower and safer doses of CXB than those currently used though linked to cardiovascular risk. Therefore, this novel liver cancer combination may represent promising adjuvant targeted chemotherapy in treating HCC patients, a finding that needs further clinical investigations. This would better follow similar preclinical research on liver cancer cell lines other than the presently used HepG2 cells.

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Author contribution statement AG, MH, MK, and FA conceived and designed research. FA and MH conducted experiments. FA, MH, MK, and AG contributed materials, technical and analytical tools. FA, MH, and AG analyzed data. AG and FA wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

The current research has followed accepted principles of ethical and professional conduct according to approval reference number (1116PO1) by the Research Ethics Committee of the Faculty of Pharmacy, Damanhour University, regarding originality, risk control, and community service.

Conflict of interest The authors declare that they have no conflicts of interest.

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