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Original Article

Evaluation of Cytotoxicity Effects of Chalcone Epoxide Analogues as a Selective COX-II Inhibitor in the Human Liver Carcinoma Cell Line

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Key Words

cancer, celecoxib, chalcone epoxide, cyclooxygenase 2, prostaglandin E2

Abstract

Objectives: Study of the mechanisms involved in cancer progression suggests that cyclooxygenase enzymes play an important role in the induction of inflammation, tumor formation, and metastasis of cancer cells. Thus, cyclooxygenase enzymes could be considered for cancer chemotherapy. Among these enzymes, cyclooxygenase 2 (COX-2) is associated with liver carcinogenesis. Various COX-2 inhibitors cause growth inhibition of human hepatocellular carcinoma cells, but many of them act in the COX-2 independent mechanism. Thus, the introduction of selective COX-2 inhibitors is necessary to achieve a clear result. The present study was aimed to determine the growth-inhibitory effects of new analogues of chalcone epoxide as selective COX-2 inhibitors on the human hepatocellular carcinoma (HepG2) cell line.

Methods: Estimation of both cell growth and the amount of prostaglandin E2 (PGE2) production were used to study the effect of selective COX-2 inhibitors on the hepatocellular carcinoma cell. Cell growth determination has done by MTT assay in 24 h, 48 h and 72 h, and PGE2 production has estimated by using ELYSA kit in 48 h and 72 h.

Results: The results showed growth inhibition of the HepG2 cell line in a concentration and time-dependent manner, as well as a reduction in the formation of PGE2 as a product of COX-2 activity. Among the compounds those analogues with methoxy and hydrogen group showed more inhibitory effect than others.

Conclusion: The current *in-vitro* study indicates that the observed significant growth-inhibitory effect of chalcone-epoxide analogues on the HepG2 cell line may involve COX-dependent mechanisms and the PGE2 pathway parallel to the effect of celecoxib. It can be said that these analogues might be efficient compounds in chemotherapy of COX-2 dependent carcinoma specially preventing and treatment of hepatocellular carcinomas.

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1. Introduction

A hepatocellular carcinoma (HCC) is considered as one of the most common malignant tumors in the world [1, 2]. The incidence of HCC differs based on strain and geography. It occurs more often in Asia and Africa, but its incidence is increasing in industrial societies such as The United Kingdom and France [3-5]. Based on late diagnosis, therapy for patients with a

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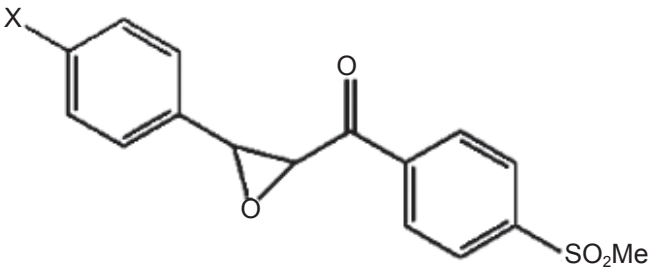
hepatocellular carcinoma is difficult and often involves treatment such as surgery and liver transplantation [6]. Thus, the lack of a definite treatment for patients with a HCC that is not diagnosed early highlights the needs for other therapies such as chemotherapy [7].

Cyclooxygenase isoenzymes (COXs), as restriction enzymes in the prostaglandin production pathway, catalyze the conversion of arachidonic acid into prostaglandins. Two isoforms of COXs have been identified: Cyclooxygenase-1 (COX-1) is expressed continuously while cyclooxygenase-2 (COX-2) is an inducible enzyme that is associated with inflammatory diseases and cancer [7-10]. Increased expression of COX-2 occurs in many malignancies, including colorectal, liver, pancreatic, breast, and lung tumors [11-15]. It seems that COX-2 activity leads to angiogenesis, tumor progression, and resistance to apoptosis [16-18]. Cyclooxygenase enzymes are responsible for the production of eicosanoids, which regulate the different responses of the cells and tissues, such as thrombocytes, renin release and inflammation. Due to their role, eicosanoids are involved in cardiovascular diseases, inflammatory diseases, cancer, and Alzheimer's disease [19-21]. For the first time in the nineteenth century (19th), Rudolf Virchow observed the presence of white blood cells within a tumor and mentioned a possible link between inflammation and cancer [22]. According to the role of COX-2 and this finding that many carcinogenic environmental factors and risk factors are associated with chronic inflammation, recent scientific observation has clearly shown that inflammation plays an essential role in tumor initiation [23].

The use of COX-2 inhibitors has been shown to have good inhibitory effects on many malignant tumors [18, 24-28], although debate continues over whether or not those inhibitory effects are mediated through COX-2 activity and production of prostaglandins. The first selective COX-2 inhibitors that were identified were Dup-697 and NS-398 compounds [29, 30]. These compounds, which have been investigated, along the COX-2 isoenzyme, have shown better selectivity. However, the most selective COX-2 inhibitors frequently belong to a class of diarylheterocycles that possess two vicinal rings attached to a central heterocyclic scaffold associated with a COX-2 pharmacophore, such as a *para*-SO2Me substituent, on one of the rings [31]. Evaluation of the *in-vitro* ability of the synthesized compounds to inhibit the COX-1 and the COX-2 isoenzymes (SAR data) has shown that COX-2's inhibitory potency and selectivity depend on the position of the COX-2 SO_2Me pharmacophore and the type of the *para*-substituent on the C-2 or the C-4 phenyl ring [32]. These results also reveal that incorporation of a methoxy (OMe) substituent at the *para*-position of the C-2 or the C-4 phenyl ring increases the selectivity of COX-2. For the above reasons, in this study, we investigated the growth inhibitory effects of new analogues of chalcone epoxide, as specific COX-2 inhibitors, on the human hepatocellular carcinoma (HepG2) cell line.

2. Materials and Methods

The HepG2 cell line was obtained from Pasteur Institute of Iran. Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum (FBS), and penicillin streptomycin-fungizone were obtained from Gibco BRL. Trypan blue, MTT, and Trypsin-ethylenediaminetetraacetic acid (EDTA) (5x) were obtained from Sigma Chemicals Co. (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was supplied by Merck (Darmstadt, Germany). The new analogues of chalcone epoxide with a chalcone-based structure and SO_2Me attachment, which have a COX-2 inhibitory effect, and celecoxib were kindly provided by the Medicinal Chemistry Department, Shahid Beheshti University of Medical Science (Fig. 1). These compounds, named as D1, D2, D3 and D4, had different substituents attached to the benzene ring. All of compounds and celecoxib were dissolved in DMSO such that the final concentration of DMSO was < 0.1%.



X = OMe, H, NHCOMe and F

Figure 1 Structure of compounds with a chalcone-based structure and a SO_2Me attachment that has OMe (D1), H (D2), NHCOMe (D3) and F (D4) as substituent groups.

The hman HepG2 cell line was cultured in DMEM and supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cultures were passaged at pre-confluent densities by using a solution of 0.25% trypsin and 1-mM EDTA 4Na.

The proliferation status of the human HepG2 cells was determined by using an MTT assay. The cells were seeded in flat-bottomed 96-well microplates. Twelve hours after incubation, the cells were treated with different concentrations of the chalcone-epoxide analogues and with celecoxib. After an incubation of 1, 2, or 3 days, 20 μL of MTT (5 g/L) were added to each well, after which the cells were incubated again for 4 h. The supernatant was then removed, and 150 μL of DMSO were added. The solution was shaken for 5 min until the crystals had dissolved. The value of the optical density at 570 nm (OD570 nm) was measured by using an ELISA reader (ELx808, BioTek, USA). The negative control well had no cells and was used as the zero point of absorbance.

The PGE2 levels in the supernatants from the cultured cells were quantified by using an ELISA reader. Cells from the HepG2 cell line were seeded at 2.0×10^4/well into the wells of microplates and were allowed to adhere overnight. The cells were then incubated with 25 or 50 μM of the D1, D2, D3, or D4 compounds and with celecoxib for 48 and 72 h. The supernatants were then aspirated and centrifuged

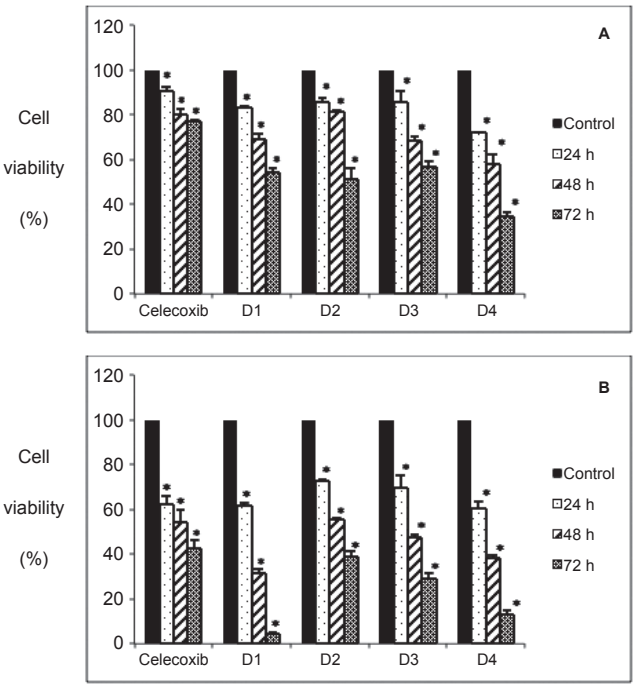


Figure 2 Effect of new analogues of chalcone epoxide on the growth of the HepG2 cell line. Cells were treated with (A) 25 μM and (B) 50 μM of celecoxib and new chalcone-epoxide analogues for 24, 48 and 72 h. The MTT assay was employed to measure the cell viability in both cases. Data are means ± standard errors of six determinations per experiment from three independent experiments ($^{*}P < 0.05$).

HepG2, human hepatocellular carcinoma

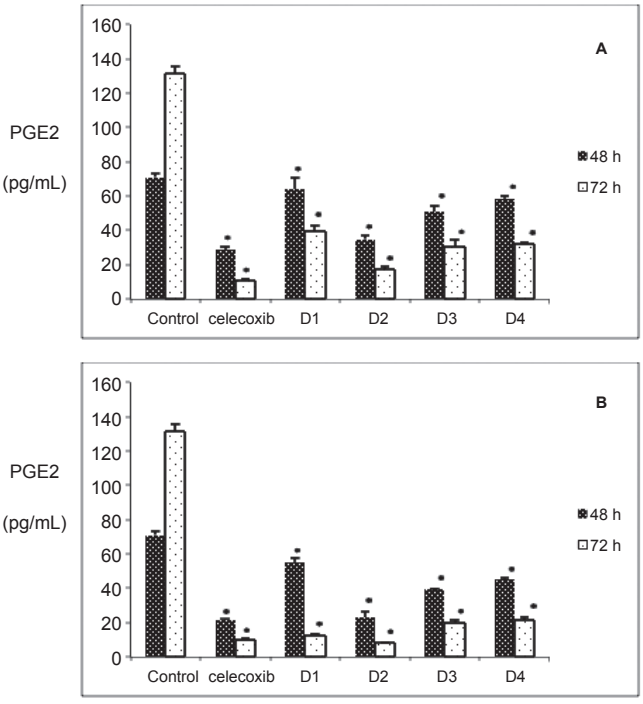


Figure 3 Effect of celecoxib and the new analogues of chalcone epoxide on PGE2 production in the HepG2 cell line. The cells were treated with celecoxib and the new analogues at (A) 25 μM and (B) 50 μM (B) 48 and 72 hours. Media were collected, and PGE2 was measured using the PGE2ELISA Kit. ($^{*}P < 0.05$ compared with the vehicle control).

PGE2, prostaglandin E2; HepG2, human hepatocellular carcinoma.

in preparation to measure the PGE2 by using the PGE2-based kit protocol.

The descriptive statistic was recorded as a mean ± standard deviation (SD). Data were analyzed using ANOVA followed by Tukey´s test as a post-hoc analysis for One-Way ANOVA and repeated measures (Bonferroni) for Two-Way ANOVA. The significance level was set at $P < 0.05$.

3. Results

The aim of the MTT assay was to evaluate cell growth inhibition due to cyclooxygenase-2 inhibition caused by the new analogues of chalcone epoxide. Results are shown in Fig. 2. All compounds at concentrations of 25 and 50 mM for incubation times of 24, 48 and 72 hours showed significant reductions in the growth of cells in the HepG2 cell line compared to the control ($P < 0.05$). In all cases, the reduction in cell growth depended on the time and the concentration so that as the concentration and the treatment time were increased, the cell viability was decreased. The times at which all the compounds were most effective in inhibiting cell growth were 48 and 72 hours, and for all compounds the concentrations of 25 and 50 mM were considered as the most effective doses; these results provide information that will be useful for follow-on experiments.

To evaluate the effect of the chalcone-epoxide analogues on the CoX-2 enzyme activity, we measured the production of prostaglandin E2 (PGE2) by using enzyme immunoassay kits (immunoassay PGE2). The PGE2 levels in the cells from the HepG2 cell line were reduced after 48-h, and especially 72-h, treatment (Fig. 3). Significant reductions in the PGE2 production was observed in all groups and in 48 h and 72 h ($^{*}P < 0.05$) compared to the control ($^{*}P < 0.05$), although the evaluated chalcone-epoxide analogues showed lower inhibitory effects than celecoxib. Significant reductions in the PGE1 production.

4. Discussion

For many years, cancer, which is one of the common causes of death among humans, has been suggested to be induced by different chemical and physical factors. Liver carcinomas, which are usually diagnosed late and have no definite treatment, are the fifth most common cancer and

the third cause of deaths due to cancer. Among the various mechanisms that can induce cancer, cyclooxygenase enzymes are the therapeutic targets of many drugs due to their involvements in various stages of cancer onset and progression. The role of the cyclooxygenase enzymes in carcinogenesis is characterized by their increased expressions in tumor formations [7, 10, 33]. Furthermore, previous studies showed an association between COX and carcinogenesis in the liver [12, 34, 35]. According to recent reports, nonsteroidal anti-inflammatory drugs (NSAIDs), including selective and nonselective inhibitors of COX-2, had significant growth inhibition effects on a small number of liver carcinoma cells [36, 37]. The NSAIDs also have important roles in the molecular pathways of cell growth in colon carcinomas [38]. The NSAIDs exert their effects via different mechanisms, such as regulation of the signal transmission pathway *via* Ras proteins, activation of the mitogen-activated protein kinase and nuclear factor κB, enablement of the sphingomyelin/ceramide pathway, expression of cyclin, and mutation of P53 [38-41]. This study showed cell growth inhibition in a time- and dose-dependent manner when new specific COX-2 inhibitors, called chalcone-epoxide' analogues, were introduced to the cells in a liver carcinoma cell line.

Park and colleagues demonstrated that inhibition of COX-2 by NS-398 led to time- and dose-dependent inhibition of cell growth in the HepG2 cell line; it also reduced the percentage of cells in the S phase of HepG2 cell cycle and increased the percentage in the G0-G1 phase [42]. Hillebrand *et al* also reported a time-dependent inhibition of cell growth that was due to a reduction in the production of PGE2, induction of apoptosis, and changes in the G1-S phase of the cell cycle in a liver cancer cell line treated with selective COX-2- inhibitors [35]. However, in another study, cell growth inhibition and induction of apoptosis were found to be independent of the expression of COX-2, indicating that COX-2-independent mechanisms may also be involved [43].

In this study, the chalcone-epoxide analogues had effects on cell growth inhibition similar to those of celecoxib. These growth inhibitory effects probably occur through the inhibition of PGE2 production, a process that depends on COX-2 activity. Among the studied analogues, the D2 compound, especially at high concentration, showed the greatest inhibitory effect on PGE2 production. However, the D1 compound induced more cell growth inhibition compared to the other compounds. The decrease in PGE2 production in a time- and dose-dependent manner confirmed the COX-2 inhibitory effects of these compounds. Overall, the chalcone-epoxide analogues exerted greater inhibitions of cell growth than of PGE2 production. These results demonstrate that one of the mechanisms involved in cell growth inhibition may work through COX-2 inhibition, as well as other ways. A comparison of the total effects of these chalcone-epoxide analogues with those of celecoxib demonstrated that they had acceptable efficacies comparable to that of celecoxib. According to another report, a reduced cell growth, a change in the cell cycle process, and non-induction of apoptosis after treatment with inhibitors of COX-2 demonstrate the roles of other mechanisms, apart from COX, in cell-growth inhibition [24]. One can assume that the lower inhibition of PGE2 production compared to that of cell growth might be due to COX-2-independent pathways that are involved in the regulation of cell growth.

5. Conclusion

The results of this study showed that the use of chalcone-epoxide analogues as selective COX-2 inhibitors may be an effective method to prevent and treat hepatocellular carcinomas. The compounds D2 and D1 showed greater cell growth inhibition and decreased PG2 production, respectively, compared to the other compounds. Due to the effects of the tested compounds, one may hypothesize that the presence of groups, such as methoxy and hydrogen groups, in the structures of chalcone-epoxide analogues may contribute to their anti-cancer properties. However, future studies show focus on an analysis of these properties, especially the ability to create hydrogen bonds.

Conflict of interest

The authors declare that there are no conflicts of interest.

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