A Study on the DNA Cleavage, Morphological Changes and Cytotoxicity Activities of the Two Synthetic COX-2 Inhibitors

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ABSTRACT

Celecoxib, a specific cyclooxygenase-2 (COX-2) inhibitor, has been proven to possess antitumor activity in a variety of cancer cells. However, the antitumor activity of two synthesized COX-2 inhibitor derivatives (A and B) on human myeloid leukemia (K562) and breast adenocarcinoma (MCF-7) cancer cells has not been well established. The present study is aimed to investigate the morphological changes of cells treated with compounds A and B as well as DNA cleavage activity of these compounds. The DNA cleavage experiments were performed by agarose gel electrophoresis. Plasmid pTZ57 DNA was treated with the compounds A and B at various concentrations. Then, bands visualized by UV light and photographed to determine the extent of cleavage of the supercoiled (SC) to Nicked (NC) DNA. Furthermore, the apoptotic activities of the two compounds were assessed using cells treated with DAPI staining method. The results obtained from DNA cleavage assay demonstrated that with the increasing concentration of compound A, SC DNA is gradually converted to NC DNA. Cells were also exposed to various concentrations (0.1-100 μM) of each compound for 24 h. Two compounds demonstrated the cytotoxic effects on MCF-7 and K562 cell lines in a concentration-dependent manner with the IC50 values ranging from 6.5 to 22.23 μM. Treatment of the cells with compounds A and B significantly cause morphological changes after 16 h. Collectively, our data indicate that compounds A and B as two COX-2 inhibitor derivatives may present promising chemotherapeutic activities, possibly targeting DNA and inducing cell death in the selected cancer cell line which needs further research.
Introduction
Non-steroidal anti-inflammatory drugs (NSAIDs) are therapeutic groups widely used for the treatment of pain, inflammation and fever [1]. Modifications of the established NSAIDs have been strategies for the design of cyclooxygenase-2 (COX-2) selective inhibitors [2]. Many lead compounds reported to have selective COX-2 inhibitory activity have been clinically introduced to reduce inflammation with very little gastrointestinal (GI) side effects. [3] There is also growing experimental and clinical evidence indicating COX-2 inhibitors have anti-cancer activity and reduce the incidence and mortality of several types of human cancer [1, 4]. Celecoxib, a specific cyclooxygenase-2 (COX-2) inhibitor, is widely prescribed under the trade name Celebrex for relief of symptoms of osteoarthritis and rheumatoid arthritis and was also approved as an adjunct to standard care for patients with familial adenomatous polyposis [5]. Several studies demonstrated that celecoxib suppressed the proliferation of various cells by inducing apoptosis, suggesting that the pro-apoptotic action of celecoxib may be useful in the chemoprevention of tumorigenesis [6,7]. Celecoxib has a 1,2-di-aryl heterocyclic structure and should be an ideal lead compound for developing novel derivatives with more potent apoptosis-inducing activity [8,9]. Previous study performed by Gauthier et al. showed that human recombinant COX-2 has been also inhibited by 1, 3-diaryl heterocyclic compounds suggested enhancing the selectivity through some structural modifications. Their model also proposed an additional aryl group adjoin to central heterocycle ring to increase the interaction between compounds and COX-2 active site [10]. Therefore, triaryl heterocycles have been introduced as more potent COX-2 inhibitors which better fit in COX-2 active site and showed a wide range of activities such as cytotoxic effects [11]. Considering all of data, it was our interest to investigate the cytotoxic effects as well as the DNA cleavage of two synthesized COX-2 inhibitors derivatives on human leukemia (K562) and breast cancer (MCF-7) cell lines.

Materials and Methods
Chemicals and reagents
Dulbecco’s Modified Eagle’s Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, IN, USA). Agarose (molecular biology grade) was obtained from Sigma–Aldrich and stored at 4 °C. Tris(hydroxymethyl)-aminomethane (Tris) buffer and ethidium bromide (3,8-diamino-5-ethyl-6 phenylphenanthridinium bromide, EthBr) were of analytical reagent grade and obtained from Merck (Darmstadt, Germany). All other chemicals were in high purity and prepared by Merck.

Chemistry
Two COX-2 inhibitor derivatives, 3-(4-chlorophenyl)-5-(4-fluorophenyl)-4-phenyl-4,5-dihydro-1,2,4-oxadiazole (A) and 3,5-bis(4-chlorophenyl)-4-phenyl-4,5-dihydro-1,2,4-oxadiazole (B) (Fig.1), were synthesized, purified and fully characterized according to our previous works [7].

Cell culture and in vitro cytotoxicity assay
Human breast adenocarcinoma (MCF-7) and erythroblastoid leukemia (K562) cell lines were obtained from the cell bank of Pasteur Institute of Iran (NCBI), and routinely cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin under the conditions of 5% CO2 at 37°C. Cell cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MCF-7 (7 × 10^3) and K562 (2 × 10^4) cells/well were cultured in 96-well plates. Then, cells were treated with various concentrations of each compound (0.1–100 µM) and incubated for 24 h, afterwards 20 µL of MTT (5mg/mL in PBS) was added to each well and the cells were incubated for another 4 h at 37 °C. The supernatants were then aspirated carefully and 200 µL of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for an additional 15 min and the absorbance values were read by the Microplate Reader (StarFax-2100, ST. Louis, USA) at 545 nm. Solvent control (DMSO) was included to check that...
the DMSO had no effect at the concentration used. The cytotoxicity of the complex was measured from the spectrophotometric data by means of this equation:

\[
\% \text{ cell cytotoxicity} = \left[1 - \frac{\text{Abs}_{\text{drug}}}{\text{Abs}_{\text{control}}}\right] \times 100.
\]

The IC_{50} value was measured by plotting the percentage cytotoxicity versus concentration on a logarithmic graph. To compare the cytotoxic effect of compounds reported here with that of the reference drugs, we also examined the cytotoxicity of doxorubicin, paclitaxel and celecoxib on MCF-7 and K562 cells under similar conditions.

**DNA cleavage**

The DNA cleavage experiments were performed by agarose gel electrophoresis, which was done by incubation at 37°C as follows: pTZ57 plasmid DNA (100 ng/µL) in 50 mM Tris–HCl/NaCl buffer (pH=7.2) was treated with compounds A and B at various concentrations (5-200µM). The samples were incubated for 3 h, and loading buffer was added. Then the samples were electrophoresed for 1 h and 20 min at 135 V on 0.7% agarose gel using Tris–boric acid–EDTA buffer. After electrophoresis, bands were visualized by UV light and photographed. The extent of cleavage of the supercoiled (SC) DNA was determined by measuring the intensities of the bands using Image J software.

**Effect of complexes on cell morphology**

The MCF-7 and K562 cells were incubated with compounds A and B at their IC_{50} concentrations for 16 h. The treated and negative-control cells were stained by DAPI (Diamidine phenyl dihydrochloride, Roche Applied Science, Indianapolis, USA) and their morphology was observed under a Zeiss Fluorescence microscope (Zeiss, Germany) and photomicrographs were taken with an Olympus digital camera (Tokyo, Japan).

**Results**

**Complexes inhibits cancer cell growth in vitro**

To investigate the anti-cancer effects of compounds A and B in vitro, we examined the inhibitory effect of these compounds on the growth of MCF-7 and K562 cell lines using MTT assay. Both compounds demonstrated remarkable cytotoxic effect on MCF-7 and K562 cells in a concentration-dependent manner [7] with IC_{50} values ranging between 6.5–22.23 µM (Table 1). Celecoxib was used as a selective COX-2 inhibitor. Doxorubicine and Paclitaxel were used as reference cytotoxic drugs.

| Table 1. IC_{50} values (µM) for antiproliferative activity of compounds A and B towards MCF-7 and K562 cells. |
| Compounds | MCF-7 | K562 |
| A | 6.50 (5.4-7.9) | 21.66 (15.5-30.3) |
| B | 10.10 (6.8-14.9) | 22.23 (18.5-26.7) |
| Celecoxib | 0.4 (0.15-0.9) | 2.3 (1.6-3.4) |
| Doxorubicin | > 100 | 4.49 (2.16-9.33) |
| Paclitaxel | 1.86 (0.87-3.98) | > 100 |

**DNA cleavage activity**

The interaction of plasmid with compounds A and B was studied using pTZ57 plasmid DNA as a substrate in the medium of 50 mM Tris–HCl/NaCl buffer (pH=7.2) in the absence and presence of external agents under physiological conditions. This intention was carried out by detecting the transition from the naturally occurring, covalently closed circular form (Form I) to the nicked circular relaxed form (Form II) and linear form (Form III) by means of gel electrophoresis of the plasmid. When DNA plasmid is conducted by electrophoresis, the fastest migration will be observed for the supercoiled form (Form I). If one strand is cleaved, the supercoiled will relax to create a slower-moving nicked circular form (Form II). If both strands are cleaved, a linear form (Form III) will be produced which migrates in between Form I and Form II [12]. As shown in Fig. 2a with the increasing concentrations of compound A, SC DNA (Form I) is gradually converted to NC DNA (Form II) at a concentration of 5 (1%), 10 (1%), 30 (1%), 60 (1%), 80 (1%), 100 (1%) and 200 µM (32%) (Fig 2a; Lane 1-8). However, compound B demonstrates no significant effect on the cleavage of SC DNA (Fig 2b; Lanes 1–8). The mechanistic studies using various additives indicate the oxidative nature of the cleavage by compound A. DNA cleavage was investigated by gel electrophoresis in the presence of various radical scavengers such as NaN_{3}, SOD (superoxide dismutase) and DMSO (Fig 2a, Lane 9-14). The DNA cleavage of the plasmid was inhibited significantly in the presence of NaN_{3} suggesting
that \( ^1\text{O}_2 \) (siglet oxygen) is likely to be the reactive species responsible for the nuclease activity (Lane 9, 11). SOD also inhibited the DNA cleavage, however not as much as NaN\(_3\) (Lane 13, 14). When hydroxyl radical scavenger, DMSO was added to the reaction mixture, it was found to decrease the nuclease activity slightly which was indicative of the weak involvement of OH\(^-\) radicals in the cleavage process (Lane 10, 12).

![Gel electrophoresis diagrams showing the cleavage of pEGFP-N1 DNA (0.1 µg/µL) at different concentrations of compounds A and B in Tris–HCl/NaCl buffer (pH=7.2) at 37 °C for 3 h. a) Lane 1: DNA control; Lane 2: DNA + A (5 µM); Lane 3: DNA + A(10 µM); Lane 4: DNA + A (30 µM); Lane 5: DNA + A (60 µM); Lane 6: DNA + A (80 µM); Lane 7: DNA + A (100 µM); Lane 8: DNA + A (200 µM); Lane 9: DNA + A (100 µM) + NaN\(_3\) (400µM); Lane 10: DNA + A (100 µM) + DMSO(400µM); Lane 11: DNA + A (200 µM) + NaN\(_3\)(400µM); Lane 12: DNA + A (200 µM) + DMSO(400µM); Lane 13: DNA + A (100 µM) + SOD(15U); Lane 14: DNA + A (200 µM) + SOD(15U). b) Lane 1: DNA control; Lane 2: DNA + B (5 µM); Lane 3: DNA + B (10 µM); Lane 4: DNA + B (30 µM); Lane 5: DNA + B (60 µM); Lane 6: DNA + B (80 µM); Lane 7: DNA + B (100 µM), Lane 8: DNA + B (200 µM).]

**Fig. 2.** Gel electrophoresis diagrams showing the cleavage of pEGFP-N1 DNA (0.1 µg/µL) at different concentrations of compounds A and B in Tris–HCl/NaCl buffer (pH=7.2) at 37 °C for 3 h. a) Lane 1: DNA control; Lane 2: DNA + A (5 µM); Lane 3: DNA + A(10 µM); Lane 4: DNA + A (30 µM); Lane 5: DNA + A (60 µM); Lane 6: DNA + A (80 µM); Lane 7: DNA + A (100 µM); Lane 8: DNA + A (200 µM); Lane 9: DNA + A (100 µM) + NaN\(_3\) (400µM); Lane 10: DNA + A (100 µM) + DMSO(400µM); Lane 11: DNA + A (200 µM) + NaN\(_3\)(400µM); Lane 12: DNA + A (200 µM) + DMSO(400µM); Lane 13: DNA + A (100 µM) + SOD(15U); Lane 14: DNA + A (200 µM) + SOD(15U). b) Lane 1: DNA control; Lane 2: DNA + B (5 µM); Lane 3: DNA + B (10 µM); Lane 4: DNA + B (30 µM); Lane 5: DNA + B (60 µM); Lane 6: DNA + B (80 µM); Lane 7: DNA + B (100 µM), Lane 8: DNA + B (200 µM).

**Apoptosis detection in MCF-7 and K562 cells through DAPI dye staining**

The ability of compounds A and B to induce apoptosis was evaluated on MCF-7 and K562 cells stained with DAPI after 8 and 16 h incubation. Compared with the untreated cells, the majority of the compound A and B-treated MCF-7 and K562 cells indicated considerable chromatin condensation after 16 h (Fig. 3; 1g, 1h and 2g, 2h). These morphological changes demonstrate cell damage after both compounds treatments for 16 h. In addition, nominal changes observed after 8 h incubation of compound A with K562 and MCF-7 cells (Fig.3, 1c, 2c).
A study on the DNA cleavage, morphological changes and cytotoxicity activities of …

Fig. 3. Apoptotic morphological changes in MCF-7 and K562 cells.(1a) MCF-7 Control cells; (1b) MCF-7 Cells treated with DMSO for 8 h; (1c) MCF-7 Cells treated with compound A at 6.5 µM for 8 h; (1d) MCF-7 Cells treated with compound B at 10.1 µM for 8 h; (1e) MCF-7 Control cells; (1f) MCF-7 Cells treated with DMSO for 16 h; (1g) MCF-7 Cells treated with compound A at 6.5 µM for 16 h; (1h) MCF-7 Cells treated with compound B at 10.1 µM for 16 h. (2a) K562 Control cells; (2b) K562 Cells treated with DMSO for 8 h; (2c) K562 Cells treated with compound A at 21.66 µM for 8 h; (2d) K562 Cells treated with compound B at 22.23 µM for 8 h; (2e) K562 Control cells; (2f) K562 Cells treated with DMSO for 16 h; (2g) K562 Cells treated with compound A at 21.66 µM for 16 h; (2h) K562 Cells treated with compound B at 22.23 µM for 16 h. Cells were stained by DAPI and observed under Zeiss fluorescent 100 × 10 microscopy.

Discussion

Celecoxib is a selective COX-2 inhibitor that was developed to diminish the incidence of gastrointestinal side effects associated with non-selective long-term inhibition of COX-1 and COX-2 by traditional NSAIDs (Non-steroid Anti-inflammatory Drugs). There is accumulating experimental evidence that celecoxib and its derivatives have a protective effect in the development of certain malignancies such as leukemia and breast cancer.\[13, 14\] Celecoxib also reduces cell proliferation and initiates apoptosis in different types of cancer cells \textit{in vitro} \[15\]. We recently reported that two COX-2 inhibitor derivatives (A, B) showed significant biological features such as cytotoxicity activity (Table 1) \[7\]. On the basis of these observations, in this study we have examined the morphological changes as well as DNA cleavage of the two COX-2 inhibitor compounds, A and B on chronic myeloid leukemia, K562 and breast adenocarcinoma, MCF-7 cell lines. To confirm that prominent antiproliferative effects of two compounds is due to induction of apoptosis, the morphological changes of MCF-7 and K562 cells treated with compounds A and B at their IC\textsubscript{50} concentrations for 8 and 16 h were evaluated using fluorescence microscopy. The morphological changes of apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, and formation of apoptotic bodies. \[16\] DAPI staining of K562 and MCF-7 cells treated with the compounds for 16 h clearly showed apoptotic bodies (Fig. 3).
DNA cleavage is regarded as another biochemical hallmark of apoptosis which evaluated by gel electrophoresis. \[17\] Change in the electrophoretic mobility of plasmid DNA on agarose gel is commonly taken as an evidence for direct DNA interactions. \[18\] Alteration of the DNA structure leads to retardation in the migration of supercoiled DNA and a slight increase in the mobility of open circular DNA to a point where both forms comigrate. \[19\] The DNA-cleaving ability of compounds was revealed initially by a plasmid relaxation assay, in which the conversion of supercoiled form (Form I, SC) to nicked circular (Form II, NC) DNA was monitored. A concentration-dependent DNA cleavage by compound \textit{A} was carried out (Fig. 2a). Significant DNA cleavage was shown at 200 µM concentration of compound \textit{A} (Fig. 2a, Lane 8). In addition, the mobility of DNA decreased with increase in concentration of compound \textit{A} suggesting that this compound probably has interaction with DNA which may lead to DNA cleavage. The comparative cleavage reactions were performed to declare the mechanism of pTZ57 plasmid DNA by compound \textit{A} in the presence of various radical scavengers (Fig. 2a) such as DMSO, as OH radical scavenger, NaN3 as \(^1\)O\(_2\) scavenger, and Superoxide Dismutase (SOD) as superoxide anion radical (O\(_2^−\)) scavenger. When the hydroxyl radical scavenger, DMSO (Lane 10, 12) was added to the reaction mixture, the nuclease activity slightly diminished which was indicative of the minor involvement of OH radicals in the cleavage process. The DNA cleavage of the plasmid was inhibited in the presence of NaN3 (Lane 9, 11), suggesting that \(^1\)O\(_2\) is likely to be the most reactive species responsible for the nuclease activity. Similarly, in the presence of SOD (Lane 13, 14), the cleavage was somehow inhibited, which indicated that O\(_2^−\) might be another activator in the cleavage process and reducing the amount of O\(_2^−\) can improve the cleavage effect. As the nuclease activity of the compound \textit{A} is diminished either in the presence of singlet oxygen or super oxide quenchers, some DNA is probably cleaved by oxidative mechanism; however, another mechanism other than oxidative is suggested due to the weak involvement of hydroxyl radical in the DNA nuclease activity.

**Conclusion**

We report herein two COX-2 inhibitor derivatives as chemotherapeutic agents. Among them, compound \textit{A} exhibited better IC\(_{50}\) value of 6.5 µM against the growth of MCF-7 cells and 21.66 µM against the K562 cells. The potency of both compounds on MCF-7 cell is more favorable than K562. The morphological changes results showed that the cytotoxic response to cancer cell lines tested may be through apoptotic pathway. Apoptotic cell death may require interaction with DNA (direct or indirect). According to our results, there is no notable evidence for a direct interaction at intermediate pH values (7.2), subsequently the anticancer activity of compounds \textit{A} and \textit{B} may not involve direct interaction with DNA at physiological conditions. Therefore, further experiments need to clarify its apoptotic mechanism.

**Conflict of interest**

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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