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Effects of Curcumin Analogue, 2, 6-Bis (2, 5-Dimethoxybenzylidene) Cyclohexanone (BDMC33) on the Activities of Drug-Metabolizing Enzymes in Cultured Caco-2 Cell Model

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ABSTRACT

Poor systemic delivery of curcumin outside the gut due to its rapid metabolism has severely limited its application to many chronic diseases. Previously, our research group synthesized curcumin analogues 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33) that has potent anti-inflammatory activities. Therefore, the aim of this study is to evaluate the effects of curcumin analog (BDMC33) on the activities of drug metabolizing enzymes in Caco-2 cells, which was compared with that of curcumin and 3-(2-Fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24). BDMC-33 was synthesized through the appropriate reaction of the aromatic aldehyde with cyclohexanone, under base catalyzed aldol condensation, at the ratio of ketone: aldehyde (1:2). Activity of drug metabolizing enzymes such as NADPH-cytochrome p450 reductase (CPR), UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and Sulfotransferase (SULT) in Caco-2 cells were evaluated upon exposure to 50µM of BDMC33, curcumin, and EF-24, separately, for 4 hours. The BDMC33, EF-24, and curcumin treatments did not affect the activities of UGT, GST, SULT, and CPR in respect to their controls (29.45, 27.18, 23.64 and 2.08µmol/mg), respectively, at all periods of incubation. Hence, BDMC33 was able to maintain the activities of both phases I and II drug metabolizing enzymes, and therefore it could be a potential lead, anti-inflammatory agents.

Keywords: Caco-2 cells, curcumin, drug metabolizing enzymes, glutathione-S-transferase, NADPH-cytochrome c reductase, sulfotransferase, UDP-glucuronosyltransferase.

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INTRODUCTION

Curcuma longa Linn (turmeric) has been used in traditional Asian cooking and medicine like Ayurveda since time immemorial. [1] Curcumin (a dietary polyphenolic compound) is a major component of the perennial herb (*Curcuma longa*), locally known as

kunyit, which had been reported to possess a wide spectrum of biological activities, proved to be saved and effective in animal and human clinical trials. [1-2] These were reported to be due to its several health promoting activities like antioxidant, anti-inflammatory, anti-bacterial, anticarcinogenic, antiproliferative, and anti-angiogenic agents [3-4] which have made curcumin a recognized therapeutic agent in the treatment of human diseases. [2, 5] Repeated oral administration of 12 g of curcumin per day has been reported to have no toxic effect. [5] Clinically, the systemic availability of curcumin in human patients after oral doses was found to be negligible. This was suggested to be due to its insolubility [6], rapid metabolism and elimination [7] as well as poor membrane permeation. [8] In animal studies, only 1.3µg/mL was observed in the serum after oral doses (4-8 g) of curcumin, and the low bioavailability was due to rapid metabolism of curcumin. The phase I (hydrocurcumin, dihydrocurcumin, tertrahydrocurcumin) and phase II (curcumin-glucuronide, curcumin-sulfate, and curcumin-glutathionide) metabolites of curcumin were formed. [9-10] The phase I metabolites were formed through successive reduction of four double bonds of the heptadiene-3, 5-dione system by the activities of enzymes (NADHP cytochrome P450 reductase or alcohol dehydrogenases) found in the cytosol of stomach and liver. [11] Free curcumin and its phase I metabolites were reported to be readily conjugated by phase II enzymes and rapidly eliminated out of the system without being absorbed outside the gut. [11] To overcome this, our research group has synthesized a curcumin analogue, which is 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33) by eliminating the unstable methylene group and β-diketone moiety (Figure 1b). BDMC33 was reported to exhibit high antioxidant and anti-inflammatory activities than the curcumin. [12-14]

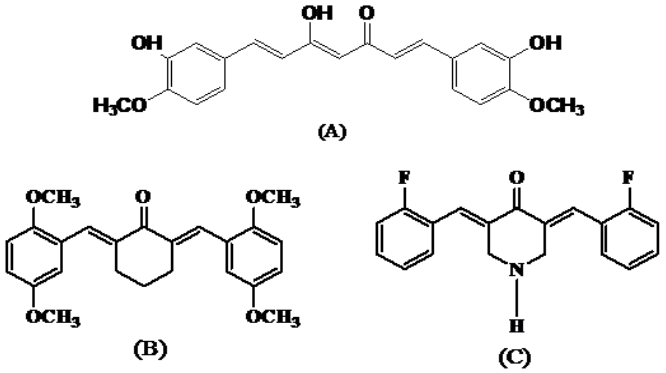

Fig. 1: Chemical structure of (A) 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione (curcumin) (MW: 368.38 g/mol) (B) 2, 6-bis (2,5-dimethoxybenzylidene) cyclohexanone (BDMC33) (MW: 394.46 g/mol), and (C) 3-(2-Fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24) (MW: 311.33 g/mol).

In this study, Caco-2 cells (derived from a human colorectal carcinoma) were used as the experimental model. It has been considered as a standard cell based tool in predicting the drug permeation across the human intestine. [15] On differentiation after cultured on semi permeable membranes, it develops a highly functionalized epithelial barrier with biochemical and morphological similar to that of humans. Transport proteins, efflux proteins, and phase II conjugation enzymes are expressed on the differentiated Caco-2 cells. [16-18] Therefore, the present study aimed to evaluate the activities of phase I metabolizing enzyme which is NADPH-cytochrome p450 reductase (CPR) and phase II metabolizing enzymes which are UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and sulfotransferase (SULT) in Caco-2 cells after exposure to BDMC3 for 4 hours. Similarly, all the experiments on curcumin (reference compound) and 3-(2-Fluoro-benzylidene)-5-(2-fluorocyclohexyl methylene)-piperidin-4-one (EF-24) (check control) were also performed for comparison.

MATERIALS AND METHODS

Chemicals and Reagents

Curcumin (Figure 1a), EF24 (Figure 1c), Eagle's minimal essential medium (EMEM), Hank's buffer salt saline (HBSS), nonessential amino acid (NEAA), UDP-glucuronic acid, UDP-glucuronidase, Glutathione, CDNB and 4-methyl umbelliferone were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA). Dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), phosphate buffered saline (PBS), and trypsin-ethylenediamine tetra acetic acid (trypsin-EDTA) solutions were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA).

Chemical Synthesis of 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33)

Curcumin analogue (BDMC33 or 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone) (Figure 1b) was chemically synthesized at the Institute of Bioscience, Universiti Putra Malaysia. The synthesis was done by coupling the appropriate aromatic aldehyde with cyclohexanone, under base catalyzed aldol condensation, using the ratio of ketone: aldehyde (1:2). [12]

BDMC33 or 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone Preparation and Dilution

The test compounds (curcumin, EF-24, and BDMC33), were weighed separately, and dissolved in DMSO to have 50 mM/L of each compound as stock solutions. Treatment concentration (50µM) of each analog (curcumin, EF-24, and BDMC33) was prepared by measuring 10µL of the stock solutions and diluted in 10 mL of Eagle's Minimal Essential Medium (EMEM), separately. The final concentration of DMSO was 1%.

Caco-2 cell Cultures

The Caco-2 cells having cell passage number 18 was purchased from American Type Culture Collection (ATCC, HTB-37). Cells were maintained in EMEM containing 20% of FBS, 1% penicillin-streptomycin solution and cultured in 75 cm^2 tissue culture flasks (Corning, USA) at 37°C in a 5% CO_2 incubator with an atmosphere of 95% humidity. After 5 - 7 days, the cells

were 80-85% confluence and harvested using trypsin-EDTA. The cells with 85% and above viable cells were seeded and grown in 6-well tissue culture plate (Corning, USA) containing EMEM with 20% of FBS, 1% penicillin-streptomycin solution. The seeding density used is 2.6×10^5 cells cm^2 (300,000 cells per well), then maintained at 37°C in a 5% CO_2 incubator with an atmosphere of 95% humidity for 21 days and the growth medium was changed every two days.

Caco-2 cell Treatment

After 21 days of culturing cells, the growth media in each well of 6-well tissue culture plate (Corning, USA) were discarded, and Caco-2 cells were rinsed three times with phosphate buffer solution (PBS) at pH 6.5. Then, 5 mL of HBSS (pH 6.5) was introduced into the wells and 25µL of stock solution of each test compound was added, separately, into each well to have 50µM as the treatment concentration of each compound (BDMC33, EF-24, and curcumin). The final concentration of DMSO in the media was 0.1%. In a separate well of cultured Caco-2 cells, the same volume of HBSS with 1% DMSO was added, which serve as the control (untreated group). Then, all plates were incubated at 37°C in a 5% CO_2 inhibitor at an hour interval for 4 hours.

Whole Caco-2 cell Protein Extractions (lysates)

At the time intervals of 1, 2, 3 and 4 hours, attached Caco-2 cell monolayers in 6-well plate were removed from the incubator and placed on ice cold water for 10 min. The media in all wells were discarded and rinsed three times with cold, sterile phosphate buffer solution (PBS) at pH 7.4. Then, 500µL of ice-cold lysis buffer (1 M PBS, 1M NaCl, 1M Triton X-100, and 1M EDTA) at pH 7.4 was added and a cold plastic cell scraper was used to scrape off the adherent cells in the 6-well plate. Detached cells were transferred and suspended in separate pre-cooled microfuge tubes (1.5 ml) containing 500µL of cold PBS (pH 7.4), maintained under constant agitation for 30 min at 4°C. The suspended cells were then centrifuged at 10,000 × *g* for 30 min at 4°C in a Microcentrifuge to separate the total protein (supernatant) from the cell debris. Supernatants were transferred into fresh tubes, separately, and stored at -80°C for the analysis of total protein contents and drug-metabolizing enzyme activity.

Determination of Protein Concentration

Bradford reagent for 1-1,400µg/mL bought from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA) was used to measure total protein content in Caco-2 cell homogenates (lysates), and protein standard curve (0-400µg/mL) at 11 concentrations by 2-folds serial dilution was developed using BSA. [19]

DRUG METABOLIZING ENZYMES ASSAYS

UDP-Glucuronosyltransferase Activity

UDP-Glucuronosyltransferase activity in Caco-2 cells was determined using 4-methyl umbelliferone as the substrate. UDP-glucuronosyltransferase activity against 4-methyl umbelliferone was determined using spectrophotometric methods [20-21] with some modification. Briefly, 100µL total protein extract (cell homogenate) was added to 1.4 mL of phosphate buffer (pH 7.4) containing 0.1 M UDP-glucuronic acid and 0.1 M umbelliferone (4-methyl umbelliferone) and incubated at 37°C for 60 min. The glucuronide formed was hydrolyzed by the addition of 0.5 mL of 0.01 M UDP-glucuronidase. The absorbance of glucuronide-conjugate formed was measured at 355 nm for 3 min using a Shimadzu UV-1240 spectrophotometer (SSI, Japan). The absorbance of a separate reaction mixture (1.4 mL) and 0.1 mL of protein extract (cell homogenate) treated with HBSS containing only 1% DMSO was also taken as control. The difference in absorbance between the control (untreated) and treated cells was the amount of 4-methyl umbelliferone consumed through glucuronide formation. The absorbance of three replicate were performed, and the activity of UGT was calculated using the molar coefficient (18.1 $m^{-1}cm^{-1}$). The unit of the UGT activity was expressed as µMol/min/mg.

Glutathione-S-transferase Activity

Glutathione-S-transferase activity in Caco-2 cells was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. Glutathione-S-transferase activity against conjugation reaction between reduced glutathione and 1-chloro-2, 4-dinitrobenzene (CDNB) was determined using spectrophotometric methods [22] with some modification. Briefly, 100µL of total protein extract (cell homogenate) was mixed with 1.9 mL of 0.1 M phosphate buffer (reaction mixture) at pH 7.4, containing 50 mM of CDNB in 2 % acetonitrile and incubated at 37°C for 60 min. The absorbance of a glutathione conjugate formed was measured at 340 nm using a Shimadzu UV-1240 spectrophotometer (SSI, Japan). The absorbance of a separate reaction mixture (1.4 mL) and 0.1 mL of protein extract (cell homogenate) treated with HBSS containing only 1% DMSO was also taken as control. The difference in absorbance between the control (untreated) and treated cells was the amount of CDNB consumed through the glutathione-conjugate formation. The absorbance of three replicate were performed, and the activity of GST was calculated using the molar coefficient (9.6 $m^{-1}cm^{-1}$). The unit of the GST activity was expressed as µMol/min/mg.

Sulfotransferase Activity

Sulfotransferase activity in Caco-2 cells was determined using 4-methyl umbelliferone (4-UM) as a substrate acceptor and phosphate adenosine-5′-phosphosulfate generating system (PAPS) as a donor of the sulfate group. The ability of sulfotransferase activity to transfer a sulfate group from 3′ phosphoadenosine 5′-phosphosulfate (PAPS) to the substrate acceptor (4-MU) to form a conjugate of sulfate derivative was determined using spectrophotometric methods [23] (Frame *et al*., 2000) with some modification. A reaction mixture of 1 mL of phosphate buffer (pH 7.4) containing 0.06 M of 3′-phosphate adenosine-5′-phosphosulfate generating system, 0.1 M 4-methyl-

umbelliferone and 100µL of total protein extract (Caco-2 cell homogenate) was prepared. Then, 0.9 mL of 1 M sodium sulfite was added to the mixture to inhibit the hydrolysis of sulfate conjugate, and the mixture was incubated at 30°C for 30 min. The color change was monitored at 420 nm using a Shimadzu UV-1240 spectrophotometer (SSI, Japan). The absorbance of a separate reaction mixture (1.4 mL) and 0.1 mL of protein extract (cell homogenate) treated with HBSS containing only 1% DMSO was also taken as control. The difference in absorbance between the control (untreated) and treated cells was the amount of substrate acceptor (4-MU) consumed through sulfate conjugate formation. The absorbance of three replicate were performed, and the activity of SULT was calculated using the molar coefficient (9.6 $m^{-1}cm^{-1}$). The unit of the SULT activity was expressed as µMol/min/mg.

NADPH-cytochrome P450 reductase Activity

The activity of NADPH-cytochrome P450 reductase (CPR) in Caco-2 cells was conducted, and the ability of NADPH-cytochrome p450 reductase to transfer a pair of electrons from NADPH using FAD and FMN as prosthetic groups to cytochromes P450 was determined using spectrophotometric methods described by Iwata *et al*. [24] with some modification. Briefly, in a reagent mixture of 1.0 mL of phosphate buffer (pH 7.4) containing 0.1 M EDTA, 1 M KCN, and 0.1 M cytochrome-c, 100µL of total protein extract (Caco-2 cell homogenate) was added, in which 0.9 mL of 4.2 mM NADPH was introduced to initiate the reaction. The mixture was then incubated at 30°C for 3 min, and the absorbance of reduced cytochrome c was measured at 550 nm using the Shimadzu UV-1240 spectrophotometer (SSI, Japan). The absorbance of a separate reaction mixture (1.4 mL) and 0.1 mL of protein extract (cell homogenate) treated with HBSS containing only 1% DMSO was also taken as control. The absorbance of three replicate were performed, and the activity of CPR was calculated using the molar coefficient (0.021 $m^{-1}cm^{-1}$). The unit of the CPR activity was expressed as µMol/min/mg.

Statistical Analysis

A one-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test (Graph Pad Prism version 5) was used to compare the means of the treated group to the control (untreated). The significant difference was considered at $p<0.05$. Data were presented as the mean ± SD (n = 3).

RESULTS

Effects on CPR Activities

Effects of curcumin (reference compound), EF-24 (check control), and BDMC33 (synthesized analogues) on CPR activities in Caco-2 cells upon incubation for 4 hrs are shown (Figure 2). Activities of CPR in Caco-2 cells treated with curcumin and its analogues throughout the period of experiments were ranged as 2.13 ± 1.23 – 2.20 ± 1.25µmole/min/mg (curcumin), 1.65 ± 1.20 – 1.95 ± 1.20µmole/min/mg (EF-24), and 1.70 ± 2.0 – 1.82 ± 1.25µmole/min/mg (BDMC33) compared to that of untreated cells (control) with 2.08 ± 1.24µmole/min/mg (Figure 2). These results have shown that activities of CPR in Caco-2 cells treated with curcumin, EF-24, and BDMC33, separately, were not differed significantly, compared to that of untreated cells.

Effects on UGT Activities

Effects of curcumin (reference compound), EF-24 (check control), and BDMC33 (synthesized analogue) on GST activities in Caco-2 cells upon incubation for 4 hrs are presented (Figure 3). Results revealed that the activities of GST in Caco-2 cells treated with curcumin and its analogues at all periods of experiments can be ranged as 19.70 ± 1.23 – 20.70 ± 1.20µmole/min/mg (curcumin), 18.00 ± 1.20 – 19.55 ± 1.25µmole/min/mg (EF-24), and 19.02 ± 1.20 – 19.65 ± 1.20µmole/min/mg (BCDMC33), while the untreated cells had 19.68 ± 1.24µmole/min/mg (Figure 3). These results have suggested that the activities of GST in all treated Caco-2 cells were not different significantly, compared to that of untreated cells throughout the period of experiments.

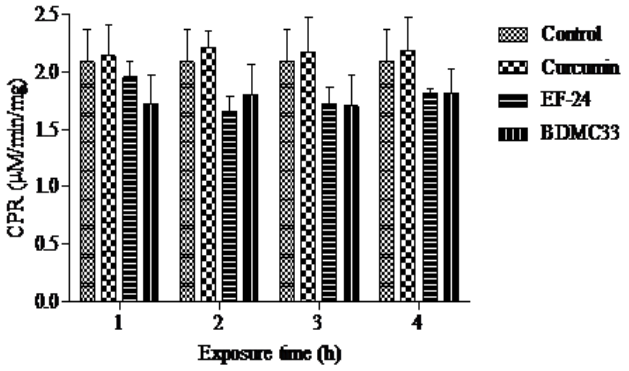

Fig. 2: NADPH-cytochrome P450 reductase (CPR) activities in Caco-2 cells exposed curcumin and its analogues. Data are presented as mean ± SD of three sets of independent experiments. Curcumin and EF-24 were served as reference compound and check control, respectively. A significant difference was observed at $p<0.05$ for the treatment groups against the untreated groups.

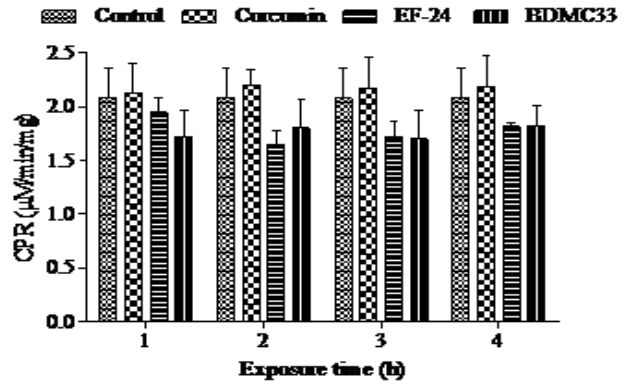

Fig. 3: UDP-glucuronosyltransferase (UGT) activities in Caco-2 cells exposed to curcumin and its analogues. Data are presented as mean ± SD of three sets of independent experiments. Curcumin and EF-24 were served as a reference and check control, respectively. A significant difference was observed at $p<0.05$ between the treatments and untreated groups.

Effects on GST Activities

Effects of curcumin (reference compound), EF-24 (check control), and BDMC33 (synthesized analogue) on GST activities in Caco-2 cells upon incubation for 4 hours are presented (Figure 4). Results revealed that the activities of GST in Caco-2 cells treated with curcumin and its analogues at all periods of experiments can be ranged as 19.70 ± 1.23 – 20.70 ± 1.20µmole/min/mg (curcumin), 18.00 ± 1.20 – 19.55 ± 1.25µmole/min/mg (EF-24), and 19.02 ± 1.20 – 19.65 ± 1.20µmole/min/mg (BCDMC33), while the untreated cells had 19.68 ± 1.24µmole/min/mg (Figure 4). Interestingly, the activities of GST in all treated Caco-2 cells were not different significantly, compared to that of untreated cells throughout the period of experiments.

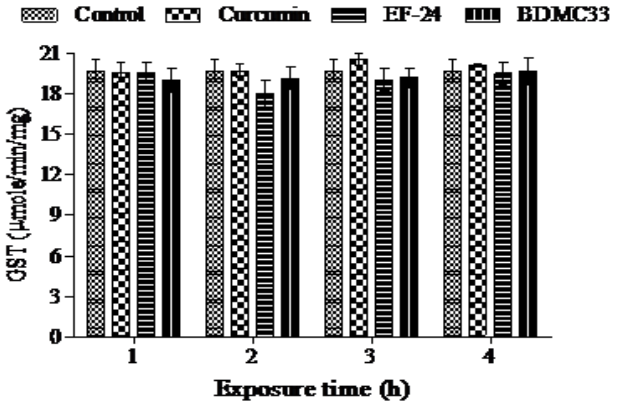

Fig. 4: Glutathione-S-transferase (GST) activities in Caco-2 cells exposed to curcumin and its analogues. Data are presented as mean ± SD of three sets of independent experiments. Curcumin and EF-24 served as a reference and positive control, respectively. A significant difference was observed at $p<0.05$ between treated and control groups.

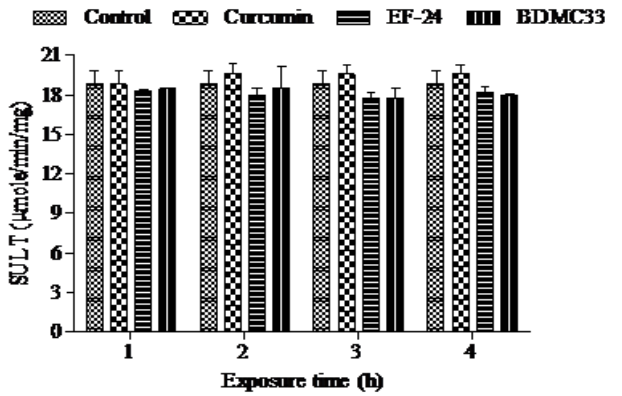

Fig. 5: Sulfotransferase (SULT) activities in Caco-2 cells exposed to curcumin and its analogues. Data are presented as mean ± SD of three sets of independent experiments. Curcumin and EF-24 served as a reference and positive control, respectively. A significant difference was observed at $p<0.05$ between treated and control groups.

Effects on SULT Activities

Effects of curcumin, EF-24, and BDMC33 on SULT activities in Caco-2 cells upon incubation for 4 hours are shown (Figure 5). In untreated cells, the activity of SULT was 18.87 ± 2.10µmole/min/mg. While effects of curcumin and its analogues on activities of SUL in Caco-2 cells throughout the period of experiments were ranged as 18.80 ± 1.26 – 19.60 ± 1.23µmole/min/mg (curcumin), 17.75 ± 1.25 – 18.30 ± 1.20µmole/min/mg (EF-24), 17.72 ± 1.23 – 18.52 ± 1.20µmole/min/mg (BDMC33) (Figure 5). These results have demonstrated that in all treatments of BDMC33, curcumin, and EF-24, activities of SULT were not differed significantly, compared to that of untreated cells.

DISCUSSIONS

Despite the high promising effects on biological activities of curcumin, its clinical progress has not been achieved due to its poor systemic delivery, and this has severely limited its application to many chronic diseases. To overcome this, our research group previously synthesized curcumin analogues; 2, 6-bis (2, 5-dimethoxybenzylidene) cyclohexanone (BDMC33) that have potent anti-inflammatory activities than curcumin. [12-14] Therefore, in this study the effects of BDMC33 on the activities of phase I metabolizing enzyme which is NADPH-cytochrome p450 reductase (CPR) and phase II metabolizing enzymes which are UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and sulfotransferase (SULT) in Caco-2 cells upon exposure for 1, 2, 3, and 4 hours were investigated and compared with that of curcumin (reference compound) and EF-24 (check control). The exposure periods of 4 hours were based on our observations that high stability of BDMC33 in HBSS solution (pH 6.5) was maintained after 3 hours of incubation.

The results of this study have demonstrated that activities of CPR in treated Caco-2 cells treated with curcumin were increased, but not affected significantly compared to that of the untreated group. Likewise, in Caco-2 cells treated with EF-24 and BDMC33, separately, had their CPR activities dropped, but not differed significantly, compared to that of untreated cells. The maintenance of CPR activities in all treatment cells compared to untreated cells could reflect the biological benefits of BDMC33 during the periods of incubation. NADPH-cytochrome P450 reductase (CPR) is classified under phase I enzymes (CYP) in the biological systems. It plays a major function in detoxification of most of the drugs used currently by catalyzing the transfer of electron from NADPH to all cytochrome P450 enzymes. [25-26] Inactivation and activation of CYP gene expression by drugs are the most important cause of drug-drug interactions. [26]

The effects of these new novel analogues on the activity of CYP enzymes are very vital to ascertain their possible drug-drug interaction. CYP enzymes have known to play a vital role in carcinogenesis. The activation of procarcinogens metabolism in the system can be catalyzed by the activity of CYP enzymes. For example, CYP1A1, an isoform for one of the CYP enzymes converts polycyclic aromatic hydrocarbons (PAHs) to highly reactive metabolites. Then, an increase of these enzymes in the system serves as a risk

in cancer generations. [27] Hence, the ability of newly synthesized analogue (BDMC33) to maintain the activities of CPR in Caco-2 cells is an indication of its higher anti-inflammatory activities. Therefore, BDMC33 may be classified as anti-inflammatory agents due to its ability to maintain the activity of NADPH-cytochrome P450 reductase throughout the period of experiments compared to control. According to Lee *et al*. [12-13] BDMC33 exhibited potent biological activity against the markers of oxidative stress and promising nitric oxide inhibitory activities in activated macrophages. Anti-inflammatory activities of BDMC33 on iNOS blockage, PGE_2 and COX-1 production and COX-2 depression *in vitro* have been reported. [12-14] Lee *et al*. [14] has suggested that BDMC33 prevents the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) that are the major pro-inflammatory mediators in activated macrophages and microglial cells. It also blocked lipopolysaccharides (LPS) signaling by suppressing the secretion of CD-14 accessory molecules. [14] The prolonged NO production is caused by inducible NOS (iNOS) expression in activated macrophages [14] and nude mice. [28] The inhibition of NO production causes the suppression of NF-kB activation and lowers the expression of COX-2 and many pro-inflammatory genes (IL-1b, IL-8, and TNF-α^3). [29]

The current study also demonstrated that Caco-2 cells treated with curcumin had their UGT activities increased but was not differed significantly, compared to untreated one. However, UGT activities in Caco-2 treated with EF-24 and BDMC33 were dropped, but, not differed significantly, compared to the untreated ones (Figure 3). Increase levels of UGT activities in Caco-2 cells treated with curcumin that were not differed compared to the control may reflect its biological activities by preventing the toxicity effects of drugs/xenobiotics that was detoxified by the enzyme. This increase in UGT activity by curcumin may also be connected with drug interaction which leads to inactivation of drugs/xenobiotics that are metabolized via this enzyme.

UGT plays a vital function in drug/xenobiotics detoxification. UGT metabolizing agents include coumarin, acetaminophen, naphthols, estrogens, morphine, etc. [30] According to Odenthal *et al*., [31] curcumin elevated the UGT protein expression in Caco-2 cells. It was hypothesized that increased activities of UGT signified a protective effect against the drug's toxicity effects (peroxidase, proinflammation cytokines, and ROS generation). [31-33] It was recently demonstrated that genetic polymorphism in UGT was found to be associated with reduced *in vivo* phase II enzymes, resulting in drug risk-related toxicity in patients. [31, 34-35] In cells free of cancer illness, increase activity of UGT may prevent the generation of oxidative stress and procarcinogens related agents. Compounds that can elevate the activity of phase II enzymes may be classified as agents of cancer diseases. [31] However, maintenance of UGT activities observed in both Caco-2 treated with EF-24 and BDMC33, separately, compared to those in control may be connected to their ability to neutralize the toxicity effects of drugs/xenobiotics detoxified by this enzyme. Potent anti-inflammatory activities of EF-24 and BDMC33 than curcumin in macrophage and nude cells were established. [8, 12-13] Hence, BDMC33 may be classified as an anti-inflammatory agent for its ability to maintain the activity of UGT throughout the period of experiments compared to control. According to Lee *et al*. [12-13], BDMC33 exhibited potent biological activity against the markers of oxidative stress and promising nitric oxide inhibitory activities in activated macrophages.

Anti-inflammatory activities of BDMC33 on iNOS blockage, PGE_2 and COX-1 production and COX-2 depression *in vitro* have been reported. [12-14] Lee *et al*. [14] has suggested that BDMC33 prevents the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) that are the major pro-inflammatory mediators in activated macrophages and microglial cells. It also blocked lipopolysaccharides (LPS) signaling by suppressing the secretion of CD-14 accessory molecules. [14] The prolonged NO production is caused by inducible NOS (iNOS) expression in activated macrophages [37] and nude mice. [37] The inhibition of NO production causes the suppression of NF-kB activation and lower expression of COX-2 and many pro-inflammatory genes (IL-1b, IL-8, and TNF-α^3). [29]

In addition, it was suggested in this study that GST activities in Caco-2 cells treated with curcumin were increased, but not differed significantly, compared to that in untreated Caco-2 cells. However, in Caco-2 cells treated with EF-24 and BDMC33, GST activities were decreased, but not differ significantly, compared to that of untreated cells (Figure 4). GST belong to a family of enzymes that play a vital function in the modification of almost all electrophilic metabolites from phase I metabolism. Most active metabolites which are detoxified by GST through glutathione conjugation reaction are benzpyrene diols, aflatoxin B1-epoxide, etc. [30, 38]

GST metabolizing agents is included acetaminophen, ethacrynic acid, tetracycline, etc. [26, 38] Increased activities of GST in Caco-2 cells treated with curcumin with indication of not differed significantly, compared to untreated ones may be associated with its potent biological benefits against the toxicity effects of most drugs and phase I reactive metabolites that are inactivated by this enzyme. According to Odenthal *et al*. [31], curcumin elevated the GST protein expression in Caco-2 cells. It was hypothesized that increased activities of GST signified a protective effect against the drug's toxicity effects (peroxidase, proinflammation cytokines, and ROS generation). [31-33] According to

Shao *et al*. [39] and Odenthal *et al*. [31], an increase GST activity inhibits tumor generation while its decrease promotes tumor risk in the gastrointestinal tract. It was demonstrated by Jurek *et al*. [40] and Odenthal *et al*. [31] that inhibition of GST protein expression in Apc Min/- mice caused 6-folds increase of colon adenoma production than in wild-type Apc Min/-mice. Lower responses to liver cancer illness were observed in transgenic rats with excess GST gene compared to wild-type rats. [31, 41] However, no alteration of GST activities in Caco-2 cells as compared to control could reflect the potent biological activities (antioxidant and anti-inflammatory properties) of BDMC33 against toxicity effects of drugs detoxified by this enzyme.

The antioxidant and anti-inflammatory activities of BDMC33 in cell line studies have been reported. [12-14] Compounds that can elevate or maintain the normal levels of phase II enzymes may be classified as agents of anti-cancer related diseases. [31] Hence, BDMC33 may be classified as agents of anti-cancer related diseases. According to Lee *et al*. [12] and Lee *et al*. [13], BDMC33 exhibited potent biological activity against the markers of oxidative stress and promising nitric oxide inhibitory activities in activated macrophages. Anti-inflammatory activities of BDMC33 on iNOS blockage, PGE_2 and COX-1 production and COX-2 depression *in vitro* have been reported. [12-13]

Moreover, this study has also demonstrated that SULT activities in Caco-2 cells treated with curcumin were increased throughout the period of experiments with an indication of not differ significantly, as compared to that in untreated one. However, decreased levels of SULT activities were observed in Caco-2 cells treated with EF-24 and BDMC33 throughout the period of experiments with an indication of no significance difference compared to that of untreated one. Increased levels of SULT recorded in curcumin treatments which were not differed significantly, compared to untreated one may be connected with higher biological advantages of curcumin against the toxicity risk of various drugs that are modified by the enzyme.

In general, SULT belongs to a group of enzymes that are responsible for the modification of various drugs and their metabolites from phase I metabolism. SULT metabolizing agents include nitrophenol, acetaminophen, dopamine, estrogens, eicosanoids, thyroid hormone, etc. [26, 38] However, the ability of newly synthesized analogues (BDMC33) to maintain the activities of SULT in Caco-2 cells throughout the period of experiments are evidence of its potential benefits against risks from chemical-induced oxidative stress and proinflammatory cytokines. Therefore, BDMC33 may be classified as an anti-inflammatory agent for maintaining the activity of SULT throughout the period of experiments compared to control. According to Lee *et al*. [12] and Lee *et al*. [13], BDMC33 exhibited potent biological activity against the markers of oxidative stress and promising nitric oxide inhibitory activities in activated macrophages. Anti-inflammatory activities of BDMC33 on iNOS blockage, PGE_2 and COX-1 production and COX-2 depression *in vitro* have been reported. [12-14] Lee *et al*. [14] has suggested that BDMC33 prevents the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) that are the major pro-inflammatory mediators in activated macrophages and microglial cells.

In conclusion, both phase I drug-metabolizing enzyme (NADPH-cytochrome p450 reductase) and phase II drug-metabolizing enzymes (UGT, GST, and SULT) activities in Caco-2 cells treated with curcumin (reference compound), EF-24 (check control), and BDMC33 (synthesized analogues) were not altered in respect to control. Thus, this study had proven that BDMC33 could be a potential leading feature anti-inflammatory agent. Therefore, *in vivo* further elucidation on the activities of both phase I and II drug metabolizing enzymes by BDMC33 is currently going on in our laboratory.

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