
**UNIVERSITI PUTRA MALAYSIA**

# *TOXICITY, PERMEABILITY AND DRUG-METABOLIZING ENZYME ACTIVITIES OF CURCUMIN ANALOGUES*

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# TOXICITY, PERMEABILITY AND DRUG-METABOLIZING ENZYME ACTIVITIES OF CURCUMIN ANALOGUES

By

**NDATSU YAKUBU**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy**

**November 2015**

**DEDICATION**

**To my family**

**(Mrs. Yakubus and children)**
**"Alhamdulillah,**
**In Allah We Trust,**
**By Love We Stand"**

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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**November, 2015**

**Chairman: Syahida Binti Ahmad, PhD**
**Faculty : Biotechnology and Biomolecular Sciences**

Curcumin (a dietary polyphenolic compound) derived from turmeric (*Curcuma longa*) possesses potent biological activities. However, curcumin's clinical application is severely limited due to its rapid metabolism and poor bioavailability. Hence, two curcumin analogues, which are 2,6-bis(2,5-dimethoxybenzy-lidene)cyclohexanone (BDMC33) and 2,6-bis(2-fluorobenzylidene)cyclohexanone (MS65) with potent anti-inflammatory activities than curcumin were synthesized by our research group. To ascertain the toxicity, permeability and drug-metabolizing enzyme activities of BDMC33 and MS65, their *in vitro* toxicity test at (0-400 µM) on Caco-2 cells using MTT assay, the *in vivo* toxicity test on zebrafish embryos and larvae at (0-50 µM), and acute toxicity effects (0-30 µM) on adult male zebrafish were investigated for 5 hr, 5 days, and 48 hr of exposure, respectively. The sub-chronic toxicity (14 days of exposure) of aspirin (control) and both compounds (8, 10 and 20 µM), respectively, on adult male zebrafish and the histopathological examinations (transverse sections) of intestine and liver of adult male zebrafish using hematoxylin and eosin (H and E) staining were evaluated. The permeability effects of both compounds (50 µM) in differentiated Caco-2 cells after 180 min of exposure were measured based on its apparent permeability coefficient ($P_{app}$) values of the apical site (A) to basolateral site (B) and basolateral site (B) to the apical site (A) and also the absorption rates of both compounds (20 µM) on adult male zebrafish were also measured after 1-5 hr of exposure. Furthermore, the effect of both compounds on drug-metabolizing enzyme activities, which were NADPH-cytochrome p450 reductase (CPR), UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and sulfotransferase (SULT) in differentiated Caco-2 cells and adult male zebrafish were measured using colorimetric methods. Similarly, toxicity, permeability effects and drug-metabolizing enzyme activities of curcumin (reference compound) and 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24) (positive control) in differentiated $Caco_2$ and adult male zebrafish were conducted for comparison. The results showed that the 5 hr $LC_{50}$ for all test compounds in Caco-2 cells were 50 µM, the 5 days $LC_{50}$ values on zebrafish embryos and larvae were 6.25 µM (BDMC33), 12.5 µM (MS65), 5 µM (curcumin and EF-24), and the 48 hrs $LC_{50}$ values on the adult male zebrafish were 20 µM (BDMC33 and MS65) and 10 µM (curcumin and EF-24). The heartbeats of zebrafish larvae subjected to BDMC33 and MS65, separately, for 5 days were 113±0.05 $min^{-1}$(BDMC33), 112±0.12 $min^{-1}$ (MS65), 109.3±0.14 $min^{-1}$ (curcumin), and 110±1.10 $min^{-1}$ (EF-24), while that of the normal zebrafish larvae was 117±0.15$min^{-1}$. The normal zebrafish embryos hatched after 2-3 days with >50% hatchability rates, which is similar to those exposed to <6.25 µM (BDMC33) and <12.5 µM (MS65) as compared to curcumin and EF-24 (<5 µM) treatments, separately. The results of

the apparent permeability coefficient ($P_{app}$) in Caco-2 cells after 120 min incubation and their absorption (uptake) rates in adult male zebrafish after 4 hr suggested that MS65>BDMC33>EF-24>curcumin. The activities of drug-metabolizing enzymes (CPR, UGT, GST and SULT) in cells and adult male zebrafish subjected to all test compounds, separately, as compared to that of normal cells and zebrafish demonstrated that MS65 is better than BDMC33, followed by EF-24 and then curcumin. Therefore, both MS65 and BDMC33 could be potential lead compounds to address the problems and issues of rapid metabolism, and poor bioavailability of curcumin when consumed orally.

Abstrak yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagi memenuhi keperluan untuk Ijazah Doktor Falsafah

**KETOKSIKAN, KADAR RESAPAN DAN AKTIVITI ENZIM METABOLISME DRUG BAGI ANALOG KURKUMIN**

Oleh

**NDATSU YAKUBU**

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Kunyit (Curcuma longa) telah digunakan dalam perubatan tradisional Asia sejak zaman dahulu lagi. Kurkumin (satu pemakanan polyphenolic kompaun) berasal dari kunyit memiliki aktiviti biologi yang mujarab. Walau bagaimanapun, aplikasi klinikal kurkumin adalah teruk terhad disebabkan oleh metabolisme cepat dan bioketersediaan miskin. Oleh itu, dua analog kurkumin, iaitu 2,6-bis(2,5-dimetoksibenzilidena) sikloheksanon (BDMC33) dan 2,6-bis(2-fluorobenzilidena) sikloheksanon (MS65) menunjukkan aktivitii anti-radang poten berbanding kurkumin telah disintesis oleh kumpulan penyelidikan kami. Oleh itu, objektif umum kajian ini adalah untuk menilai ketoksikan, kadar resapan dan aktiviti enzim metabolisme-drug BDMC33 dan MS65 dalam sel Caco-2 (sel terbit daripada karsinoma kolorektal manusia) dan zebrafish (*Danio rerio*). Ujian ketoksikan dalam vitro dua analogue (0-400 µM) sel menggunakan MTT assay, ujian ketoksikan dalam vivo zebrafish embrio dan larvae kedua-dua sebatian (0-50 µM), dan kesan ketoksikan akut (0-30 µM) zebrafish lelaki dewasa telah dinilai setiap hari selama 5 jam, 5 hari, dan 48 jam pendedahan, masing. Di samping itu, kesan ketoksikan sub-kronik (14 hari pendedahan) aspirin (kawalan) dan analog kedua-duanya (µM 8, 10 dan 20), masing, pada zebrafish lelaki dewasa (>6 bulan) telah dinilai dan Histologi usus dan hati tisu yang menggunakan hematoksilin dan eosin (H dan E) telah dikaji. ktiviti kadar resapan kedua-dua analog kurkumin (50 µM) dalam sel Caco-2 dan kadar serapan mereka di zebrafish lelaki dewasa selepas 180 min dan 5 jam, masing, telah dikaji. Tambahan pula, kesan kedua-dua analog kurkumin terhadap aktiviti enzim metabolisme-dadah, iaitu sitokrom p450 reduktase (CPR), UDP-glukuronosiltransferase (UGT), glutation-S-transferase (GST), dan sulfotransferase (SULT) dalam sel Caco-2 yang dan zebrafish jantan dewasa (>6 bulan) dikira menggunakan kaedah enzim kalorimetri.Dengan cara yang sama, analisis terakhir kurkumin (rujukan kompaun) dan 3-(2-Fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one (EF-24) (kawalan positif) dalam CaCo2 diperbezakan dan zebrafish lelaki dewasa yang sama telah dinilai untuk perbandingan.Keputusan menunjukkan bahawa 5 jam $LC_{50}$ untuk semua ujian sebatian dalam sebatian-2 sel telah 50 µM, 5 hari $LC_{50}$ nilai pada zebrafish embrio dan larva adalah 6.25 µM (BDMC33), 12.5 µM (MS65), 5 µM (kurkumin dan EF-24), dan nilai 48 jam $LC_{50}$ dalam zebrafish lelaki yang dewasa telah 20 µM (BDMC33 dan MS65) dan 10 µM (kurkumin dan EF-24).Denyutan jantung larva zebrafish tertakluk kepada BDMC33 dan MS65, secara berasingan, untuk 5 hari telah 113±0.05 $minit^{-1}$ (BDMC33), 112±0.12 $minit^{-1}$ (MS65), 109.3±0.14 $minit^{-1}$ (kurkumin), dan 110±1.10 $minit^{-1}$ (EF-24), manakala yang larvae biasa zebrafish 117±0.15 $minit^{-1}$. Embrio normal zebrafish penetasan selepas 2-3 hari dengan kadar penetasan >50%,

yang menyerupai orang-orang yang terdedah kepada <6.25 µM (BDMC33) dan <12.5 µM (MS65) berbanding kurkumin dan EF-24 (<5 µM) rawatan secara berasingan. Seterusnya, tahap normal MDA (Indeks kerosakan oksidatif) dan MPO (Indeks epitelium kecederaan) dalam zebrafish lelaki dewasa yang dirawat dengan BDMC33 (6.25 µM), MS65 (12.5 µM), kurkumin (10 µM) dan EF-24 (10 µM) selepas 14 hari pendedahan dikekalkan berbanding dengan ikan yang biasa dengan 0.04 µmole/mg dan 0.03 U/mg, masing.. Pekali kadar resapan yang jelas (Papp) semua Kata majmuk (50 µM) dari A→B penyerapan, secara berasingan, selepas 120 minit inkubasi sel Caco-2 telah adalah dari 0.75x106-3.4x106 cm/s, nisbah pengambilan (Papp, A→B)/Papp B→A) adalah dalam lingkungan 0.3 3.0%, nisbah efluks (Papp B→A/Papp A→B) telah semua <1%, dan % Imbangan jisim (% pemulihan) dikira adalah dalam lingkungan 18.79-47.67%, dan perintah incrementing parameter kadar resapan adalah MS65>BDMC33> EF-24>kurkumin.Aktiviti enzim metabolisme-dadah (CPR, UGT, GST dan SULT) dalam sel-sel tertakluk kepada semua Kata majmuk (50 µM) dan di zebrafish lelaki dewasa yang tertakluk kepada ujian semua Kata majmuk (20 µM), secara berasingan,yang tidak berbeza berbanding dengan sel Caco-2 normal (2.08-21.53 µmole/min/mg) dan zebrafish (20,33-30,78 µmole/min/mg). Penemuan ini telah mencadangkan bahawa MS65 adalah lebih baik daripada BDMC33, diikuti oleh EF-24 dan kemudian curcumin. Oleh itu, kedua-duanya MS65 dan BDMC33 boleh menjadi sebatian plumbum berpotensi untuk menangani masalah dan isu-isu tentang ketaklarutan, metabolisme cepat, dan bioketersediaan kurkumin apabila dimakan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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| | |
|---|---|
| A | Apical |
| ADH | Alcohol dehydrogenase |
| B | Basolateral |
| BDMC33 | 2,6-bis(2,5-dimethoxybenzylidene)cyclohexanone |
| CPR | NADPH-cytochrome p450 reductase |
| DMEs | Drug metabolizing enzymes |
| DMSO | Dimethyl sulfoxide |
| dph | Day of post fertilization |
| DNPH | 2, 4-Dinitrophenyhydrazine |
| E3M | Embryo medium |
| EDTA | Ethylenediaminetetraacetic acid |
| EF-24 | 3-(2-fluoro-benzylidene)-5-(2-fluorocyclohexylmethylene)-piperidin-4-one |
| EMEM | Eagle's minimal essential medium |
| ER | Efflux ratio |
| FAD | Flavin adenosine dinucleotide |
| FBS | Fetal bovine serum |
| FMN | Flavin mono nucleotide |
| g | Gram |
| GSH | Reduced glutathione |
| GST | Glutathione-S-transferase |
| hpf | Hour of post fertilization |
| HBSS | Hank's balanced salt solution |
| IACUC | Institutional Animal Care and Use Committee |
| KCN | Potassium cyanide |

| | |
|---|---|
| kg | Kilogram |
| $LC_0$ | Maximum concentration that causes 0% mortality |
| $LC_{20}$ | Maximum concentration that causes 20% mortality |
| $LC_{50}$ | Medium concentration that causes 50% mortality |
| $LC_{80}$ | Maximum concentration that causes 80% mortality |
| $LC_{100}$ | Minimumconcentration that causes 100% mortality |
| LOEC | Low observable effect concentration |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| mg | Milligram |
| mg/L | Milligram/litre |
| mL | Milliliter |
| mm | Millimeter |
| mM | Millimolar |
| µ | Micron |
| µg | Microgram |
| µg/L | Microgram/litre |
| µL | Microliter |
| µM | Micromolar |
| MPO | Myeloperoxidase |
| MS65 | 2,6-bis(2,5-dimethoxy-benzylidene) cyclohexanone |
| MTT | 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide |
| MW | Molecular weight |
| NOEC | No observable effect concentration |
| nm | Nanometer |
| NADPH | Reduced nicotinamide adenosine dinucleotide phosphate |

| | |
|---|---|
| OECD | Organization for Economic Co-operation and Development |
| PAPS | 3-Phosphoadenosine-5′-phosphosulfate |
| $P_{app}$ | Apparent permeability coefficient |
| PBS | Phosphate buffer saline |
| ROS | Reactive oxygen species |
| SULT | Sulfotransferase |
| SD | Standard deviation |
| TEP | 1,1,3,3-Tetraethoxypropane |
| U | Unit |
| UGT | Uridine diphosphate glucuronosyltransferase |

# CHAPTER 1

## INTRODUCTION

Treatment of many diseases using the medicinal plant is related to folk medicine from different parts of the world. Natural products obtained from plants, fungi, bacteria and other organisms, have been utilized in pharmaceutical industries as pure compounds or as extracts (Zhang et al., 2010). There are different compounds like curcumin that can be extracted and characterized from plants called *Curcuma longa* Lin (Turmeric). Turmeric (*Curcuma longa*), locally known as kunyit, has been used in traditional Asian medicine since time immemorial. It is majorly cultivated in the Asian countries, like Malaysia, Indonesia, and Thailand. Curcumin (a dietary polyphenolic compound) derived from turmeric possess higher biological activities (Ammon et al., 1992, Mullaicharam and Maheswaran, 2012). It occurs naturally as flavonoid (polyphenol) in the turmeric, which has been reported to possess some pharmacological activities for the treatment of many diseases in humans, such as anti-inflammatory, antioxidant, antiviral and antifungal, anticancer actions (Aggarwal et al., 2007). Additionally, the hepatic and nephroprotective (Kiso et al., 1983, Venkatesan et al., 2000), thrombosis suppressing and myocardial infarction protective (Dikshit et al., 1995, Nirmala and Puvanakrishnan, 1999), hypoglycemic (Arun and Nalini, 2002), and antirheumatic (Deodhar et al., 1980) effects of curcumin are also reported.

The most important rationale for the therapeutic use of curcumin is its high safety profile. To date, no studies in either animals (Shanker et al., 1980) or humans (Lao et al., 2006) have shown any side effects associated with the use of curcumin even at very high doses. In different animal models (Shankar et al., 1980; Qureshi et al., 1992) or human studies (Lao et al., 2006), it has been shown that curcumin is highly safe even at 800 mg/kg/day for 3 months. However, the molecule remains overlooked due to lack of a suitable delivery system that can result inadequate therapeutic levels *in vivo*. In comparing with other polyphenolic compounds derived from diets and anti-cancer drugs, the structural instability, rapid metabolism and elimination of curcumin has been contributed to its low bioavailability (Hoehle et al., 2006; Garcea et al., 2005; Sharma et al., 2007). The structural instability of the curcumin has been reported to be due to the presence of active methylene groups and a β-diketone moiety (Lee et al., 2009).

The pharmacokinetic studies of curcumin in rodents and humans after oral doses have been reported over more than three decades. Collectively, these studies have shown that curcumin metabolized rapidly, which severely prevents its absorption outside the stomach (Sharma et al., 2005). It has shown in animal studies that curcumin metabolism was by glucuronidation, sulphation and glutathionylation to curcumin glucuronide, curcumin sulfate and curcumin glutathione, respectively (Ireson et al., 2001), and enzymatic reduction to tetrahydrocurcumin, hexahydrocurcumin and hexahydrocurcuminol (Holder et al., 1978; Ireson et al., 2001). In clinical studies, oral doses of curcumin in humans have shown the presence of excreted curcumin and its metabolites in both feces and urine. (Sharma

et al., 2005). In animal study, a dietary dose (1 g/kg) of curcumin orally administered, about 75% of compounds similar to curcumin were being found in feces, whereas none or little amounts were found in the urine (Wahlstrom and Blennow, 1978). All these were the evidence which supported the hypothesis that curcumin pass through rapid metabolism and easily eliminated in the feces after oral doses in the guts (Rabindranath and Chandrasekhara, 1980). These reports have shown that curcumin can be administered safely to patients, but has low oral bioavailability due to its rapid metabolism in the intestinal tract (Garcea et al., 2005, Sharma et al., 2005). Therefore, the curcumin clinical trial is yet to be progressed, which has prevented its physiological activities to be transformed into clinical benefit.

To overcome these shortcomings of curcumin, our research group previously synthesized two curcumin analogues, which are 2,6-bis(2,5-dimethoxybenzy-lidene)cyclohexanone (BDMC33) and 2,6-bis(2-fluorobenzylidene)cyclohexanone (MS65) by eliminating the unstable methylene group and β-diketone moiety. This was done by reacting an aromatic aldehyde with cyclohexanone, through base-catalyzed aldol condensation, using the ratio of ketone: aldehyde (1:2) (Lee et al., 2009). In *in vitro* screening, higher pharmacological activities of these compounds due to their potent antioxidant and anti-inflammatory properties have been reported (Lee et al., 2009, Lee et al., 2011). Inhibition of NO production in IFN-γ/LPS-challenged macrophage cells (RAW 264.7), suppression of NF-kB activation and AP-1 activities by blockade of ERK/JNK signaling pathways by these compounds have been reported (Lee et al., 2009, Lee et al., 2011 and Lee et al., 2012). Therefore, the need for *in vitro* and *in vivo* investigations on toxicity, permeability and drug metabolizing enzyme activities of these novel compounds is of utmost necessary. In this study, Caco-2 cells (derived cells from human colorectal carcinoma) and zebrafish (*Danio rerio*) were the experimental models selected for this study.

Caco-2 cells are obtained from a human colorectal carcinoma, which has been considered as a useful cell-based model in predicting the drug permeation across the human intestine. On differentiation after cultured on semipermeable membranes, the developed epithelial linings possess similar biochemical and morphological characteristics to that of humans. Differentiated Caco-2 cells also expressed protein transporters, efflux proteins, and phase II metabolizing enzymes (Van Breemen and Li, 2005). The apparent permeability coefficients ($P_{app}$) calculated from Caco-2 cell permeation studies have shown to correlate with human intestinal absorption (Van Breemen and Li, 2005). It was also demonstrated that the permeation of drugs/chemicals across Caco-2 cells correlated very well with the oral absorption in humans.

Zebrafish (*Danio rerio*), a freshwater tropical species has become a useful biomedical and toxicological models, which allow *in vivo* or *in vitro* toxicity testing using zebrafish embryos (Li et al., 2011). The embryos are small enough to be accommodated in 96-well plates, developed rapidly, very transparent for easy imaging and reproduced rapidly (Li et al., 2011). It cardiovascular, nervous systems and metabolic pathways are similar to those of humans at anatomical, physiological, and molecular levels, and it has about 80% correlation to high animal models (Dubey et al., 2013). Zebrafish has almost the same number of chromosomes with

humans (25 vs. 23 pairs), respectively, and that about 71.4% of human genes have at least one zebrafish orthologue (Postlethwait et al., 2000, Dubey et al., 2013). Zebrafish possess homologs for both cyclooxygenase (COX) isoforms, which function and demonstrate similar responses to pharmacological inhibitors as observed in mammals (Aswan et al., 2011). The expressions of phase I and II key enzymes that involved in drug metabolism pathway have been identified in zebrafish (McGrath and Li, 2008). Among the key enzymes identified in zebrafish are cytochrome P450, epoxide hydrolases (Phase I) and UDP-glucuronosyltransferase, Sulfotransferase, and glutathione-S-transferase (Phase II). These similar characteristics have made zebrafish the most useful model organism for the assessments of screening and pharmacological studies of drugs, especially, in the field of pharmacokinetic studies of drugs (Li et al., 2011, Dubey et al., 2013, Jason et al., 2013).

Thus, the hypothesis of this study states that the two synthesized curcumin analogues could be relatively less toxic, have higher bioavailability activities than EF-24 and curcumin.

The general objective of this study was to evaluate the toxicity, permeability and drug-metabolizing enzymes, which were NADPH-cytochrome P450 reductase (CPR), UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST), and sulfotransferase (SULT) activities of BDMC33 and MS-65 in Caco-2 cells and zebrafish. The specific objectives of this study are:

1. To evaluate the toxicity effects of curcumin analogues in Caco-2 cells, zebrafish embryos and larvae, and adult male zebrafish

2. To assess the permeability and absorption of curcumin analogues in Caco-2 cells and adult male zebrafish, respectively

3. To evaluate the effects of curcumin analogues on the activities of drug-metabolizing enzymes (NADPH-reductase, UDP-glucuronosyltransferase, Sulfotransferase, and Glutathione-S-transferase) in Caco-2 cells and adult zebrafish

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