UNIVERSITI PUTRA MALAYSIA

EVALUATION OF CURCUMIN DERIVATIVES AS NEW CYCLOOXYGENASE-2 INHIBITORS VIA In Silico AND In Vitro ANALYSES

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FBSB 2016 7

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

April 2016

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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April 2016

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Prostaglandin E_2 (PGE_2) is one of the lipid mediators of inflammation. Chronic inflammation drives overproduction of PGE_2 that leads to development of chronic inflammatory diseases. PGE_2 is synthesized by cyclooxygenase (COX) enzyme that exists in isoforms of COX-1, which is constitutively expressed; and COX-2, which is expressed upon induction. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COXs to control excessive production of PGE_2 during inflammation but, most of commercialized NSAIDs selectively inhibit COX-1 or being non-selective which compensate for limitations and detrimental side effects of the medicine. Hence, deciphering the mechanisms of selectively inhibiting COX-2 is of great interest. Curcumin was known as remedy to treat the inflammatory-related diseases, but suffers from poor bioavailability and instability. Synthesis of curcumin derivatives was carried out to overcome the limitations. Thus, the objectives of this study are to investigate the effects of 43 curcumin derivatives towards activated cellular PGE_2 production and COX's activity, as well as to understand its mechanism of actions *in silico* and *in vitro*. In this study, effects of curcumin derivatives on PGE_2 production in murine macrophage (RAW264.7) cells which was stimulated by combination of interferon-gamma (IFN-γ) and lipopolysaccharide (LPS), were evaluated using immunoassay procedures. Quantitative structure-activity relationship (QSAR) analysis was performed to correlate between the structure and PGE_2 inhibition activity of curcumin derivatives. Enzymatic assay and molecular docking analysis were performed to decipher the mechanism of inhibition on COX activity by curcumin derivatives. Effects of active curcumin derivatives on gene expression of COX-1 and COX-2 were also determined. Results demonstrated that 3 out of 43 compounds significantly inhibited PGE_2 production in IFN-γ/LPS-stimulated RAW264.7 cells dose-dependently which were 2,6-bis(2-fluorobenzylidene)cyclohexanone (compound 25), 2,6-bis(4-fluorobenzylidene)cyclohexanone (compound 27), and 2,5-bis(3,4,5-trimethoxybenzylidene)cyclopentanone (compound 43) with IC_{50} values of 6.15 ± 0.48 μM, 5.78 ± 1.67 μM and 12.15 ± 1.88 μM respectively which were higher than that of curcumin. Furthermore, these three compounds were not toxic to the cells (cytotoxicity IC_{50}>500 μM). The PGE_2 inhibitory effect was contributed by the suppression of the IFN-γ/LPS-stimulated COX-2 gene expression, without affecting the phorbol myristate

acetate (PMA)-stimulated COX-1 gene expression in RAW264.7 cells by these three compounds. Arene substitution patterns and substituents of electron withdrawing groups may contribute to the PGE_2 inhibition activity of the compounds. Besides, QSAR study recommended that positive contribution of lipophilicity and numbers of rotatable bonds, and negative contribution of kappa_2 descriptor of the compounds were crucial for their anti-inflammatory properties. The enzymatic assay showed that most curcumin derivatives tested selectively inhibited COX-1 activity rather than COX-2. However, compounds 25 and 43 selectively inhibited COX-2, unlike compound 27 which favours towards COX-1 activity. Moreover, docking study revealed that compounds 25 and 43 interacted with COX's active site receptors that favour towards COX-2 inhibition. Arg120, His90, Phe518 and Arg513 are important receptors involved in COX-2 inhibition, while Arg120 and Ser530 are important receptors in COX-1 inhibition. In conclusion, the experimental data have provided mechanistic insights into properties of compounds 25, 27, and 43 as COX-inhibitors. Compounds 25 and 43 could be potential lead compounds for development of new COX-2 selective inhibitors.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Master Sains

PENILAIAN TERHADAP TERBITAN KURKUMIN SEBAGAI PERENCAT SIKLOOKSIGENASE-2 YANG BAHARU MELALUI ANALISIS *In Silico* DAN *In Vitro*

Oleh

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Prostaglandin E_2 (PGE_2) merupakan satu daripada pengantara lipid bagi radang. Keradangan kronik telah mendorong lebihan pengeluaran PGE_2 yang membawa kepada perkembangan penyakit radang kronik. PGE_2 disintesis oleh enzim siklooksigenase (COX) yang wujud dalam isoform COX-1, yang diekspres sebagai penyelenggara; dan COX-2 yang terinduksi. Dadah anti-radang bukan steroid (NSAIDs) ialah dadah yang merencat COX-2 untuk mengawal lebihan pengeluaran PGE_2 semasa radang, tetapi NSAIDs komersil kebanyakannya secara terpilih merencat COX-1 atau tidak berdaya memilih yang mana memampas kepada pembatasan dan kesan sampingan yang memudaratkan oleh ubat tersebut. Oleh itu, dengan mentafsirkan mekanisme yang berdaya memilih bagi merencatkan COX-2 adalah menjadi keutamaan. Kurkumin telah dikenalpasti sebagai ubat bagi merawat penyakit-penyakit berkaitan keradangan, tetapi menderita daripada bioketersediaan yang lemah dan ketakstabilan. Sintesis terbitan kurkumin telah dilakukan bagi mengatasi keterbatasannya. Jadi, objektif kajian ini adalah bagi menyiasat kesan 43 terbitan kurkumin terhadap penghasilan PGE_2 sel teraktif dan aktiviti COX, serta bagi memahami mekanisme tindakannya *in silico* dan *in vitro.* Dalam kajian ini, kesan terbitan kurkumin terhadap penghasilan PGE_2 dalam sel makrofaj murin (RAW 264.7) yang diransang oleh kombinasi interferon-gama (IFN-γ) dan lipopolisakarida (LPS), dinilai melalui prosedur imunoasai. Analisis hubungan struktur-aktiviti kuantitatif (QSAR) telah dilakukan untuk mengaitkan antara struktur dan aktiviti perencatan PGE_2 oleh terbitan kurkumin. Asai enzim dan analisis mengedok molekul dilakukan bagi mentafsir mekanisme perencatan aktiviti COX oleh terbitan kurkumin. Kesan terbitan kurkumin yang aktif terhadap ekspresi gen COX-1 dan COX-2 juga ditentukan. Keputusan menunjukkan 3 daripada 43 sebatian merencat penghasilan PGE_2 dalam sel yang diransang IFN-γ/LPS dengan ketara mengikut dos iaitu 2,6-bis(2-fluorobenzilidena)sikloheksanon (sebatian 25), 2,6-bis(4-fluorobenzilidena)sikloheksanon (sebatian 27), dan 2,5-bis(3,4,5-trimetoksibenzilidena)siklopentanon (sebatian 43) dengan nilai IC_{50} masing-masing 6.15 ± 0.48 µM, 5.78 ± 1.67 µM dan 12.15 ± 1.88 µM yang mana lebih tinggi berbanding kurkumin. Tambahan pula, tiga sebatian ini tidak toksik kepada sel

(kesitotoksian IC_{50}>500 µM). Kesan perencatan PGE_2 disumbangkan oleh penindasan gen COX-2 yang diransang IFN-γ/LPS, tanpa memberi kesan terhadap ekspresi gen COX-1 yang diransang forbol miristat asetat dalam sel RAW264.7 oleh tiga sebatian tersebut. Pola penggantian arin dan kumpulan penarikan electron mungkin menyumbang kepada aktiviti merencat PGE_2 sebatian tersebut. Selain itu, kajian QSAR telah menganjurkan bahawa sumbangan positif lipofilik dan nombor ikatan boleh putar, dan juga sumbangan negatif penerang kappa_2 sebatian tersebut adalah penting untuk sifat anti-radangnya. Asai enzim menunjukkan kebanyakan terbitan kurkumin yang diuji dengan terpilih merencat aktiviti COX-1 berbanding COX-2. Bagaimanapun, sebatian 25 dan 43 dengan terpilih merencat COX-2, tidak seperti sebatian 27 yang cenderung kepada aktiviti COX-1. Selain itu, kajian mengedok mendedahkan bahawa sebatian 25 dan 43 berinteraksi dengan reseptor tapak aktif COX yang cenderung kepada perencatan COX-2. Arg120, His90, Phe518, dan Arg513 adalah reseptor penting yang terlibat dalam perencatan COX-2, manakala Arg120 serta Ser530 adalah reseptor penting dalam perencatan COX-1. Kesimpulannya, data eksperimen ini telah menyediakan pemahaman mekanisme bagi ciri-ciri sebatian 25, 27 dan 43 sebagai perencat COX. Sebatian 25, dan 43 boleh menjadi sebatian utama yang berpotensi untuk pembangunan perencat COX-2 yang baharu.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my thankful to ALLAH s.w.t for all His blessings that empower me to complete this Master's project and thesis successfully. There are plentiful individuals towards whom I owe sincere gratitude and without their contributions, this study could not have been completed.

I would like to thank my supervisor, Dr. Syahida Ahmad for giving me the opportunities to explore the wonder of sciences. I am driven by her enthusiasm in research and I am thankful that she has always positively encouraged me to come out with my own ideas in performing this project. Her expert guidance and mentorship at all levels have made my thesis completion possible. My heartfelt gratitude is also extended to my co-supervisors, Assoc. Prof. Dr. Faridah Abas and Dr. Lam Kok Wai. With their support for the project, I have benefited greatly from their insightful knowledge and advices.

Nonetheless, I would like to express my appreciations to all my labmates especially Naimah, Amiza, Atika, Najwa, Madam Yu, Yakubu and Ibrahim in Laboratory of Drug Discovery, FBSB for their willingness to share knowledge with me. Special thanks to research officer, Dr Tan Sheau Wei from Laboratory of Vaccine and Immunotherapeutic, IBS for her expert guidance which helped me a lot in carrying out Real Time PCR from the start to its completion.

I would also like to thank Graduate Research Fellowship (UPM) and MyMaster Scholarship (Ministry of Education Malaysia) for funding me during my Master of Science programme. I am beyond grateful to my dear mother, Hasnah binti Mohamad as well as my parents in law, Mohd Sakeh bin Abd Jabar and Badariah binti Jaafar whom have always been proud of me and believe in me. May Allah grant tranquility to my late father, Abdul Bahari bin Abdul Rani whom I have admired most. My love and pray to my family members whom have always being courteous, supportive and thoughtful towards me. Their endless love, understanding, patience and sacrifices have been an enormous source of strength and inspiration for me. May Allah bless them.

Ultimately, I would like to dedicate this thesis to my other half, Nurshafika binti Mohd Sakeh. She has inspired me with great patience, responsibility and honesty in everything I do. She has never failed to lift up and polishes all the good values in me. Her intense love, support, motivation, ideas, humor and limitless encouragement from the very start of this project have made the completion of this thesis a memorable, painless one. May Allah grant her every happiness she deserves till Jannah.

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

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AD	Alzheimer's disease
Adj-R^2	adjusted R^2
ANOVA	one-way analysis of variance
AP	activator protein
APC	Adenoma Prevention with Celecoxib
APC^{min}	Adenomatous polyposis coli
APPROVe	Adenomatous Polyp Prevention on Vioxx
ARG	Arginine
ATCC	American Type Culture Collection
B_o	maximum binding
β-actin	beta actin
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
cAMP	Cylic adenosine monophosphate
$CDCl_3$	deuterated chloroform
cDNA	complementary Deoxyribonucleic acid
CLASS	Celecoxib Long Term Arthritis Safety Study
CNS	Central nervous system
COX	Cyclooxygenase
CO_2	Carbon dioxide
CRE	cAMP response element
C_T	Threshold cycle
DAMP	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dsDNA	double strand DNA
E.C	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EIA	enzyme immunoassay
EIMS	Electron Ionization Mass Spectroscopy
ERK	Extracellular signal-regulated kinase
EtOH	ethanol
Ev	electronvolt
FBS	fetal bovine serum
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GFA	Genetic Function Approximation
GI	gastrointestinal
Gln	Glutamine
Glu	Glutamine
GPCRs	G protein-coupled receptors
h	Hour/s
HCl	Hydrochloric acid
His	Histidine
HPLC	high-performance liquid chromatography

ICAM	Intercellular adhesion molecule 1
IC_{50}	Inhibitory concentration 50%
IFN-γ	Interferon-gamma
IgG	immunoglobulin G
IL	Interleukin
IL-β	interleukin 1 beta
Ile	Isoleucine
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
kb	kilobase
KBr	Potassium bromide
Kcal	kilocalorie
LOF	Friedman's lack of fit
LOX	lipoxygenase
LPS	Lipopolisaccharide
LSE	least-squares error
M	Molar
MAPK	Mitogen-activated protein kinase
MBD	membrane binding domain
mCOX	murine cyclooxygenase
mg	miligram
MHz	megahertz
min	Minute
ml	Millilitre
mM	Millimolar
mm	millimeter
mmol	milimol
mPGES	microsomal prostaglandin E synthase
mRNA	Messenger ribonucleic acid
MS	Mass spectra
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaOH	Sodium hydroxide
NF-κB	Nuclear factor-kappa B
Nm	nanometer
NMR	Nuclear Magnetic Resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
NSB	non-specific binding
NS-398	*N*-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide
NTC	Non-template control
O_2	Oxygen
oCOX	ovine cyclooxygenase
OD	optical density
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline
PD	Parkinson's disease
PG	Prostaglandin
pg	picogram
Phe	Phenylalanine
PLA_2	Phospholipase A_2
PMA	Phorbol myristate acetate

ppm	parts per million
Pred-R^2	predicted R^2
QSAR	Quantitative structure-activity relationship
RNA	Ribonucleic acid
RA	Rheumatoid arthritis
RFU	relative fluorescence unit
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
R^2	Extinction of coefficient
s	second
S.E.M	Standard error of mean
Ser	Serine
SUCCESS	Successive Celecoxib Efficacy and Safety Study
TA	Total activity
TLC	thin layer chromatography
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Tyr	Tyrosine
U	Unit
UV	Ultraviolet
V	Volt
Val	Valine
VCAM	Vascular cell adhesion molecule 1
VIGOR	Vioxx Gastrointestinal Safety of Rofecoxib
VSMC	Vascular smooth muscle cells
Å	Angstrom
ºC	Degree celcius
µg	Microgram
µL	Microlitre
µM	Micromolar
ηM	nanomolar

CHAPTER 1

INTRODUCTION

Inflammation is a complex biological response. As part of immune system, the inflammation response not only serves as protective frontline towards harmful stimuli, but also as convergence point towards progression of severe inflammatory defects. Inflammation is an intrinsically beneficial event for biological host in fighting against offending factors such as pathogens, toxins, injuries, and chemical irritants with aims to eliminate the injurious factors, promote healing process and tissue restoration, as well as to build up memories for a faster and more specific counteractions in future occurrence (Stables and Gilroy, 2011).

The beneficial effects of inflammation were normally implemented during acute phase which is the initial stage of inflammation. In this stage, innate immunity takes place through recruitment of activated cells like neutrophils, dendritic cells, lymphocytes and macrophages to the site of injury. They integrate to phagocytize the pathogens and promote healing process. However, persistence of pro-inflammatory stimuli will lead to sustainment of inflammation for weeks, months and even years if the stimuli cannot be eliminated or if there are problems with healing process such as auto-immunity (Stables and Gilroy, 2011).

The persistence of inflammation has been reported to be associated with pathophysiology of chronic stage. Extended period of inflammation is problematic which can harm physiological systems due to tremendous increase of multiple reactive oxygen species and pro-inflammatory mediators such as prostaglandins, cytokines, inflammatory enzymes, and protein kinases (Kellog *et al*., 2015). Damage on cells/tissues is the initial process which may lead to several serious chronic inflammatory diseases like rheumatoid arthritis (RA), Alzheimer's disease (AD) and cancers (Ricciotti and Fitzgerald, 2011).

One of the important regulators in inflammatory response is prostaglandin. This physiologically active 20-carbon lipid compound in normal condition, engages in various homeostasis regulation such as blood pressure, gastrointestinal integrity, cardiovascular system, central nervous system (CNS) activity, and fertility (Ricciotti and Fitzgerald, 2011). Prostaglandin also involves in onset of cardinal signs of inflammation: pain, heat, redness, and swelling. Thus it has been referred as classical pro-inflammatory mediator (Funk, 2001; Harris *et al*., 2002).

Derived from plasma membrane arachidonic acid (AA), prostaglandin exists in several isoforms, and most abundant isoform in human is PGE_2 (Serhan and Levy, 2003). PGE_2, through different binding receptors, termed EP1 to EP4, exerts multiple homeostasis and inflammatory signals (Sugimoto and Narumiya, 2007). PGE_2 can also repeatedly cooperate with cytokine and pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) during multiple inflammatory events and amplifies cytokine and PAMP/DAMP signaling by boosting the expression of inflammation-related genes induced by these stimuli (Aoki and Narumiya, 2012). Alleviated PGE_2

levels in human have been linked specifically with pancreatic, colorectal, breast, and lung cancer (Wang *et al*., 2007).

Biosynthesis of PGE_2 from AA is catalyzed by prostaglandin G/H synthase, or cyclooxygenase (COX). The enzyme exists in two isoforms. The first isoform is cyclooxygenase-1 (COX-1), that constitutively and ubiquitously expresses prostaglandin at basal level to coordinate physiological conditions. COX-1 is present in almost every cells in human body. On the other hand, the second isoform is an inducible enzyme known as cyclooxygenase-2 (COX-2). COX-2 is produced only in injured tissues relatively at high levels and subsequently produces large amount of PGE_2 upon triggered by inflammatory stimuli (Lee[a] *et al*., 2009).

Activated macrophages serve as one of the central producers of COX-2 in the course of inflammation (Bowdish *et al*., 2007). Combination of lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) synergistically stimulates the activation of macrophage (Chan and Riches, 2001). Persistence of stimulation by these pro-inflammatory mediators however may resulted in over-expression of COX-2 which in turn leads to excess production of PGE_2 and consequently ends up with various chronic inflammatory diseases and tumors (Greenhough *et al*., 2009; Wang *et al*., 2007).

Production of prostaglandin particularly through inflammation can be controlled by targeting COX activities. Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs which have been reported to deliver pharmacological effects such as analgesic, anti-pyretic, and anti-inflammatory properties (Vonkeman and Laar, 2010). The mechanisms of most NSAIDs are particularly either blocking the production of prostaglandin via competitively bind and inhibit the activity of COX enzyme, suppress the COX gene expression itself or inhibition of transcription factors (Liggett *et al*., 2014). Although plenty of successful NSAIDs have been produced, marketed and administered for a long time, mounting of health and clinical issues are raised over time.

Epidemiological studies on several traditional NSAIDs like acetylsalicylic acid, indomethacin, ibuprofen and naproxen have revealed that these medicines are non-selective on COX isoforms, reflecting both clinical efficacy and deleterious effects, which in turn associated with gastric ulceration and renal failure (Liu *et al*., 2001). Unfortunately, benefit-risk calculations have been biased on continuingly marketing these drugs due to their efficacy in treating chronic inflammation diseases.

Recently, NSAIDs that preferentially block COX-2, namely 'coxibs' family were considered as new generation of safe and effective drugs (Rao and Knaus, 2008). Unfortunately, clinical trials on prevention of colorectal cancer by rofecoxib and celecoxib resulted in myocardial infarction and stroke, and even death, which were far more serious than side effects of traditional NSAIDs (Bresalier *et al*., 2005; Solomon *et al*., 2005). The trials had led to the withdrawal of rofecoxib (Vioxx) from the market. Therefore, discovery of alternative anti-inflammatory agents is of utmost important.

On natural preference of the treatment, a wide spectrum of phytochemicals and their derivatives have been identified for development as anti-inflammatory agents. Interestingly, curcumin which is widely presents as secondary metabolite in plants has been recognized as potent inhibitor against inflammation (Bukhari *et al*., 2013). However, curcumin suffers from major drawbacks due to poor bioavailability. Once consumed, it will go through hepatic conjugation, resulted in production of glucuronides and sulphates whereas systemic administration caused it to be eliminated (Anand *et al*., 2007). The problem is due to unstable 7-carbon spacer of ß-diketone moiety (diarylheptanoid) of curcumin, which is a specific substrate for liver aldo-keto reductases (Anand *et al*., 2007).

Synthesizing a new class of curcumin related compounds is one of researchers' approaches (Leow *et al*., 2014). In addition, computational analysis has become another powerful tools in modern research area which can be utilized to understand the structure-activity relationship of the potential compounds. Recently, a few studies have proved the pharmacological interest of curcumin derivatives due to its effectiveness against inflammation (Jantan *et al*., 2012). These findings have enormously encouraged the development of better amendment of newly synthesized curcumin derivatives. Nevertheless, the effects and mechanism of actions of curcumin derivatives towards PGE_2 in normal and inflammatory conditions are poorly understood.

Here, a series of curcumin derivatives were assayed for prostaglandin E_2 inhibition in IFN-γ/LPS-stimulated RAW264.7 (macrophage) cells as well as on pure COX-1 and -2 enzyme activities. Their potential in PGE_2 inhibition was analyzed quantitatively related to their molecular geometry. While molecular docking study was exploited to find a theoretical mechanism of inhibition of curcumin derivatives in COX active sites in comparison with their effects on the enzymatic activity. Finally, samples were tested on *COX* genes expression level.

Objectives of study

The general objective of this study is to elucidate the anti-inflammatory properties of curcumin derivatives in IFN-γ/LPS-stimulated macrophage cells.
The specific objectives are:

1. To evaluate the inhibition activity of 43 curcumin derivatives towards PGE_2 production in IFN-γ/LPS-stimulated RAW264.7 cells.
2. To measure geometric and chemical characteristics of curcumin derivatives in which related to their PGE_2 inhibition activity using quantitative structure-activity relationship (QSAR) analysis.
3. To determine the binding orientation of curcumin derivatives in COX active sites using molecular docking approach and correlate with COX enzymatic assay.
4. To determine the effect of selected curcumin derivatives on mRNA expression of COX-1 and -2 in IFN-γ/LPS-stimulated RAW264.7 cells.

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