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Design, Synthesis and Biological Evaluation of Novel Curcumin Analogues as Inhibitors of Matrix Metalloproteinases and Pro-inflammatory Cytokines

A Dissertation Presented

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Yu Zhang

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We, the dissertation committee for the above candidate for the

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Abstract of the Dissertation

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There are two principal reasons why we age: 1) the toxicity of oxygen species; 2) the continuous degradation of collagen by groups of enzymes such as the matrix metalloproteinases (MMPs). The MMPs are a group of more than 25 structurally zinc-containing enzymes that are involved in the degradation of numerous extracellular, pericellular and non-matrix proteins. In many disease conditions their levels and proteinase activity rise, producing pathological and serious structural damage. Tetracyclines (TCs) are known inhibitors of mammalian-derived MMPs, and non-antibiotic formulations of doxycycline are FDA-approved to treat periodontitis and the chronic inflammatory skin disease, rosacea. However, a significant limitation is that the FDA only permits the use of subantimicrobial doses of these antibiotics for these diseases to prevent antibiotic side-effects. Therefore a series of chemically-modified TCs (CMTs), which are no longer anti-bacterial at any dose, were developed, but a significant side-effect was increased

photosensitivity. Accordingly, in order to avoid these limitations, a series of chemically-modified curcumins (CMCs) were prepared and evaluated as new MMP inhibitors because they have the same zinc-binding site as the TCs, namely a polyenolic assembly. The current lead compound, CMC2.24 [a bis-(demethoxy) phenylaminocarbonyl derivative of curcumin], exhibits inhibitory IC_{50} values *in vitro*, ranging from 2-8μM against two collagenases (MMP-8 and MMP-13), two gelatinases (MMP-2 and MMP-9), MMP-3, MMP-7 and MMP-12. The zinc-binding as well as the acidity constants of these CMCs were evaluated and correlated to their potency as MMP-inhibitors *in vitro*. An *in vitro* lipophilicity study and *in vivo* pharmacokinetics were also carried out for curcumin and CMC2.24. The results show that CMC2.24 is more bioavailable in rat serum, and is retained in organ tissues such as the liver, heart, spleen, lung, kidney and brain in comparison with curcumin, which showed much lower levels of retention in these tissues. It was also found that the production of the pro-inflammatory cytokines and the chemokines including TNF-α, IL-1β and MCP-1 was significantly reduced by CMC2.24 and a related trione (CMC2.5) in cell culture. Collateral studies with CMC2.24 involving its effects on various disease models are discussed.

Dedication

谨以此论文献给我亲爱的的父母和祖父母，感谢他们的抚育，教导，支持，鼓励！

This thesis is dedicated to my parents, Mr. Zhiqiang Zhang and Ms. Wei Liang, for it is their love, support and encouragement that bring a project like this to fruition.

Also to my grandparents, Mr. Shushan Zhang and Ms. Yujie Gong, for it is the good care they have been taking of since the first day I was born.

"You raise me up,

So I can stand on mountains;

You raise me up,

To walk on stormy seas;

I am strong,

When I am on your shoulders;

You raise me up,

To more than I can be."

----Brendan Graham, Irish novelist and composer

Table of Contents

List of Figures

List of Tables

List of Schemes

List of Abbreviations

ADME: Absorption, distribution, metabolism and excretion

APMA: p-Aminophenylmercuric acetate

ARDS: Acute respiratory distress syndrome

BAM: Bis-aroyl methane

CD: Circular dichroism

CMC: Chemically-modified curcumin

CMT: Chemically-modified tetracycline

COOT: Crystallographic object oriented toolkit

CRP: C-reactive protein

DMSO: Dimethyl sulfoxide

Dnp: 2,4-Dinitrophenyl

Dpa: N-3-(2, 4-Dinitrophenyl)-L-2,3-diaminopropionyl

ECM: Extracellular matrix

ESI-MS: Electrospray ionization mass spectrometry

FDA: U.S. Food and Drug Adminstration

FPLC: Fast protein liquid chromatography

HPLC: High performance liquid chromatography

IC_{50}: The half maximal inhibitory concentration

IND: Investigational new drug

i.p.: Intraperitoneal

IPTG: Isopropyl β-D-1-thiogalactopyranoside

i.v.: Intravenous

LDL: Low-density lipoprotein

LPS: Lipopolysaccharide

MALDI: Matrix-assisted laser desorption/ionization

MBP: Maltose binding protein

Mca: (7-Methoxycoumarin-4-yl)acetyl

MIC: Minimum inhibitory concentration

MMP: Matrix metalloproteinase

MW: Molecular weight

NMR: Nuclear magnetic resonance

Nval: Norvaline

OA: Osteoarthritis

PBMC: Peripheral blood mononuclear cells

PDB: Protein data bank

PMN: Polymorphonuclear

SAR: Structure-activity relationship

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

STZ: Streptozotocin

TCs: Tetracyclines

TIMPs: Tissue inhibitors of matrix metalloproteinases

TEV: Tobacoo etch virus

TLC: Thin-layer chromatography

Xaa: Acidic amino acid

Preface

"I have never let my schooling interfere with my education."

----Mark Twain, American author

"Prediction is very difficult, especially about the future."

----Niels Henrik David Bohr, Danish physicist

Inspired by my high school teacher's remark, "Chemistry is a science of finding, representing and creating nature, and it is the one field of making full use of my logical thinking merit.", I chose chemistry as my major because it is abstruse, challenging and interesting.

I came to Stony Brook for graduate study in the fall of 2007 after I completed my bachelor degree in Jilin University, China. I still remember what my father told me before I boarded the plane from Beijing to New York, "Be confident, capable and committed!", which guided me through all my days for the past five years.

In the spring of 2008, I began my research project in Dr. Francis Johnson's lab. I have been focusing on the project *Design, Synthesis and Biological Evaluation of Novel Curcumin Analogues and Bis-Aroyl Methanes as Inhibitors of Matrix Metalloproteinases and Pro-Inflammatory Cytokines for Diseases of Tissue Loss*. This project was derived from the idea of the therapeutical use of thermorubin, a known antibiotic.

Back to 2004, Dr. Johnson came to Center for Biotechnology at Stony Brook University to further pursue the biological efficacy of thermorubin, and then he was introduced to Dr. Lorne M. Golub, the inventor of two FDA approved drugs, non-antibacterial (sub-antimicrobial dose)

tetracyclines. At that time, thermorubin was initially examined as an inhibitor against the proteinase activity of *P. acnes*. However, the antimicrobial activity and poor solubility potentially limit the long-term therapeutic use for the inhibition of cytokines and chemokines and microbial proteinases. Then a series of bis-aroyl methanes (BAMs) were synthesized, because of their similar 1,3-diketone moiety related to the tetracyclines. Preliminary data on the BAMs showed great inhibitory interest against pro-inflammatory cytokines and chemokines.

Because curcumin is structurally the vinyl homologue of BAMs, I carried out the design and synthesis of a series chemically-modified curcumins under the mentorship of Drs. Johnson and Golub, with increasing potency as proteinases inhibitors, based on the studies of structure activity relationship (SAR).

While this thesis delves into the discovery and development of novel MMP inhibitors in depth, I hope more general messages can be extracted, that have relevance well beyond this sector. Fortunately in consulting with our research team, especially Drs. Johnson and Golub, I have been on the lookout for what can be leveraged from their experience. Much has been learned, and much more will be learned.

Everything has to come to an end, meanwhile it is a beginning of another story. I would like to quote Dr. Johnson from the symposium to celebrate his 80th year birthday, “Be passionate, patient and persistent!” With this in mind, I sincerely hope the scientific discoveries we have made toward the new type of MMP inhibitor will improve human health and well being.

Acknowledgments

A thesis like this would simply not have been possible without the help and support of many dedicated people.

I really want to express my deepest appreciation to my advisor, Dr. Francis Johnson, for his guidance, support and encouragement of my research. Dr. Johnson is truly a friend, who introduced me to the drug discovery arena. Dr. Johnson has been inspiring me with his enthusiasm, knowledge and expertise all the time. The most importment thing I have learned from Dr. Johnson in the past five years is not just the solution of questions, but the way to solve problems: how to think independently and act independently.

I am particularly indebted to Dr. Lorne M. Golub, who guided me throughout the whole project, helped me formulate ideas of research work and provided me continuous helpful feedback. As my "co-advisor", Dr. Golub shaped my thinking about many of the core issues I confronted in this thesis and my publications, not only providing insight into the biology behind the whole project, but also offering me the opportunities to make decisions on my own and to build my confindence.

I was blessed with an incredibly talented and dedicated research team. I would like to thank Dr. Hsi-Ming Lee, who gave me invaluable assistance on my research work on biochemistry. I am grateful to Dr. Arnold Wishnia, who was tenacious in processing and analyzing the data on biophysics. I would like to thank Dr. Stephen G. Walker, for his assistance in the antimicrobial study. I am also grateful to Dr. Miguel Garcia-Diaz, for his guidance and suggestions through the X-ray crystallography studies. I would like to thank our collaborators in United States, Canada and Brazil for their endeavor to move this project forward.

I am sincerely grateful to my committee members, Dr. Kathlyn A. Parker, Dr. Robert C. Kerber and Dr. Carlos de los Santos for their suggestions and comments on the project. I was fortunate to benefit from the discussions on my research work. I am also sincerely grateful to Department of Chemistry and Department of Pharmacological Sciences at Stony Brook University for the support of my graduate study and research work. I would like to thank Chem-Master International, Inc. for their generous support of chemicals and reagents.

I am beholden to my family, friends and colleagues, who made my graduate study in Stony Brook a truly wonderful journey.

Vita, Publications and Fields of Study

Education

Ph.D. in Chemistry, Stony Brook University 2007-2012

B.S. in Chemistry, Jilin University, China 2003-2007

Publications

1. **Zhang, Y.**; Gu, Y.; Lee, H.M.; Hambarjieva, E.; Vrankova, K.; Golub, L.M.; Johnson, F. Design, Synthesis and Biological Activity of New Polyenolic Inhibitors of Matrix Metalloproteinases: A Focus on Chemically-Modified Curcumins. *Curr. Med. Chem.*, **2012**, *19*, 4348-4358

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Abstracts

1. Clemens, M.; Napolitano, N.; Lee, H.M.; **Zhang, Y.**; Johnson, F.; Golub, L.M.; Gu, Y. Chemically modified curcumin normalizes chronic inflammation in diabetic rats. *J. Dent. Res.*, **2012**, *91*, Special Issue 1526

2. Napolitano, N.; Clemems, M.; Lee, H.M.; **Zhang, Y.**; Johnson, F.; Golub, L.M.; Gu, Y. Novel curcumin derivatives suppress inflammatory mediators in periodontally-relevant cells. *J. Dent. Res.*, **2012**, *91*, Special Issue 1527

3. Elburki, M.; Lee, H.M.; Gupta, N.; Balacky, P.; Zhang, Y.; Johnson, F.; **Zhang, Y.**; Golub, L.M. Chemically-modified curcumins inhibit alveolar bone loss in diabetic rats with periodontitis. *J. Dent. Res.*, **2012**, *91*, Special Issue 1528

4. Yu, H.W.; Zhang, Y.; **Zhang, Y.**; Lee, H.M.; McClain, S.A.; Johnson, F.; Golub, L.M.. A novel chemically-modified curcumin (CMC2.24) improves diabetic wound-healing. *J. Dent. Res.*, **2012**, *91*, Special Issue 1529

5. **Zhang, Y.**; Lee, H.M.; Golub, L.M.; Hambardjieva, E.; Tong, S.; Pierre, B.; Vrankova, K.; Johnson, F. Synthesis of poly-enolic zinc-binding compounds, new inhibitors of matrix metalloproteinases and inflammatory mediators for periodontitis and other diseases. *Abstracts of papers, 241st ACS National Meeting*, MEDI 242, Anaheim, CA, March, **2011**

6. Golub, L. M.; Lee, H. M.; Zhang, Y.; London, L.; Grewal, J. S.; Sorsa, T.; Hambardjieva, E.; **Zhang, Y.**; Johnson, F. New collagenase inhibitors for periodontitis and other diseases of tissue erosion. *International Association for Dental Research*, Barcelona, Spain, July, **2010**

Fields of Study

Organic Chemistry: organic compound synthesis, purification and process development

Analytical Chemistry: chemical structure analysis, characterization and determination

Physical Chemistry: acidity, metal and protein binding study

Enzymology: *in vitro* optimization (IC_{50}) and *in vivo* validation

Pharmacokinetics: *in vivo* drug distribution and metabolism

Structure Biology: cloning, protein expression and purification, and X-ray crystallography

Chapter 1. Introduction

Matrix metalloproteinases (MMPs) are a family of more than 25 zinc-containing endopeptidases responsible for tissue degradation. MMP-1, also called Collagenase-1, discovered by Gross *et al.* in 1962, is an animal-derived neutral proteinase that could degrade triple helical collagen fibrils into ¼ and ¾ fragments under physiological conditions of pH and temperature.[1] Subsequently additional MMPs have been found that they can degrade almost all of the constituents of the extracellular matrix (ECM) including collagens and elastins. When the levels of MMPs are excessively expressed under pathological conditions, the degradation by MMPs in the ECM can cause the destruction of collagen, connective tissue and bone tissue.[2,3] Scientists in both academia and industry have long tried to develop compounds to inhibit pathologically excessive MMP activity for human diseases such as periodontal disease, arthritis, postmenopausal osteoporosis, cardiovascular disease and cancer.[4-6] Basal levels of MMPs are essential for normal connective tissue turnover and remodeling under physiological conditions, whereas the excessive inhibition of MMPs can cause various side effects such as musculoskeletal side-effects, including muscle aches, pain, weakness and cramps.[7,8] To date, the only governmental MMP inhibitor drugs available (USA, Canada, Europe) to clinicians for the treatment of periodontitis and chronic skin disease or any other disease are several formulations of sub-antimicrobial doses of doxycycline. This drug arose because of the discovery in 1983 by Golub *et al*. that an old family of drugs, tetracyclines (TCs), could inhibit mammalian collagenases, and several other matrix metalloproteinases (MMPs), and by non-antimicrobial mechanisms.[9-12] Studies have also shown that tetracyclines not only inhibit MMPs including collagenases and gelatinases from a variety cells including macrophages and polymorphonuclear (PMN) leukocytes, but also inhibit the activation of MMPs from their latent forms, and can decrease Pro-MMP synthesis. With this

in mind, beyond the development of two FDA-approved orally available drugs, Periostat® for the treatment of periodontitis and Oracea® for chronic inflammatory skin disease, several chemically-modified tetracyclines were developed, lacking antimicrobial activity but possessing the MMP inhibitory activity.[10-12] CMT-3, one of the chemically-modified tetracyclines, appeared to be the most potent inhibitor against a variety of cytokines and chemokines in addition to MMPs, and was found to be active in a clinical study of Kaposi's sarcoma with anti-angiogenic activity.[13,14] A significant drawback of the tetracyclines as well as the non-antimicrobial chemically-modified tetracyclines is the side-effect of increased photosensitivity at higher doses, which gave the concern in its systematic long-term use.[15,16]

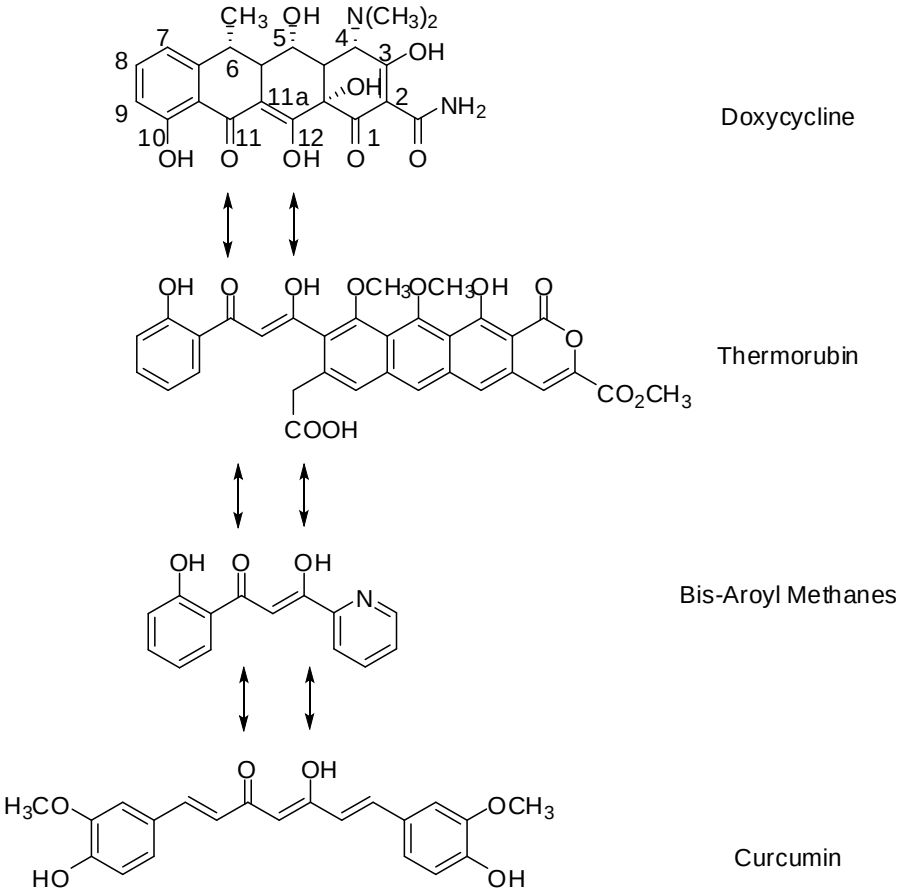
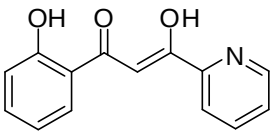
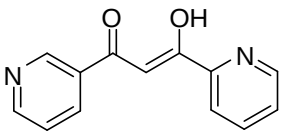


Figure 1.1 Doxycycline, Thermorubin, Bis-Aroyl Methanes and Curcumin

Since the enolic β-diketone moiety of tetracyclines at carbon 11, 11a and 12 is responsible for the inhibition of MMPs through the binding to the zinc atom in the catalytic domain of these enzymes, thermorubin, a natural product isolated from a thermophilic fungus *Thermomycetes* in the countryside near Pavia, Italy, was first examined as an MMP inhibitor using the proteinase activity of *P. acnes* as a model because the side chain functionality of thermorubin mimics the linear array of oxygen atoms in tetracycline (Figure 1.1). However, the antimicrobial activity of thermorubin potentially limits the long-term therapeutic use for the inhibition of cytokines and chemokines including MMPs.[17] Bis-aroyl methanes (BAMs) appeared to be a hydrid form of thermorubin and tetracycline since all molecules contain a β-diketone moiety (Figure 1.2). Preliminary studies (unpublished) on the BAMs as inhibitors of MMPs showed reasonable but not improved activity in contrast to the TCs. BAM 1.11 and BAM1.12 each showed *in vitro* IC_{50} of 53 μM and 60 μM against human-derived neutrophil collagenase (MMP-8), and no evidence of toxicity was observed after 5 hour incubation with monocytes. BAM1.11 and BAM1.12 each showed 90% and 100% of inhibition against interlukin-1β (IL-1β), the pro-inflammatory cytokine up to 50 μM in monocytes cells in culture, stimulated by lipopolysaccharide (LPS). However, because of the limitations on the improvement of acidity of these compounds as well as the maintenance of the planarity, we started to focus on a series of bis-vinyl homologues of BAMs, namely chemically-modified curcumins (CMCs).



BAM1.11



BAM1.12

Figure 1.2 Bis-Aroyl Methanes (BAMs)

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], is the bis-vinyl homologue of BAMs, and also possesses a β-diketone assembly responsible for the metal ion-binding. Curcumin is a natural dietary ingredient of turmeric, derived from the perennial herb *Curcuma longa L*. Curcumin has been used as an herbal treatment for various diseases including pulmonary, gastrointestinal, liver diseases, non-healing wounds and cancer due to its anti-inflammatory, anti-oxidant, anti-angiogenesis, pro-apoptotic and anti-cancer activity.[18-23] Evidence of efficacy has been shown in several *in vitro* and *in vivo* systems. However, the poor oral bioavailability, due to the poor solubility, has limited the therapeutic use of this compound in clinical studies. The current study has focused on a series of enolic bi-and tri-carbonyl compounds, chemically-modified curcumins (CMCs), with the objective of improving the solubility and metal binding characteristics, and additionally improving the biological activity against pro-inflammatory chemokines and cytokines, and principally the MMPs (Figure 1.3).

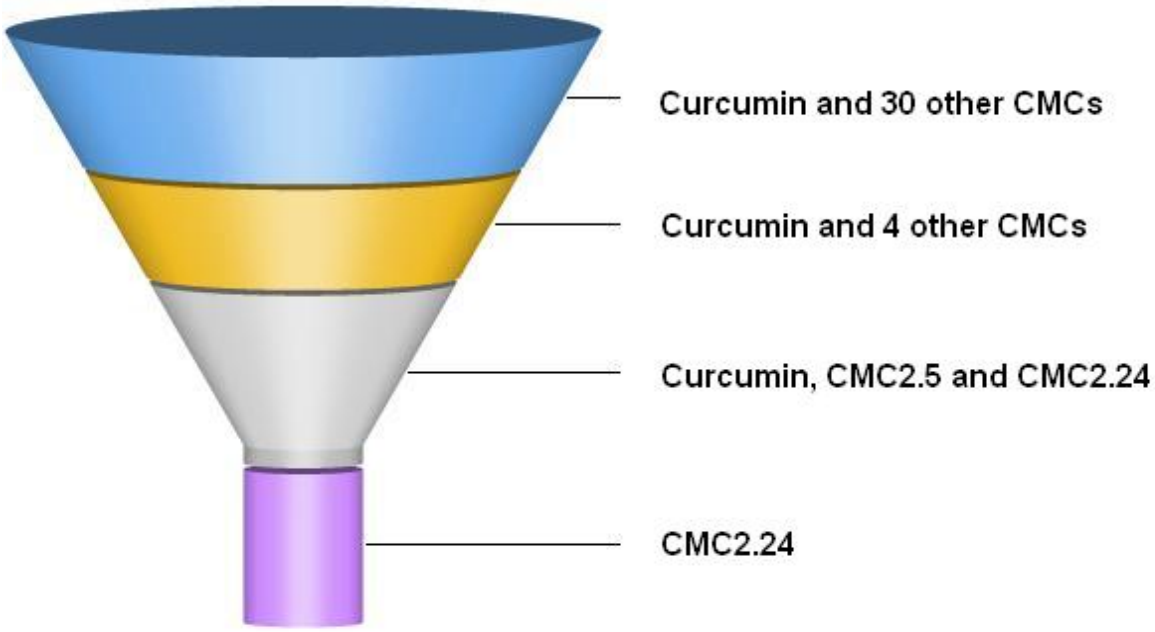


Figure 1.3 Development of novel MMP inhibitors related to curcumin

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Chapter 2. Literature review

Part 1. Matrix metalloproteinases (MMPs)

A. History

Matrix metalloproteinases (MMPs) are a group of more than 25 structurally-related zinc-containing enzymes that play an important role in the degradation of the main components of the extracellular matrix (ECM).[1,2] The first collagenolytic enzyme was discovered by Gross *et al.* in 1962.[3] In their study, the enzyme, now known as MMP-1, was recovered from the culture medium of tadpole tail resorbing fragments, and was shown to be able to degrade the triple-helical collagen molecule into two fragments, approximately one quarter distance from the C-terminus under physiological conditions of pH and temperature. Then in 1968, the inactive form of the enzyme, Pro-MMP-1, was first isolated.[4] Both the MMPs and their endogenous inhibitors of the active forms of MMPs, [mainly the tissue inhibitors of the MMPs (designated TIMPs)], play an important role in a variety of physiological and pathological conditions that involve tissue remodeling, ranging from periodontal disease, arthritis, stroke, wound healing to cancer.[5,6] TIMPs were discovered in 1975 by Bauer *et al.* during their tissue culture studies and isolated by Woolley *et al*. from serum.[7,8] Because collagen and the other constituents of the ECM exist throughout the body, and are regulated by MMPs, the development of synthetic inhibitors has been a target for academic groups and the pharmaceutical industry during the past 30 years.[9-13] Principally this thesis concerns the development of a new type of MMP inhibitor that has little toxicity and shows promise in the amelioration of a variety of diesases.

B. The Extracellular Matrix (ECM) and Basement Membrane (BM)

The extracellular matrix (ECM) is defined as the extracellular material around the cells, consisting of fibrous components (e.g., collagens, elastins, fibronectins) and non-fibrous components (e.g., proteoglycans and polysaccharides).[14] The ECM was initially considered as only providing mechanical support to tissue and cells as a static space-filling complex, but studies later showed that the ECM also controls and regulates the behaviors of cells through feedback loops, exhibiting an influence on a variety of cellular events such as adhesion, migration, proliferation, differentiation and survival.[15] Of all of the components in the ECM, collagen plays a very important role in the connective tissue of mammals. There are five common types of collagens, namely type I-V.[16] These can be subject to different types of diseases that cause connective tissue degradation, in systems such as skin, vascular ligature, bone and cartilage. Each fundamental structural unit of collagen molecule (MW about 300 kD) is a long (300 nm), thin (1.5 nm diameter) protein with three coiled subunits woven together in a characteristic right-handed triple helix: two α1 chains and one α2 chain (about 1,000 amino acids each).[17] The three α chains that form the triple helical collagen molecule comprise the structure where Glycine-X-Y is the repeating peptide triplet, in which X and Y can be any amino acid but are frequently proline and 4 (or 3)-hydroxyproline. After the synthesis of collagen molecule in fibroblasts (as pro-collagen), ascorbic acid (vitamin C) plays an important role in regulating the function of propyl hydroxylase during the posttranslational hydroxylation of proline residues.[18] Collagen cross-linking is the ability of collagen fibrils to polymerize with adjacent fibrils, while excessive levels of cross-linking leads to the deterioration of tissue function. Fibronectin is another important protein of the ECM. It is a glycoprotein which plays a prominent role in cell adhesion and migration, because it binds to matrix molecules throughout many of the organ

domains.[19] The basement membrane (BM) acts as a specialized ECM, which connects cells (e.g., epithelial cells, endothelial cells) to adjacent connective tissues.[20] BM functions as a scaffold, where cells can differentiate and migrate, and eventually regulates connective tissue-repair and remodeling processes after injury. MMPs are responsible for the physiological turnover and remodeling of nearly all components of ECM and BM, especially collagen. Unfortunately, pathological levels of these enzymes are associated with a variety of tissue-destructive diseases, including but not limited to aging, osteoarthritis and cancer.[16-20]

C. Structure and classification

1. Structural features of MMPs

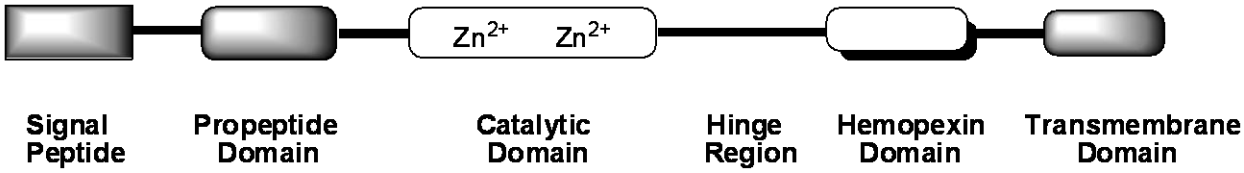


Figure 2.1 General domain structures of MMPs
(Modified from Whittaker *et al.*[21])

MMPs exhibit an almost 40% identity in their primary structures, and this includes both the prodomain and the catalytic domain (Figure 2.1).[21] The structural domains of MMPs include a signal peptide, a propeptide domain, a catalytic domain, a hinge region and a hemopexin domain, and a transmembrane domain, the latter being found only in certain types of membrane-type MMPs.[22] The prodomain usually consists of approximately 80 amino acids, and contains a conserved cysteine in the sequence -PRCGXPD- that binds to the zinc atom in the catalytic domain. The N-terminal signal peptide is responsible for the secretion and removal of the proenzyme. The propeptide domain maintains the latency of MMPs before its removal in the MMP activation process. The catalytic domain (approximately 170 residues) contains two α-

helices and five β-sheets with two zinc atoms, one of which, the catalytic zinc atom, is chelated by three adjacent histidine residues and is responsible for the proteolytic activity of the MMPs.[23,24] All MMPs except MMP-7, MMP-20, MMP-21 contain a hemopexin domain, and a hinge region which connects the latter to the catalytic domain. The function for the hemopexin domain varies with different MMPs. For example, in the collagenases, it is responsible for the TIMP and substrate binding, whereas in gelatinases, it is only responsible for the interaction with TIMPs.[25] The transmembrane domain as well as a furin-activation site is found in only certain MMPs, such as membrane-type MMPs, in which furin is one member of subtilisin-like proprotein convertase family, responsible for the activation of proenzymes.[26] In the ECM, MMPs exist as Pro-MMP forms, which are inactive. There are two steps involved in the MMP extracellular activation related to cysteine switch mechanism: 1) uncovering of the zinc ion from the propeptide in which zinc interacts with cysteine residue through the thiol group in the cysteine complex, and 2) cleavage of the propeptide by other endopeptidases such as membrane-type MMPs. The *in vitro* activation of MMPs also involves the chemical processing whereby the sulfur atom is inactivated by alkylation reagents, disulfides formation, oxidation, or mercury (or silver) salt formation.[27-30]

2. Classification of MMPs

MMP classification is based on the different substrate specificities observed during *in vitro* studies of the individual enzymes. There are five sub-groups of MMPs in total: collagenases, gelatinases, stomelysins, membrane-type, and unclassified. These are listed in Table 2.1.[11-13]

Table 2.1 Matrix metalloproteinases (MMPs) and their principle substrates (Modified from references[11-13])

Group	Enzyme	MMP	Principal Substrates
Collagenases	Fibroblast Collagenase	MMP-1	Fibrillar Collagens, PG Core Protein
	Neutrophil Collagenase	MMP-8	Fibrillar Collagens, α1 Proteinase Inhibitor (Serpin)
	Collagenase-3	MMP-13	Fibrillar Collagens, PG Core Protein
	Collagenase-4	MMP-18	Collagen I
Gelatinases	Gelatinase A	MMP-2	Gelatin, Elastin, Fibronectin, Collagens IV, V and VI, Cardiac Myocyte Intracellular Contractile Proteins
	Gelatinase B	MMP-9	Gelatin, Elastin, Fibronectin, Collagen I, IV and V
Stromelysins	Stromelysin-1	MMP-3	Fibronectin, Non-Fibrillar Collagens, Laminin
	Stromelysin-2	MMP-10	Fibronectin, Non-Fibrillar Collagens, Laminin
	Stromelysin-3	MMP-11	Gelatin, Fibrillar Collagens, α1 Proteinase Inhibitor (Serpin)
Membrane-type	MT1-MMP	MMP-14	Pro-MMP-2, Fibrillar Collagens, Gelatin, Elastin, Casein, Fibronectin, Vitronectin, Aggrecan

	MT2-MMP	MMP-15	Pro-MMP-2, Gelatin, Fibronectin, Laminin, Nidogen, Tenascin
	MT3-MMP	MMP-16	Pro-MMP-2, Collagen III, Gelatin
	MT4-MMP	MMP-17	Pro-MMP-2, Gelatin
	MT5-MMP	MMP-24	Pro-MMP-2
	MT6-MMP	MMP-25	Pro-MMP-2
Unclassified	Matrilysin	MMP-7	Fibronectin, Collagen IV and X, Laminin, Aggrecan, Casein, Decorin, Insulin
	Macrophage Metalloelastase	MMP-12	Elastin, Fibronectin, Proteoglycan, α1-Antitrypsin
	RASI-1	MMP-19	Gelatin
	Enamelysin	MMP-20	Amelogenin, Aggrecan
	CA-MMP	MMP-23	Mca-peptide
	Matrilysin-2	MMP-26	Fibrinogen, Fibronectin, Vitronectin
	Epilysin	MMP-28	Casein

a. Collagenases

There are four collagenases (1-4) in the MMP family, namely MMP-1, MMP-8, MMP-13 and MMP-18. These enzymes possess the activity of cleaving native fibrillar triple-helical type I, II and III collagens.[31,32] The Glu-Ile/Leu peptide bond is the cleavage site of the collagen α-chain, which generates two collagen cleavage fragments, one three times the size of the other on scission.[33]

MMP-1 was the first MMP discovered by Gross *et al.* as mentioned earlier.[3] This exists in two forms, one which is glycosylated having a molecular weight (MW) of 57 kD, and one

unglycosylated having a MW of 52kD.[34] MMP-1 is a constitutive enzyme, and is crucial in diverse physiological processes such as tissue morphogenesis, wound repair and remodeling of collagen extracellular matrix in various physiological and pathological situations. In addition to the role in the turnover of collagen fibrils, MMP-1 is very important in the regulation of cellular behavior for the cleavage of non-matrix substrates and cell surface molecules.[35]

MMP-8, also known as neutrophil collagenase, is an inducible enzyme mainly produced in neutrophils by polymorphonuclear (PMN) leukocytes in bone marrow maturation.[36,37] The glycosylated form of MMP-8 is stored in intracellular granules, where the external stimuli can trigger the degranulation and then release MMP-8. MMP-8 is also found in chondrocytes, odontoblasts, monocyte/macrophage, etc.[38,39] MMP-8 is able to regulate the inflammatory response induced by carcinogens, thus playing a protective role in several pathological conditions including inflammation and cancer, but excessive MMP-8 leads to diseases.[40]

ProMMP-8 can be activated by reactive oxygen species as well as endopeptidases, like other MMPs (e.g., MMP-3, MMP-14), and certain bacterial proteases.[41,42]

MMP-13 was first found in breast cancer cells, and later found to be expressed in other cells including chondrocytes, gingival epithelial cells and osteoblasts.[43,44] MMP-13 is elevated in several pathological conditions of arthritis, periodontitis and various carcinomas.[45] MMP-13 can cleave a variety of components in basement membranes, and shows relatively greater efficiency in cleaving type-II collagen in contrast to types-I and -III.[46] Also MMP-13 appears to be the major collagenase in rats and mice in contrast to other collagenases.[47]

b. Gelatinases

There are two gelatinases in the MMP family, namely gelatinases A and B, respectively MMP-2 and MMP-9.[48-51] Both of these have head-to-tail repeats located in their catalytic domains, which

are responsible for the binding of gelatin substrate to fibronectin, and which possess the specific activity to further degrade denatured collagens.[52,53] It has been found that TIMPs 1-3 can inhibit both MMP-2 and MMP-9 with different specificities: TIMP-1 is more specific for MMP-9, whereas TIMP-2 is more specific for MMP-2, and TIMP-3 shows almost the same inhibitory activity toward MMP-2 and MMP-9.[54] MMP-2 was first found by Sellers *et al*. in rabbit bone, displaying gelatinase activity, and further confirmed by Liotta *et al.* in their studies on the degradation of type-IV collagen in basement membrane.[55,56] MMP-2 has been found to be expressed in a variety cell lines, and in endothelial cells associated with different types of carcinomas.[34,56] MMP-2 plays a crucial role in the regulation of the ECM, but when the levels of MMP-2 are elevated in cancer cells, increased malignant tumor cell migration has been observed.[11] The activation of MMP-2 is involved in the membrane-type MMPs as well as TIMP-2 on cell surface.[57,58] Pro-MMP-2 has an MW of 72kD, whereas the activated form has an MW of 62kD.

MMP-9 was found in human macrophages by Vartio *et al.* in 1982.[59] It is essential for several remodeling processes such as would healing, inflammation and cancer metastasis.[60] Compared to MMP-2, MMP-9 does not have extensive degradation activity toward type-I, -II or -III collagen. The secretion of MMP-9 is also observed in PMN neutrophiles, from which it is released from tertiary granules under immune response.[61] Human Pro-MMP-9 has a larger MW (92kD) than most of the others because of the extensive N- and O- glycosylation.[51] The active form of MMP-9 ranges from 65 to 82kD due to either N- or C-termini proteolytic degradation.[62,63] Pro-MMP-9 can be activated by human trypsin-2 (serine proteinase) and reactive oxygen species.[38,61] Under the pathological conditions of various inflammatory and malignant diseases (e.g., periodontitis), the production of MMP-9 is elevated. Also several chemokines and cytokines [e.g. tumor

necrosis factor-α (TNF-α), interleukin-1 (IL-1)] play an important role in the up-stream expression and activation of this enzyme.[61]

c. Stromelysins

There are three stromelysins in the MMP family, namely Stromelysin-1 (MMP-3), Stromelysin-2 (MMP-10) and Stromelysin-3 (MMP-11). MMP-3 and -10 are responsible for a variety of physiological processes, such as the degradation of specific substrates within the ECM, and activation and inactivation of other proteinases, such as other Pro-MMP members.[64,65] The *in vivo* activation of stromelysins involves serine proteinases, such as neutrophile elastase, chymotrypsin or trypsin.[64] MMP-3 possesses higher efficacy in the proteolysis of specific substrates in contrast to MMP-10.[11] When MMP-3 is activated, it is able to degrade several structural proteins in the ECM, and also able to activate other MMPs like Pro-MMP-1, -8, -13 and -9 in contrast to its weak activity against elastin.[11] MMP-11 is always classified as a stromelysin-like MMP. It is expressed in breast carcinoma cell lines and is responsible for the degradation of the serpins (endogenous serine proteinase inhibitors).[66] There are two different glycosylated forms of Pro-MMP-3 secreted as 60kD and 57kD, the latter being the major constituent.

d. Matrilysin

MMP-7, also called matrilysin, appears to be the smallest MMP found so far, which only contains the propeptide and catalytic subunits in the domain structure. MMP-7 was first found in rat uterus.[67,68] Because MMP-7 does not have a hemopexin C-terminal domain, the latent form and active form have MWs of only 28kD and 19kD, respectively. MMP-7 is active against a variety of peptide substrates in the ECM, and responsible for the activation of Pro-MMP-1, -2 and -9. MMP-7 has been shown to be involved in early-stage carcinoma development and late-

stage cancer cell invasion (metastasis).[69] MMP-7, as well as MMP-13, is produced by the cancer cells themselves, while other MMPs are mainly expressed by the surrounding cells of the epithelial tumors.

e. Metalloelastase

MMP-12, macrophage metalloelastase, was found in the macrophages of mice and possesses degradation activity against elastin.[70] The proenzyme of MMP-12 has an MW of 54kD, and the active form has an MW of 22kD after the cleavage of the hemopexin domain from the catalytic domain at the C-terminal end. The activation of Pro-MMP-12 proceeds through one intermediate form of 45kD after the N-terminal processing.[71] The importance of MMP-12 has been shown in alveolar macrophages of lung diseases, such as acute lung injury and chronic inflammatory airway diseases, where it contributes to the exacerbation of these conditions.[72]

f. Membrane-type MMPs

There are 6 members in the membrane-type MMP (MT-MMP) class, namely, MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24) and MT6-MMP (MMT-25).[73,74] MT-MMPs are covalently linked to the cellular membrane through the transmembrane domain of each enzyme, close to the end of the hemopexin domain at the C-terminal end, whereas MMP-17 and MMP-25 are bound to the cell surface though the domain extension at the C-terminus.[58] Beside the domain structures which are similar to other MMPs, there is a furin-like motif located between the pro-domains and catalytic domains except for the case of MMP-17.[73] In addition to degrading substrates in the ECM, MT-MMPs also play an important role in the activation of other Pro-MMPs. For example, MMP-14 and MMP-16 are responsible for the activation of Pro-MMP-2 in the membrane of cells, while MMP-14 can also activate several other Pro-MMPs including Pro-MMP-8, -9 and -13.[73-75] In

addition, MT-MMPs are able to degrade substrates such as type-I, -II and -III collagens.[73] MMP-14 has a latent form of 63kD in MW and once activated, the MW of this enzyme is reduced to 60kD. The activation of MMP-14 can be achieved by the use of the intracellular neutral proteinase, furin.[76,77]

g. Others

Several other MMPs are also known. For example, MMP-19, possessing similar activity to that of stromelysins, is expressed in tissues including the lung, spleen and prostate, and is crucial in the process of angiogenesis.[78,79] MMP-20, enamelysin, was found in tooth enamel, and is responsible for the degradation of ECM substrates such as amelogenin.[80] MMP-28, epilysin, was found in the epidermis, and is also expressed in tissues such as the heart and brain.[81] Others are likely to be found given their extensive involvement in tissue remodeling.

D. MMPs involvement in diseases

The role of MMPs in tissue remodeling in the ECM comprises mainly the degradation and removal of the extracellular components as mentioned before.[82] When MMPs levels are elevated, they can exacerbate various diseases, and breakdown connective tissue including cartilage and bone.[83] The molecules in the ECM, such as proteoglycans, elastins and fibronectins, are important in cell functions including interactions between various cells and the induction of proteinase activity. As a result, in addition to their regulations of physiological turnover and remodeling, MMPs play an important role in a number of pathological conditions characterized by diseases, including but not limited to arthritis, periodontal disease, postmenopausal osteoporosis, cardiovascular disease and cancer. Because MMPs have a physiological role at low levels, excessive inhibition as a therapeutic goal has been mis-guided; accordingly, “mild”

inhibition, as advocated by Golub *et al.* and Sorsa *et al.* appears to be effective without producing side-effects in human clinical trials. [6,48]

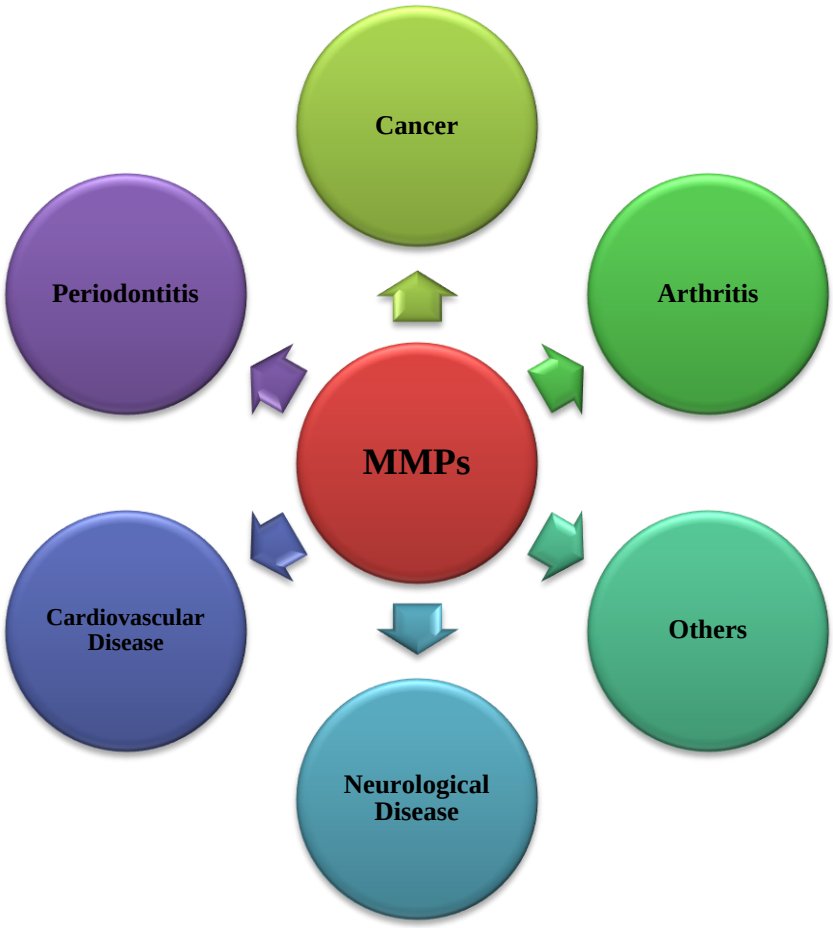


Figure 2.2 MMPs in various diseases

1. MMPs in Cancer

The degradation of extracellular substrates and basement membrane by MMPs, together with the regulation of TIMPs, is essential in tumor invasion.[84-86] Elevated levels of MMPs including MMP-1, -2, -3, -7, -9, 10, -11, -13 and -14 have been found in various human tumors including cancers of the breast, colon, lung, skin, pancreas, etc.[87,88] Studies also suggest that tumor cell growth factors, as well as the ECM components of the tumor cells, may also be the target for the MMP degradation. The invasion by tumor cells is believed to be the most critical step in tumor

growth, including mainly tumor cell adhesion (to the ECM), proteolytic degradation of the ECM and migration of tumor cells, followed by the detachment of malignant tumor cells from the ECM.[87,89] For example, MMP-9, in the initial studies, was found to be induced by chemokines and cytokines, and expressed to excessive levels by carcinoma cells.[90] MMP-2 is also responsible for the regulation of laminin-5, an ECM substrate which is essential to tumor cell migration in breast epithelium by the cleavage of residue 578 and initiates the mobility of the tumor cells.[91] MMP-12 was found to be associated with a variety of carcinomas in the degradation of microfibrils related to the elastins.[92,93] MMP-3 and MMP-7 play an important role in cancer cell apoptosis. MMP-3 is responsible for the epithelial cell pro-apoptosis, and MMP-7 is able to induce the apoptosis of epithelial cells by initiating the promoter, Fas ligand, by the cleavage of which, cancer cells are prevented from undergoing apoptosis under the condition of chemotherapeutic agents.[94,95] Angiogenesis, the formation of new blood vessels, plays a crucial role in providing nutrients and oxygen in both physiological and pathological conditions.[96] In pathological condition, the degradation of basement membranes by different types of MMPs provides the prerequisites for angiogenesis, in order to transport the essential nutrients to the cancer cells for tumor growth and metastasis.[97] For example, in addition to its role in the growth of the vascular epithelium, MMP-9 was found to be expressed and induced during the metastasis of the lung and ovarian carcinomas.[98,99]

2. MMPs in Periodontitis

The initiation of an immune response by periodontal bacteria is due to the lipopolysaccharides (LPS), which are the major components of bacterial membranes and lead to periodontal degradation. This induction causes the expression of various chemokines and cytokines, e.g. interleukin-1 (IL-1) and tumour necrosis factor-a (TNF-α).[100,101] Periodontal degradation was

found in tissues including gingiva, alveolar bone and periodontal ligament. MMP-8, a neutrophil collagenase, and MMP-13, are involved in several physiological and pathological conditions of host response initiation of periodontal disease.[102,103]

3. MMPs in Arthritis

MMPs are the major enzymes that degrade the cartilage matrix (mainly type-II collagen) in osteoarthritis and rheumatoid arthritis.[104] MMP-3 was found to be upregulated in the pathological conditions of arthritis in contrast to other MMPs, because MMP-3 can degrade type-II collagen and aggrecan (the cartilage-specific proteoglycan core protein), and can also activate several other Pro-MMPs including Pro-MMP-1, Pro-MMP-8, Pro-MMP-9, etc.[105]

4. MMPs in other diseases

MMPs are found to be highly expressed at pathological levels in several other diseases including cardiovascular disease, neurological disease (including Alzheimer's disease), and several lung conditions such as acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). MMP-9, in particular, is overexpressed by mononuclear phagocytes, and found to be associated with abdominal aortic aneurysm (AAA), the ruptured aneurysm in cardiovascular disease.[106] Both MMP-2 and MMP-9 are induced by amyloid-β peptide, the deposit of neuritic plaques, in Alzheimer's disease.[107]

E. MMP inhibitors

1. Endogenous inhibitors

There are generally two types of endogenous inhibitors of MMPs. One is α2-macroglobulin, the glycoprotein in plasma, which entraps proteinases including MMPs within the protein complex. Thus α2-macroglobulin has the ability to regulate the MMPs in the plasma and tissue fluids. In

contrast to α2-macroglobulin, the other class of MMP inhibitor, namely the tissue inhibitor of metalloproteinases (TIMPs), is more specific and comprises a family of four different proteins, namely TIMP-1, -2, -3 and -4. (Table 2.2)

Table 2.2 Classification of tissue inhibitor of metalloproteinases (TIMPs)
(Modified from Zitka *et al.*[108])

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Inhibited MMPs	All MMPs except MMP-14	All MMPs	MMP-1, -2, -3, -9, -13	MMP-1, -2, -3, -7, -9
MW (kD)	28	21	24	22
Tissues	ovary, bone, uterus	placenta, lung brain	kidney, lung, uterus, brain	brain, heart, muscle
Expression *in vitro*	inducible	constitutive	inducible	constitutive (restricted)
Localization	diffusible	diffusible	ECM associated	diffusible

The main function of the TIMPs is to control the degradation by the MMPs in the ECM.[82,109] TIMP-1, -2, -3 and -4 are similar in the structures of their domains. However, they appear to have unique roles due to their substrate specificities.[110] TIMPs bind to MMPs with 1:1 stoichiometry ratio in the MMP-TIMP complex.[11,84] TIMP-1 was first found by Bauer *et al.* as a protein that inhibits cultured skin fibroblasts in 1975, and later studies showed that TIMP-1 could bind to the hemopexin domains of MMPs including MMP-2 and MMP-9, thus it is responsible for the regulation of the activity of these enzymes.[111,112] The inhibition mechanism of TIMPs against MMPs involves the cysteine switch, where MMPs are inactivated by the down-

regulation of disulfide bond formation in cysteines, due to the reductive catalysis of TIMPs.[27] TIMP-3 was found to be expressed in embryo fibroblast cells by Blenis *et al.* in 1983, and appears to be more active in MMP inhibition in comparison to other TIMPs.[113,114] TIMP-4 is expressed abundantly in kidney and colon, and in the inhibition of Pro-MMP-2, it is similar to that of TIMP-2.[115,116]

2. Synthetic inhibitors

Because MMPs play a key role in the degradation of the ECM and are related to a variety of diseases, the development of MMP inhibitors has been a target of both academic groups and pharmaceutical industry.[117,118] Most of the synthetic inhibitors target the zinc ions in the catalytic domains of MMPs, and some of them showed great potency *in vitro*, but failed in clinical trials due to no evidence of response.[119] The only semi-synthetic MMP inhibitor approved by the FDA is subantimicrobial doses of doxycycline, developed by Golub *et al*., initially as a treatment for periodontitis, later developed as a treatment for rosacea, a chronic skin disease, under the trade names of Periostat® and Oracea® respectively.[5,6]

a. Tetracyclines

The first tetracycline possessing antibiotic activity was found in the soil organism *Streptomyces aureofaciens* by Duggar in 1948, namely chlortetracycline or Aureomycin. Chlortetracycline showed superior antibiotic activity in contrast to penicillin, the first antibiotic in the history of the world discovered by Fleming in 1928, and chlortetracycline showed antibiotic activity against both Gram-negative and Gram-positive organisms with little toxicity.[120] Since then, natural tetracyclines including chlortetracycline, tetracycline and oxytetracycline have been used in anti-microbial agents for the treatment of various infections.[121] In 1983, Golub *et al.* found that minocycline (later chemically-modified tetracyclines, 1991) could inhibit collagenase

activity in a diabetic rat model, and could also regulate several other MMPs by a non-antimicrobial mechanism of tetracycline in tissue degradation and remodeling.[5,6,48,102,103] These studies confirmed that tetracyclines can inhibit MMPs even after the removal of the dimethyamino group at Carbon-4 which is responsible for the antimicrobial activity. This discovery led to the development of more than 30 chemically-modified tetracyclines (CMTs) and their use in the treatment of various disease including periodontitis, arthritis, osteoporosis, etc., mainly by the regulations of MMPs in tissue remodeling.[122] (Figure 2.3)

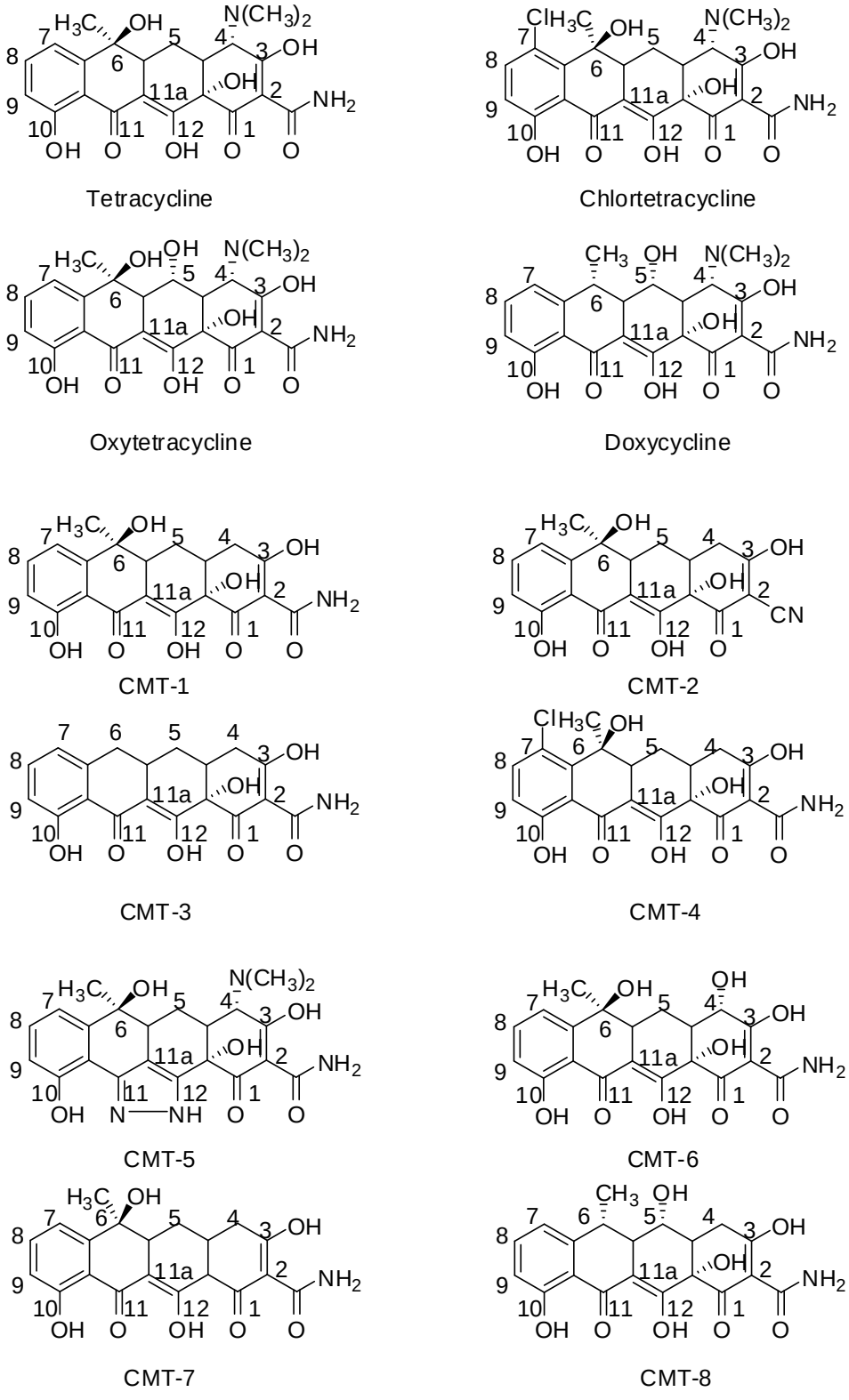


Figure 2.3 Structures of tetracyclines and chemically-modified tetracyclines

Studies were carried out on several tetracyclines (TCs) *in vitro* and *in vivo*. *In vitro* IC_{50}s of tetracyclines are summarized below. Of all the TCs and CMTs, doxycycline appeared to be the most potent MMP inhibitor.

Table 2.3 *In vitro* potency of tetracycline and doxycycline
(Modified from references[122,123])

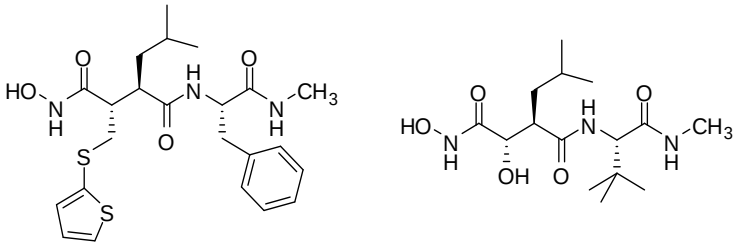
	Tetracycline	Doxycycline
MMP-1	>200 μM	15 μM
MMP-8	30 μM	30 μM
MMP-13	<1 μM	10 μM

CMT-1(4-de-dimethylaminotetracycline) was the first chemically-modified non-antibiotic tetracycline to be examined. CMTs-2, -3, -4 can inhibit the polymorphonuclear (PMN) collangenases, whereas CMT-5, the pyrazole derivative of TC (lacking the zinc binding site) was not active. Also, CMTs-1, -3, -6, -7, -8 and doxycycline were found to inhibit bone resorption *in vivo*.[123-125] From the *in vivo* data, it was found that CMT-3 possessed superior inhibitory activity against purified collagenases from tissues including those of the gingiva, osteoblasts and lung cancer cells. CMT-3 was also found to be effective as an anti-angiogenic agent in patients with Kaposi's sarcoma. Tetracyclines were not only the direct inhibitors of MMPs activity, but also the inhibitors of the activation of the MMPs from the proenzyme form.[125,126] Studies also showed TCs could inhibit the expression of MMPs in various cells, including epithelial cells. In this regard, this discovery was translated into the development of two FDA-approved, orally-administered drug substances, namely Periostat® for the treatment of chronic inflammatory oral-bone-destructive periodontitis, and Oracea® for chronic inflammatory skin disease, both

formulated as an "FDA limited sub-antimicrobial" dose of doxycycline. However, a significant side-effect of the tetracyclines and especially their non-antimicrobial congeners such as CMT-3, is increased photosensitivity, particularly at higher doses, and this limits their long-term use.[127,128]

b. Other MMP inhibitors

Because MMPs are involved in various pathological conditions including cancer, periodontitis, arthritis, etc., the development of MMP inhibitors has been a therapeutic target virtually ever since the first of these was discovered by Gross in 1962. Beside chemically-modified tetracyclines, the earlier generation of MMP inhibitors in 1980s were primarily (but not limited to) hydroxamic acids, possessing the zinc binding group derived from the cleavage site of collagen molecule as the sequence of Ile-Ala-Gly and Leu-Leu-Ala.[129,130] Batimastat (BB-94), developed by British Biotech, showed great potency against MMPs *in vitro* (data shown in Table 2.4), and has been evaluated against tumors including breast carcinoma.[131,132] Marimastat (BB-2516), the second generation hydroxamic acid derivative showed increased activity not only against MMPs but also against certain angiotensin-converting enzymes and enkephalinases including TACE (TNF-α converting enzyme). Marimastat superseded Batimastat in clinical studies for cancer and other diseases. However, the clinical study of Marimastat was negative, and it had no desirable activity against cancer in contrast to the preclinical studies, and caused musculo-skeletal side-effects.[133] The further development of Marimastat therefore was terminated.[134]



Batimastat Marimastat

Figure 2.4 Structures of Batimastat and Marimastat

Table 2.4 *In vitro* potency of Batimastat and Marimastat

	Batimastat (BB-94)	Marimastat (BB-2516)
MMP-1	3nM	5nM
MMP-8	10nM	2nM
MMP-2	4nM	6nM
MMP-9	1nM	3nM
MMP-3	Not Determined	200nM
MMP-7	Not Determined	20nM
TACE(TNF-α converting enzyme)	Not Determined	3800nM

Other MMP inhibitors including functional groups such as hydroxamate, thiol, pyrimidine, hydroxypyrone and bisphosphonates were developed mainly for cancer treatment, arthritis and cardiovascular disease. However, no other inhibitors, except doxycycline, were approved by the FDA for clinical use.[135-137]

3. Structure comparison of doxycycline, thermorubin, bis-aroyl methanes and curcumin

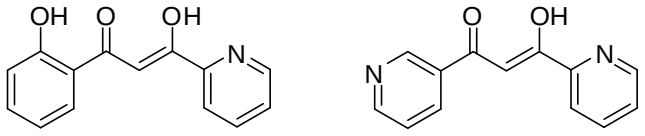
As for tetracyclines, the active site responsible for the inhibition of MMPs is the enolic β-diketone moiety at carbon 11, 11a and 12, by means of the binding to zinc atom in the catalytic domain of these enzymes, based on the studies carried out by Golub *et al*.[5,122] In a search for potential MMP inhibitors, we directed our initial studies to the antibiotic, thermorubin, which has the same β-diketone moiety in its side-chain (present in the nucleus of the tetracyclines). Thermorubin is an antibiotic substance, first discovered by Craveri *et al.* who isolated it from a thermophilic fungus *Thermomycetes antibioticus* (collected in the countryside near Pavia, Italy) which grows best at 50^{o}C rather than the normal temperature of 37^{o}C.[138] Subsequently it was then isolated by a Japanese group who initially gave it the designation BT3-3, until its identity with the Italian product was established. Of the crude fungal extract, thermorubin is the principal product and has a pigment-like character being orange-red in color and rather insoluble in most common solvents. Maximum production occurs at 20-24h in submerged culture (120-200μg/ml) and extraction was accomplished at pH 4.5 with chloroform to give thermorubin as a pure compound containing a molecule of chloroform. The purified material has activity against both Gram-positive and Gram-negative bacteria.[139,140] Against the former, its level of activity approximates that of penicillin or lincomycin, but it has lower activity against Gram-negative bacteria and is not active against yeasts or filamentous fungi. Typical MIC values are given in Table 2.5. Of great interest is the fact that its LD_{50} in mice is 300mg/kg body weight when the substance is administered by peritoneal injection. Introduction of 3mg/kg by this route over a three-day period affords 100% protection against an experimental *S. aureus* infection. *In vitro* it is less active in the presence of serum albumin. Oral or subcutaneous administration is ineffective, undoubtedly attributable to the low solubility of the drug in aqueous humour.

Table 2.5 Antibiotic activity of thermorubin

Test Organism	Minimum inhibitory Concentration (MIC) (μg/mL)
Sarcina lutea ATCC 9341	0.005
Staphylococcus aureus ATCC 6538	0.005
Staphylococcus aureus PV 43 (penicillin resistant)	0.005
Streptococcus pyogenes ATCC 12384	0.05
Streptococcus faecalis ATCC 10541	0.5
Corynebacterium simplex LE B5	0.2
Corynebacterium equi ISM 16111	0.5
Shigella flexneri ATCC 11836	1
Escherichia coli ATCC 10536	2
Klebsiella penumoniae ATCC 10031	10
Proteus vulgaris ATCC 881	10
Salmonella paratyphi ATCC 9150	20
Pseudomonas aeruginosa ATT 10145	100
Mycobacterium tuberculosis var. *hominis* H 37 RV	100

Thermorubin was first examined as an inhibitor using the proteinase activity of *P. acnes* as a model because the side chain functionality of thermorubin mimics the linear array of oxygen atoms in tetracycline. The results revealed that thermorubin possesses inhibitory activity against *P. acnes*, a causative factor in acne whereas the MMPs play a pathogenic role. However, the antimicrobial activity and poor solubility of thermorubin potentially limit the long-term

therapeutic use for the inhibition of cytokines and chemokines including MMPs. We then shifted onto a family of bis-aroyl methanes (BAMs), which possess the same 1,3-diketone moiety presented in the tetracyclines to which they are distantly related. Preliminary data on the BAMs (unpublished), show that several BAMs are able to inhibit human-derived MMPs *in vitro*. BAM1.11 (IC_{50}=53 µM) and BAM1.12 (IC_{50}=60 µM) showed great inhibitory interest against human-derived neutrophil collagenase (MMP-8). The inhibition of pro-inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6) was also carried out for BAM1.11 and BAM1.12.



BAM1.11 BAM1.12

Figure 2.5 Structures of BAM1.11 and BAM 1.12

Table 2.6 *In vitro* potency of BAM1.11 and BAM1.12

Inhibition	BAM1.11	BAM1.12
MMP-8(IC_{50})	53 µM	60 µM
Cytotoxicity (Monocytes)	2 µM-0% 25 µM-0% 50 µM-0%	2 µM-0% 25 µM-0% 50 µM-0%
TNF-α (%)	2 µM-0% 25 µM-0% 50 µM-0%	2 µM-0% 25 µM-26% 50 µM-26%

IL-1β (%)	2 μM-0% 25 μM-13% 50 μM-100%	2 μM-0% 25 μM-90% 50 μM-90%
MCP-1 (%)	2 μM-68% 25 μM-70% 50 μM-73%	2 μM-0% 25 μM-74% 50 μM-65%
IL-6 (%)	2 μM-0% 25 μM-0% 50 μM-0%	2 μM-10% 25 μM-10% 50 μM-38%

However, because of the limitations of the improving of acidity of BAM compounds as well as the maintaining of the planarity (as in the tetracycline), we switched our focus to a series of bis-vinyl homologues of the BAMs, namely the bi-phenolic chemically-modified curcumins (CMCs), which also possess the β-diketone assembly, necessary for zinc binding.

Part 2. Curcumin

F. History

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], is a natural product found in turmeric. The latter substance also known as "curry powder" is used as a flavoring spice, a coloring compound and a therapeutic agent. It is derived from the perennial herb *Curcuma longa L*. that grows in Asian countries particularly India and China. Curcumin was first isolated initially by Vogel *et al.* in 1815, and its structure was elucidated by Lampe *et al.* in

1910.[141] There are two other compounds related to curcumin that also occur in turmeric, namely demethoxycurcumin and bis-demethoxycurcumin. (Figure 2.6)

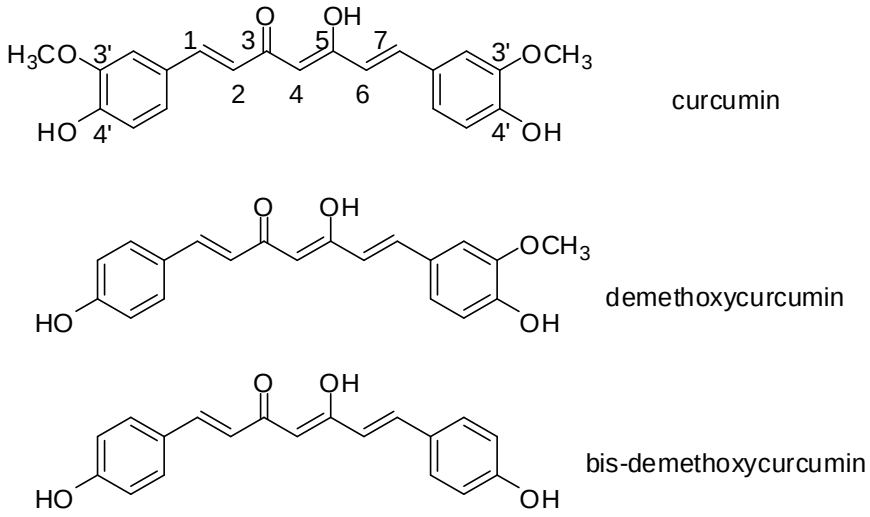


Figure 2.6 Structures of curcumin, demethoxycurcumin and bis-demethoxycurumin

G. Therapeutic use of curcumin

Curcumin has been used therapeutically as an herbal medicine for a variety of conditions including pulmonary, gastrointestinal and liver diseases, and also as a remedy for non-healing wounds and in the treatment of cancer.[142-144] Curcumin was found to possess a variety of therapeutic efficacies including the ability to inhibit proliferation and metastasis of various carcinomas, the down-regulation of transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the down-regulation of the expression of chemokines and cytokines including TNF-α, and the suppression of protein kinases and growth factors. (Figure 2.7)

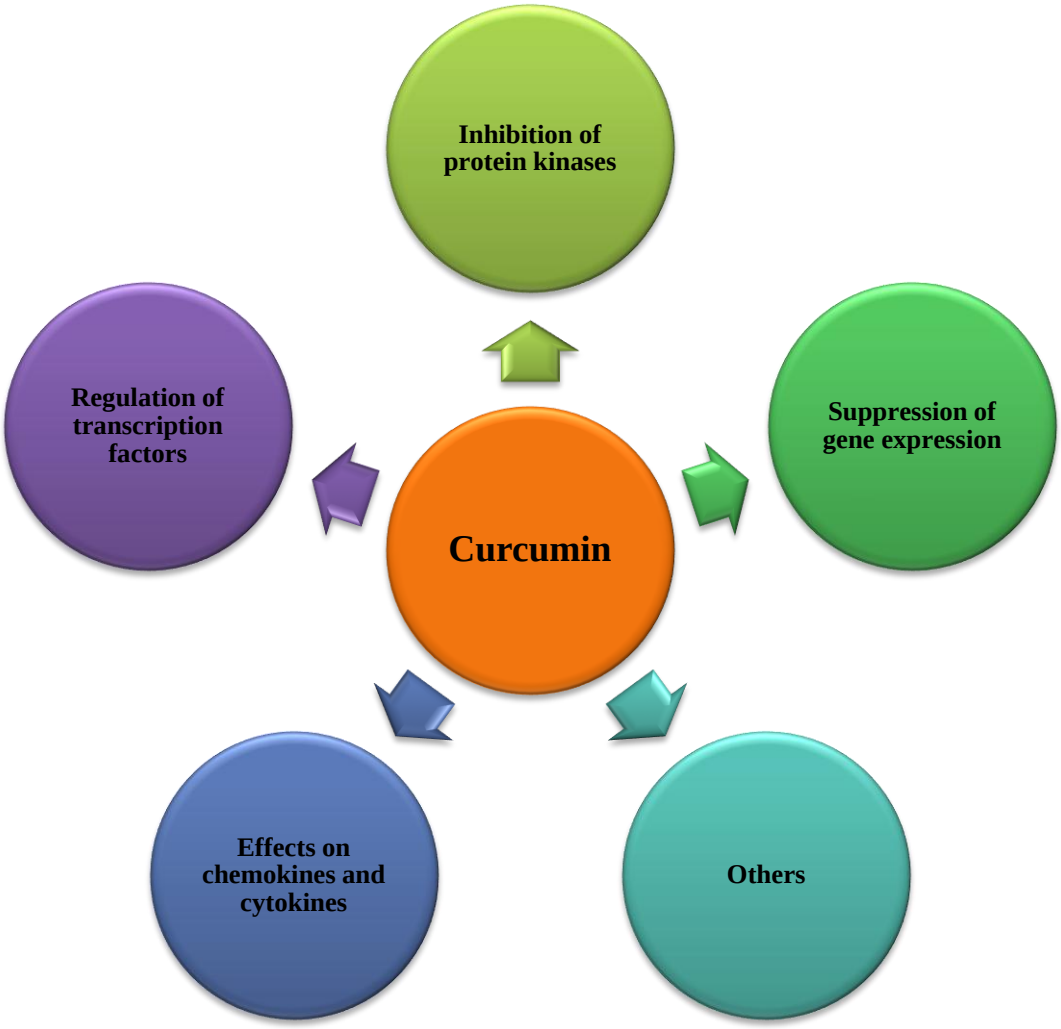


Figure 2.7 Curcumin used in various diseases

1. Anti-cancer activity

Curcumin has been evaluated in a variety of human cancer studies, including head and neck cancer, breast cancer, prostate cancer, colon cancer, pancreatic cancer and others.[145-152] The anti-cancer activity of curcumin is mainly due to its role in suppressing the cellular pathways which are responsible for carcinogenesis and the activation of apoptosis in tumor cells. Curcumin is also able to suppress the DNA mutagenesis, caused by UV irradiation.[151] It also inhibits the cyclin D1, the subunit of cyclin-dependent kinases (CDK) 4 (Cdk4) and 6 (Cdk6), that are

regulators of cellular mitosis.[150] Curcumin can also inhibit the P_{450} hepatic cytochromes that are involved in both Phase I and Phase II metabolism of xenobiotic substances.[153] Curcumin has also been shown to suppress a variety of transcription factor activation processes, which are responsible for carcinogenesis, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cyclooxygenase 2 (COX-2) and tumor necrosis factor (TNF).[149] Both preclinical and clinical studies have already shown the efficacy of curcumin in the treatment of various cancers. In the study carried out by Lal *et al*., the efficacy of curcumin in the clinical treatment of idiopathic inflammatory orbital pseudotumors is described.[154] The 2-year trial contained eight patients who received an oral dosage of 375mg thrice daily. Four patients out of five who completed the whole study recovered without any recurrence. In addition, no evidence of toxicity was observed.

2. Anti-inflammatory activity

Beside anti-cancer activity, curcumin also possess significant anti-inflammatory effects.[155] It was found to inhibit NF-κB, which regulates the gene expression of a variety of pro-inflammatory cytokines and chemokines including TNF-α, IL-6 and IL-8.[147,149] Curcumin also inhibits the inhibitor kappa B kinase (IκK), which regulates the release and activation (phosphorylation) of NF-κB. Also studies on the inhibition of prostaglandin E2 biosynthesis using curcumin revealed an inhibitory activity toward microsomal prostaglandin E2 synthase-1 enzyme. Above all, curcumin has been used as an anti-inflammatory agent in many pathological conditions including arthritis, Alzheimer's disease and cardiovascular disease.[156-159]

3. Anti-oxidant activity

Curcumin also possesses anti-oxidant activity in both *in vitro* and *in vivo* systems. The phenolic hydroxyl groups in the curcumin molecule are considered to be responsible for its anti-oxidant

acitivity.[160] The anti-oxidant activity plays an important role in both physiological and pathological conditions including cardiovascular diseases, aging, etc. In cardiovascular diseases, curcumin was found to induce the expression of endogenous anti-oxidants and to suppress the peroxidation of lipids.[161]

H. Bioavailability of curcumin

Curcumin is reasonably soluble in a variety of organic solvents including alcohols, tetrahydrafuran, dichloromethane, dimethylsufoxide and dimethylformamide. However, it is very insoluble in aqueous solution including water and physiological buffer.[162] This poor solubility contributes to the poor absorption seen in animal models and human studies, whereas it was found that most of curcumin remained in the stomach and intestine.[163-166] (Table 2.7) When curcumin was administered to humans in doses as high as 12g, a concentration of only 51.2ng/mL was found in serum after 4 hours of dosage.[165]

Table 2.7 Serum concentrations of curcumin by oral administration
(Modified from references[163-166])

Species	Oral Dosage	Concentration in Serum
Rat	1g/kg	0.5μg/mL
Rat	2g/kg	1.4μg/mL
Human	2g	6.0ng/mL
Human	10g	50.5ng/mL
Human	12g	51.2ng/mL

In addition, the rapid metabolism of curcumin represents a second concern for its bioavailability. Studies have shown that curcumin is metabolized to curcumin glucuronide and curcumin

sulphate by oral administration. Also it undergoes metabolic reduction to dihydrocurcumin, tetrahydrocurcumin, hexahydrocurcumin and hexahydrocurcuminol. These metabolites were found after intravenous and intraperitoneal adminsteration (Figure 2.8).[167,168] It has still not been determined fully, whether curcumin metabolites are active or not. However, some studies do show that curcumin glucuronides and tetrahydrocurcumin are not as active as curcumin in anti-inflammatory and antiproliferative effects including the suppression of COX-2 activity.[169,170]

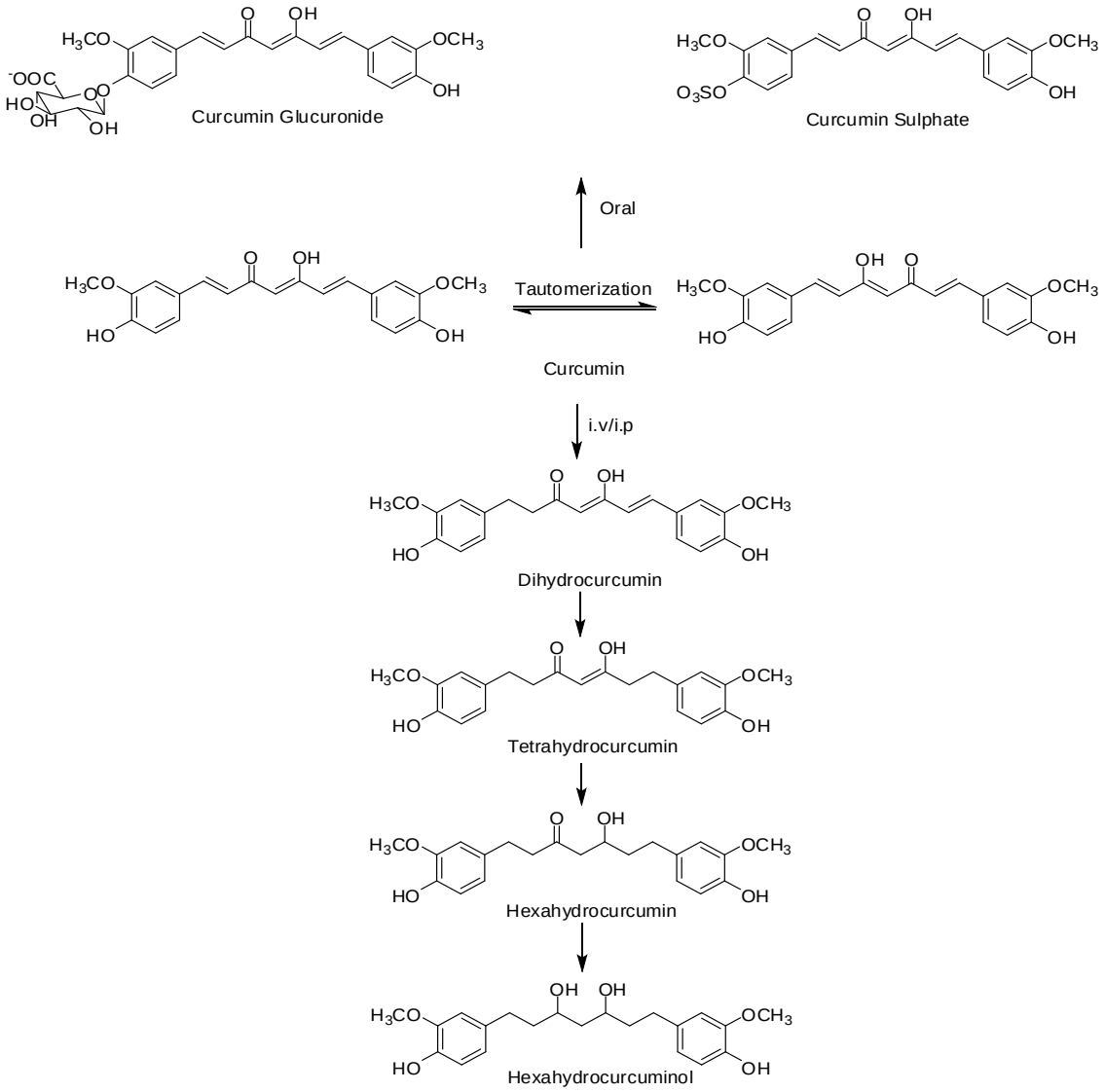


Figure 2.8 List of curcumin metabolites via different administrations

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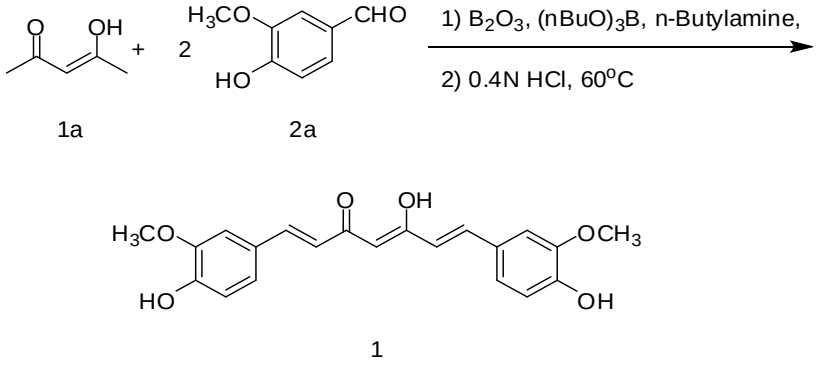
Chapter 3. Synthesis of chemically-modified curcumins by a modified Pabon reaction

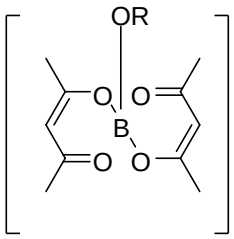
A. Introduction

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; **1**], a yellow pigment, comes from the perennial herb *Curcuma longa L*. that grows in Asian countries as mentioned in Chapter 2, and its chemical structure was not elucidated till 1910 by Lampe *et al.*[1] The first synthesis for curcumin carried out by Lampe *et al*. in 1918 contained eight steps beginning with vanillin, but as a method it appeared to lack any practical use.[2] In 1937, Pavolini *et al*. carried out a one-step synthesis of curcumin using 2,4-pentanedione (**1a**), vanillin (**2a**) and boric anhydride, but the yield was only 10%.[3] Since then, curcumin and curcumin analogues have been synthesized by several other methods with much improved yield. Of importance are the procedures of Pabon *et al.*[4] and Pedersen *et al.* [5] In Pabon's paper, curcumin was prepared in two steps: 1) generation of a boron complex from 2,4-pentadione, vanillin and boric anhydride in the presence of n-butylamine and tri-n-butyl borate, where boric anhydride was used to avoid Knoevenagel condensation[5] at C-3 position of 2,4-pentadione, because the boron complex makes the C-3 position of 2,4-pentadione not active in Knoevenagel reaction; 2) hydrolysis of boron complex into curcumin by 0.4N HCl at 60^{o}C. (Scheme3.1)



Scheme 3.1 Synthesis of curcumin by Pabon reaction

In the first step of the synthesis of curcumin reported by Pabon *et al.*, two ways were used to generate the boron complex, one was by heating the mixture of vanillin, 2,4-pentadione and boric anhydride at 100^{o}C for 30min, followed by the addition of n-butylamine, while in the second method, reactants were dissolved in ethyl acetate at 60^{o}C. The latter method gave a higher yield (73%) in contrast to the first one (45%). The proposed structure for boron complex from the view of Pabon is shown in Scheme 3.2, however, it's still not elucidated because no analysis was done on the structure of boron complex with 2,4-pentadione. The tri-alkyl borate was used to catalyze the formation of boron complex in the following equilibrium (Scheme 3.2). The first step of Pabon reaction was to generate the boron complex of 2,4-pentadione, then the decomposition of boron complex in dilute acid could give curcumin as the final product.



R=Alkyl Group

Equilibrium:

$$6\ {}^{t}BuOH + Acetylacetone/B_2O_3 \rightleftharpoons 2\ ({}^{t}Bu)_3BO_3 + Acetylacetone$$

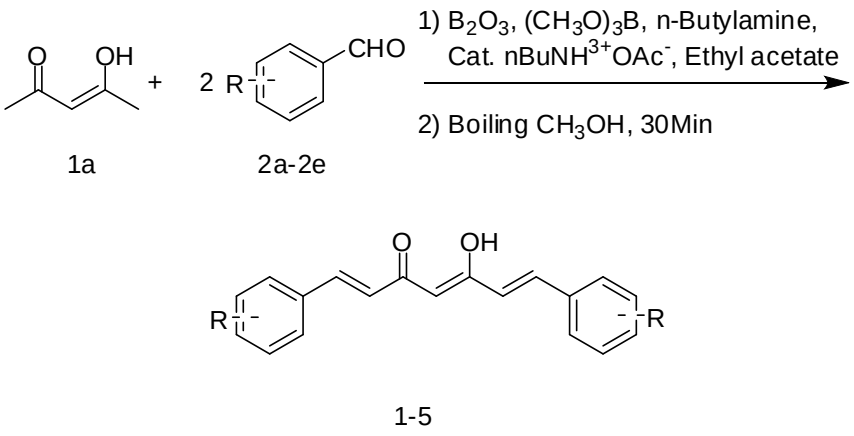
Scheme 3.2 Boron complex and equilibrium for Pabon reaction

The equilibrium indicates that alcohol could also decompose the boron complex of curcumin to tri-alkyl borate and free curcumin. Thus, in the variation of the Pabon method, boiling methanol was used instead of diluted acid in order to deprotect the boron complex because in many cases the substances are sensitive or even not stable under the acidic condition. Also trimethyl borate was used instead of tri-n-butyl borate, because the boiling point of trimethyl borate is 68-69^{o}C, lower than that of tri-n-butyl borate, 230^{o}C accordingly, which suggests that excessive trimethyl

borate could be simply removed by co-evaporation with methanol.[6,7] A catalytic amount of n-butylammonium acetate was utilized in addition to n-butylamine, in order to catalyze the formation of the double bonds by helping the dehydration.

B. Side chain modification

Following the modified Pabon reaction procedure, several compounds were prepared according to Scheme 3.3. 2,4-Pentandione and very finely powdered boron oxide were heated to 120^{o}C for 5min until a pale-yellow viscous liquid formed, then the reaction was cooled to room temperature, followed by the addition of the corresponding aldehyde and trimethyl borate in ethyl acetate. Catalytic amounts of n-butylamine and n-butylammonium acetate were then added. The mixture was stirred, and a red metallic precipitate began to appear after 1 hour. Then the reaction was held at room temperature for 48 hours to complete the process. The precipitate was filtered and collected, and then boiled with methanol to give products **1**-**5**.



Scheme 3.3 Synthesis of **1-5** by modified Pabon reaction

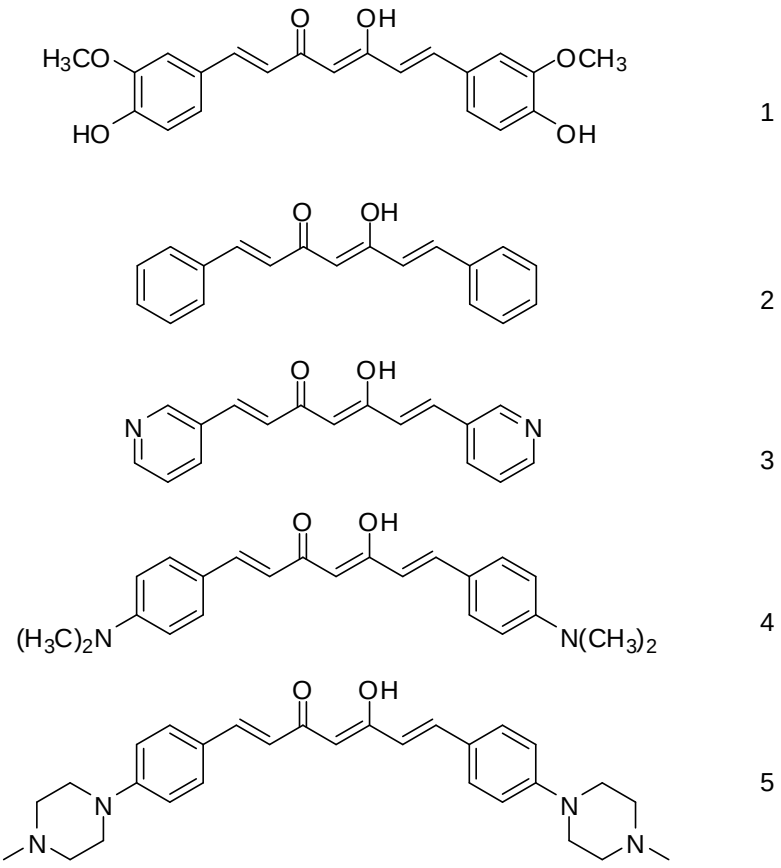


Figure 3.1 Structures of compounds **1**-**5**

Table 3.1 Symmetric curcumin analogues 1-5 by side chain modification

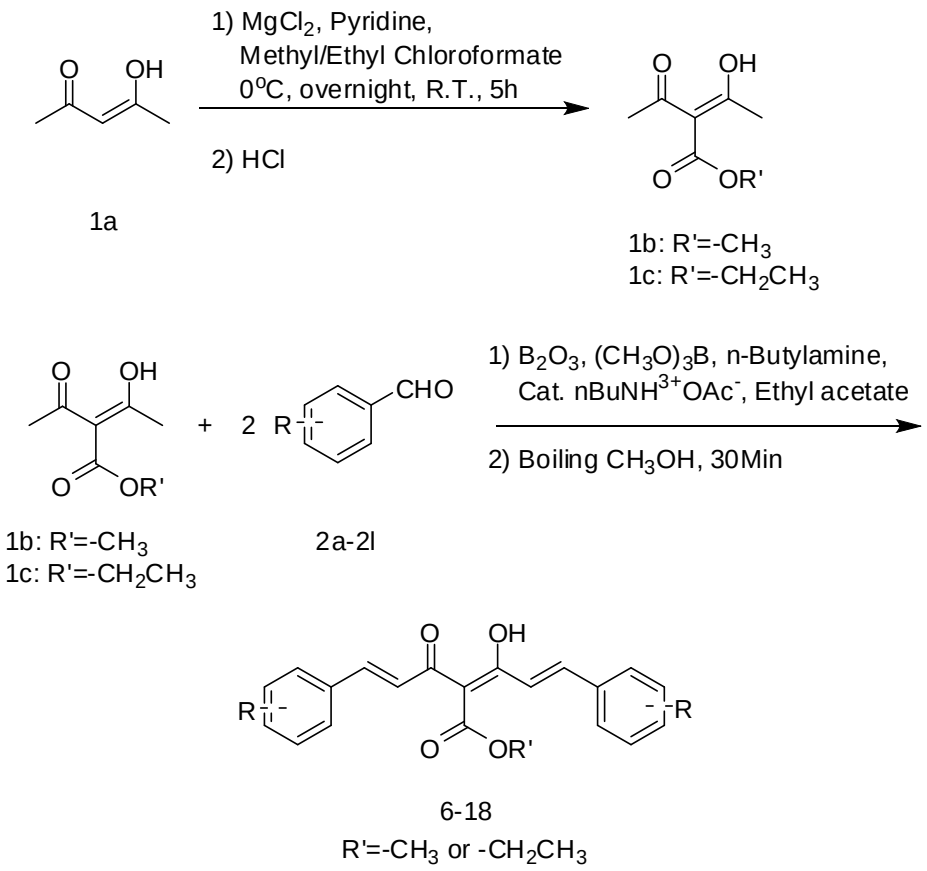
Compound	Name	Yield (%)	Melting Point (^{o}C)
1	Curcumin	77.0	175-176
2	CMC2.2	65.1	141-142
3	CMC2.3	63.2	171-172
4	CMC2.7	61.1	212-214
5	CMC2.19	46.4	239-240

As seen in the table above, compounds **1**-**5** were synthesized by a modified Pabon reaction, and were obtained in higher yield compared to Pabon's original preparation in the cases of **1** (73%

reported by Pabon), **2** (23% reported by Pabon) and **4** (36% reported by Pabon). The melting points have been repeated for these analogues.[4, 8] These compounds except curcumin did not show any desirable activity against human-derived MMPs. Biological data will be discussed in detail in Chapter 4.

C. Introduction of a methoxy/ethoxy carbonyl group on C-4

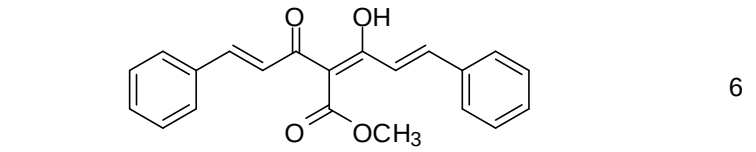
The poor solubility of curcumin limits its bioavailability for therapeutic applications, so an improvement is needed to increase the solubility.[9] Accordingly, the methoxy/ethoxy carbonyl group was introduced at the 4-position to obtain curcumin analogues. Side chain aryl group modification was also developed. The biological data in Chapter 4 reveal that some of these compounds are more potent against human-derived MMPs relative to curcumin. The pK_a studies shown in Chapter 5 also indicate that CMC2.5 (**7**), the first lead compound among these curcumin analgues, is more acidic and more ionized at physiological pH (pH=7.4-7.6), and correlates with the biological data obtained. The syntheses of these curcumin analogues **6-18** with methyl/ethyl group on C-4 starts from 3-substituted-2,4-pentadione, the precursor for the Pabon reaction.[10] 2,4-Pentandione was first added to a suspension of magnesium chloride in methylene chloride in the presence of pyridine to generate the anion at carbon-7. Then an alkyl chloroformate was added dropwisely at 0^oC, followed by the work up of dilute HCl after warming to room temperature over 5 hours. Both **1b** and **1c** were then used in the modified Pabon reaction to give compounds **6-18**. Several functional groups were placed on the phenyl rings including nitro, dimethylamino, pyridinyl and additional methoxy groups.



Scheme 3.4 Synthesis of Compounds **6-18**

Among the curcumin analogues **6-18**, the yields vary dramatically for different substitutions on the phenyl ring. It appears that the introduction of an electron-donating group on the phenyl group, like methoxy group, increases the yield in the Pabon reaction, as can be seen for CMC2.13 (**13**), whereas the introduction of electron-withdrawing groups such as nitro group decreases the yield, as can be seen for CMC2.12 (**12**). However, the yields for the remaining curcumin analogues are around 40-50% except a lower yield was observed for CMC2.18 (**18**), probably because of the steric hindrance in the phenyl ring during the initial condensation. CMC2.16 (**16**) with an ethoxy carbonyl group on C-4 position, was also synthesized. All the biological data for these compounds will be discussed in Chapter 4. Both CMC2.5 (**7**) and

CMC2.14 (**14**) displayed excellent inhibitory activity against human-derived MMPs *in vitro*, so further studies were focused on the modifications of these two compounds.



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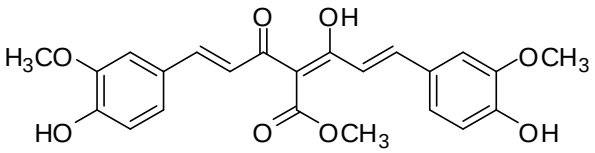

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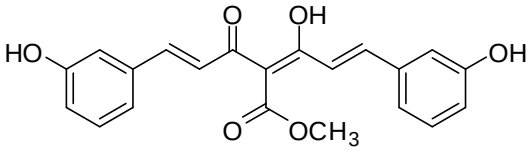

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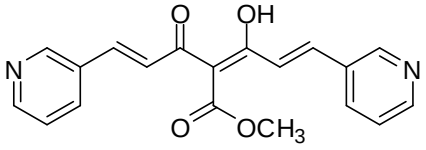

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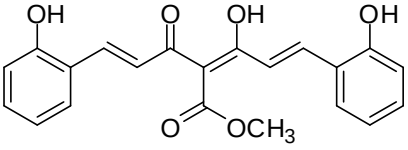

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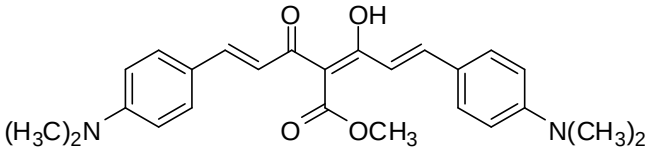

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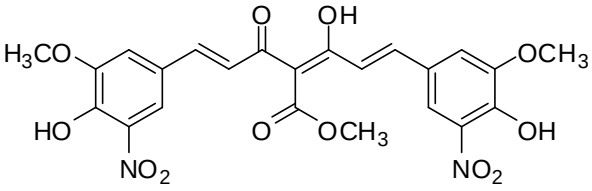

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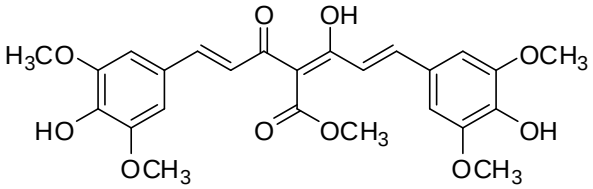

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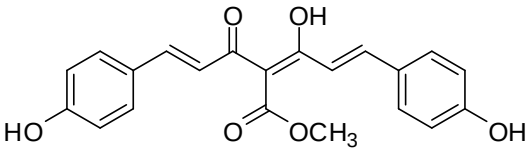

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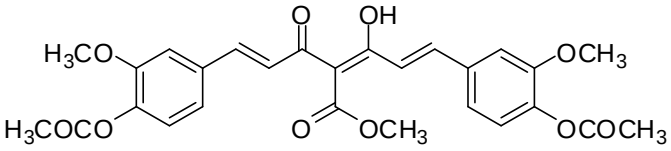

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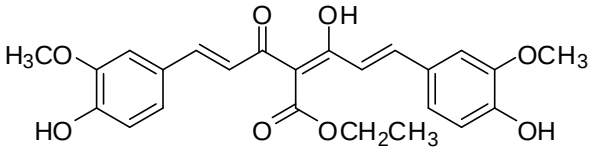

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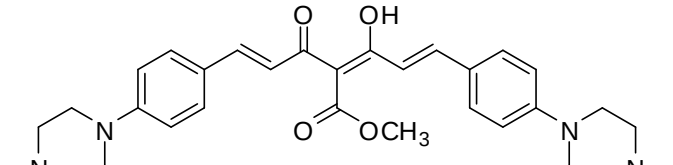

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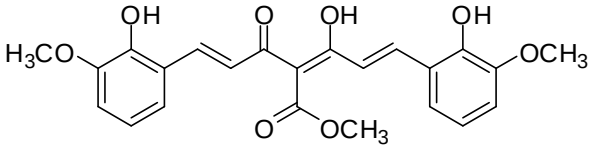


18

Figure 3.2 Structures of compounds **6**-**18**

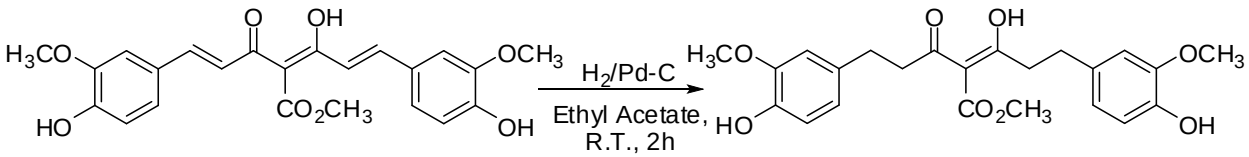
Table 3.2 Symmetric curcumin analogues **6**-**18**

Compound	Name	Yield (%)	Melting Point (^{o}C)
6	CMC2.4	52.1	126-127
7	CMC2.5	72.0	175-176
8	CMC2.6	40.2	199-189
9	CMC2.8	38.7	195-196

10	CMC2.10	46.3	165-166
11	CMC2.11	45.1	224-225
12	CMC2.12	26.0	207-208
13	CMC2.13	77.0	179-180
14	CMC2.14	49.2	214-216
15	CMC2.15	46.0	169-170
16	CMC2.16	44.6	158-159
17	CMC2.17	49.1	191-192
18	CMC2.18	25.8	201-202

D. Tetrahydro-curcumin analogue

Tetrahydro-curcumin is one of the metabolites of curcumin in rats and human by intravenous route, however, it is still not clear if the metabolites are active or not. [11, 12] One tetrahydro-curcumin analogue CMC2.9 (**19**) was prepared by hydrogenation of CMC2.5 (**7**), the first lead compound from *in vitro* test against human-derived MMPs, in the presence of palladium-on-charcoal in ethyl acetate for 2 hours. However, **19** didn't show any inhibitory activity against MMPs.

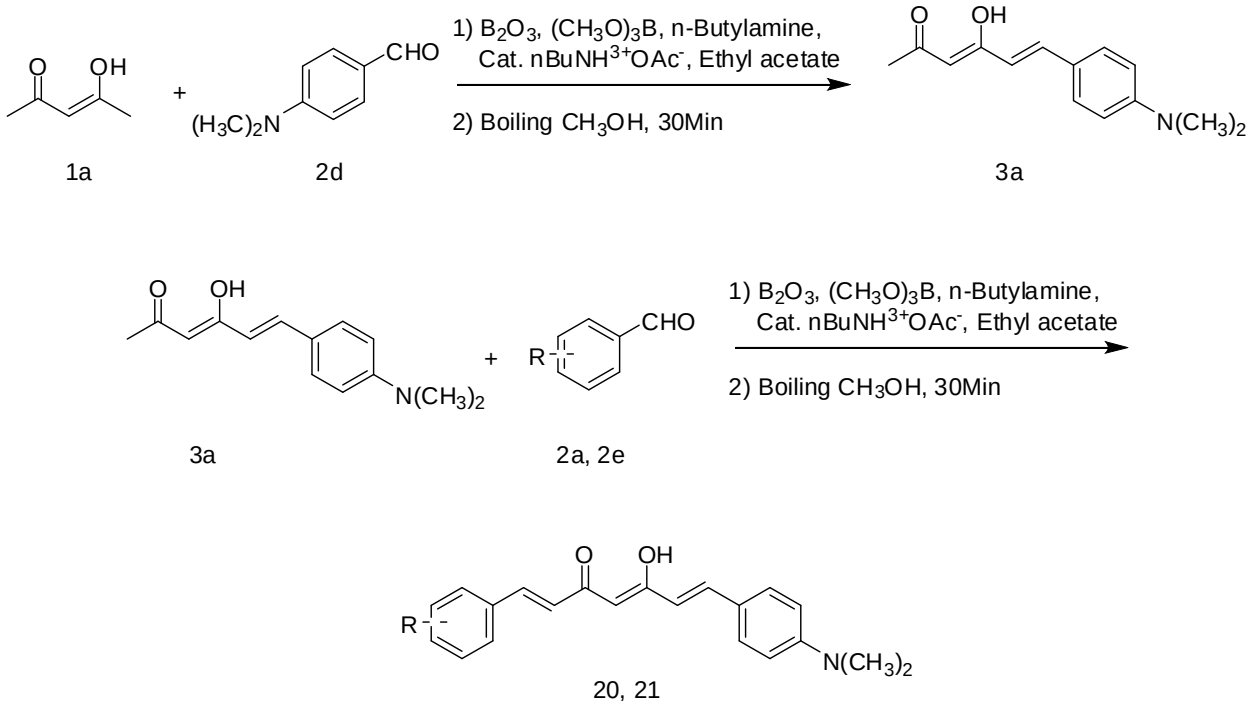


7 19

Scheme 3.5 Synthesis of tetrahydro-curcumin analogue **19**

E. Unsymmetrical curcumin analogues

Curcumin is highly symmetric and very insoluble. Modifications were made to the synthetic method to prepare the unsymmetrical curcumin analogue **20** and **21**, which were synthesized by Dr. Kveta Vrankova. Both of these contain a dimethylamino group on one phenyl ring, and 4-hydroxy-3-methoxy for **20**, 4-methyl-piperazinyl group for **21** on the other phenyl ring. The dimethylamino group was expected to increase the solubility in aqueous media, whereas the other functional group would be responsible for the MMP inhibitory activity. However, these two compounds lack potency against human-derived MMPs when tested (see Chapter 4).



Scheme 3.6 Synthesis of asymmetric curcumin analogues **20**, **21**

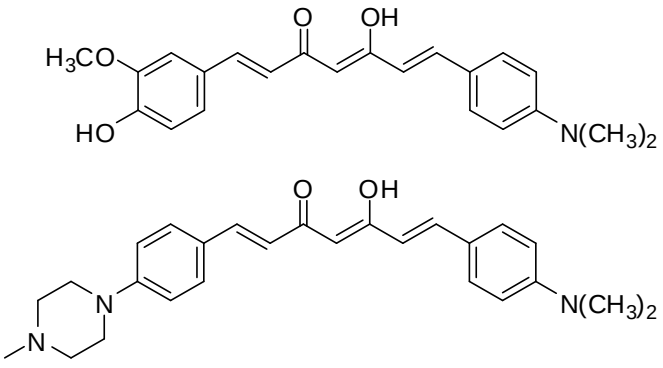
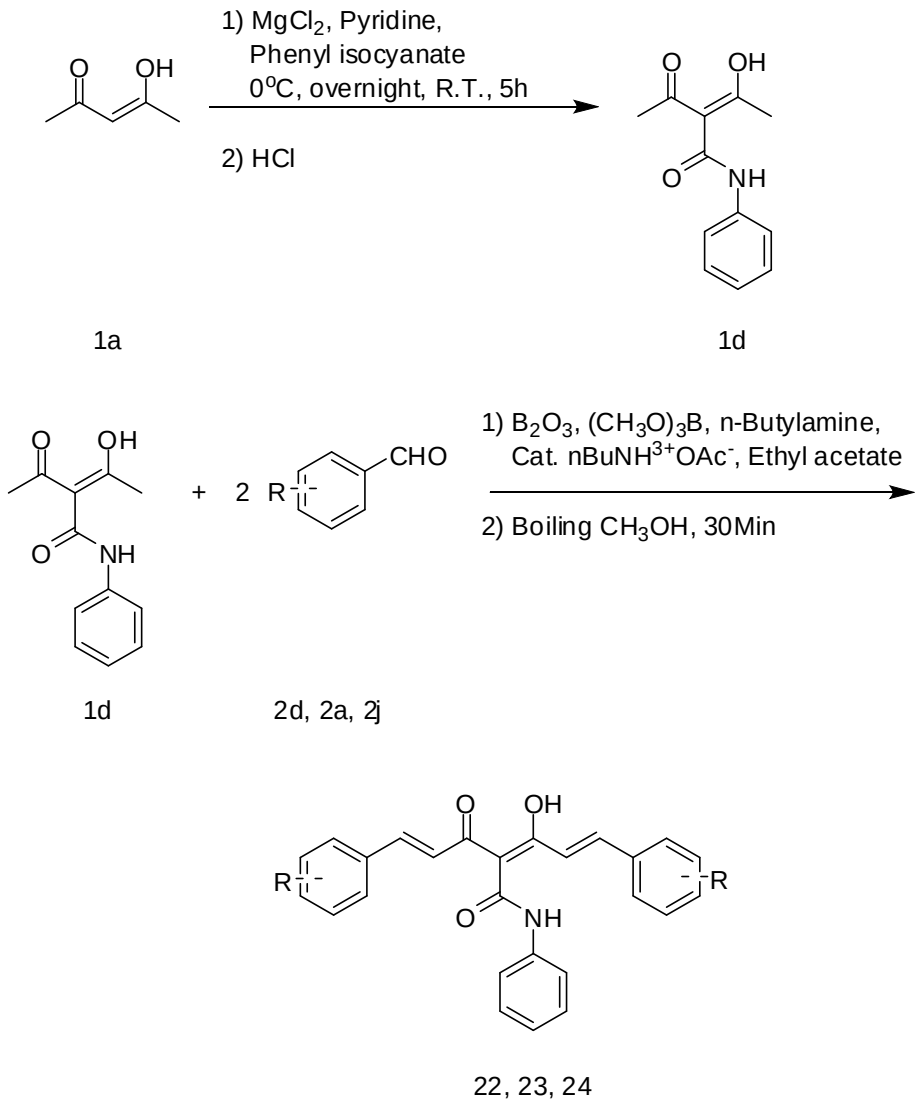


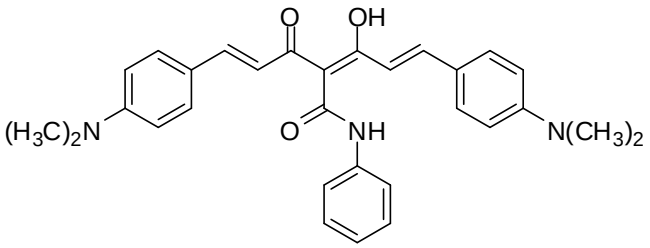
Figure 3.3 Structures of Compounds **20**, **21**

F. Introduction of an N-phenylaminocarbonyl group on C-4

Given that CMC2.5 (**7**) and CMC2.14 (**14**) showed desirable inhibitory activities against human-derived MMPs *in vitro* (data shown in Chapter 4), these two compounds were selected as the initial lead compounds as MMP inhibitors. Thereafter an N-phenylaminocarbonyl group was introduced in place of the methoxy carbonyl groups on **7** and **14**. This synthesis starts with a reaction of 2,4-pentanedione and phenyl isocyanate in the presence of magnesium chloride and pyridine to give the 3-arylaminocarbonyl-2,4-pentadione. Three N-phenylaminocarbonyl substituted chemically modified curcumins, namely CMC2.22 (**22**), CMC2.23 (**23**) and CMC2.24 (**24**) were prepared using a modified Pabon reaction as described in this chapter. Compound **23** and **24** both showed great potency against human-derived MMPs *in vitro*, and collateral studies in Chapter 8 also revealed that these two compounds are more potent compared to curcumin in cell culture (chemokine and cytokine inhibition), and *in vivo* studies (periodontitis, arthritis and wound healing).[13-19]



Scheme 3.7 Synthesis of compounds **22-24**



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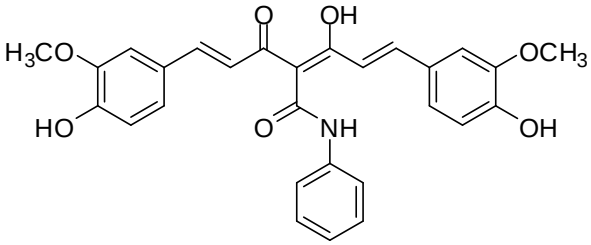
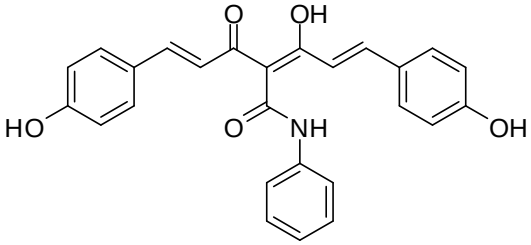


23



24

Figure 3.4 Structures of compounds **22-24**

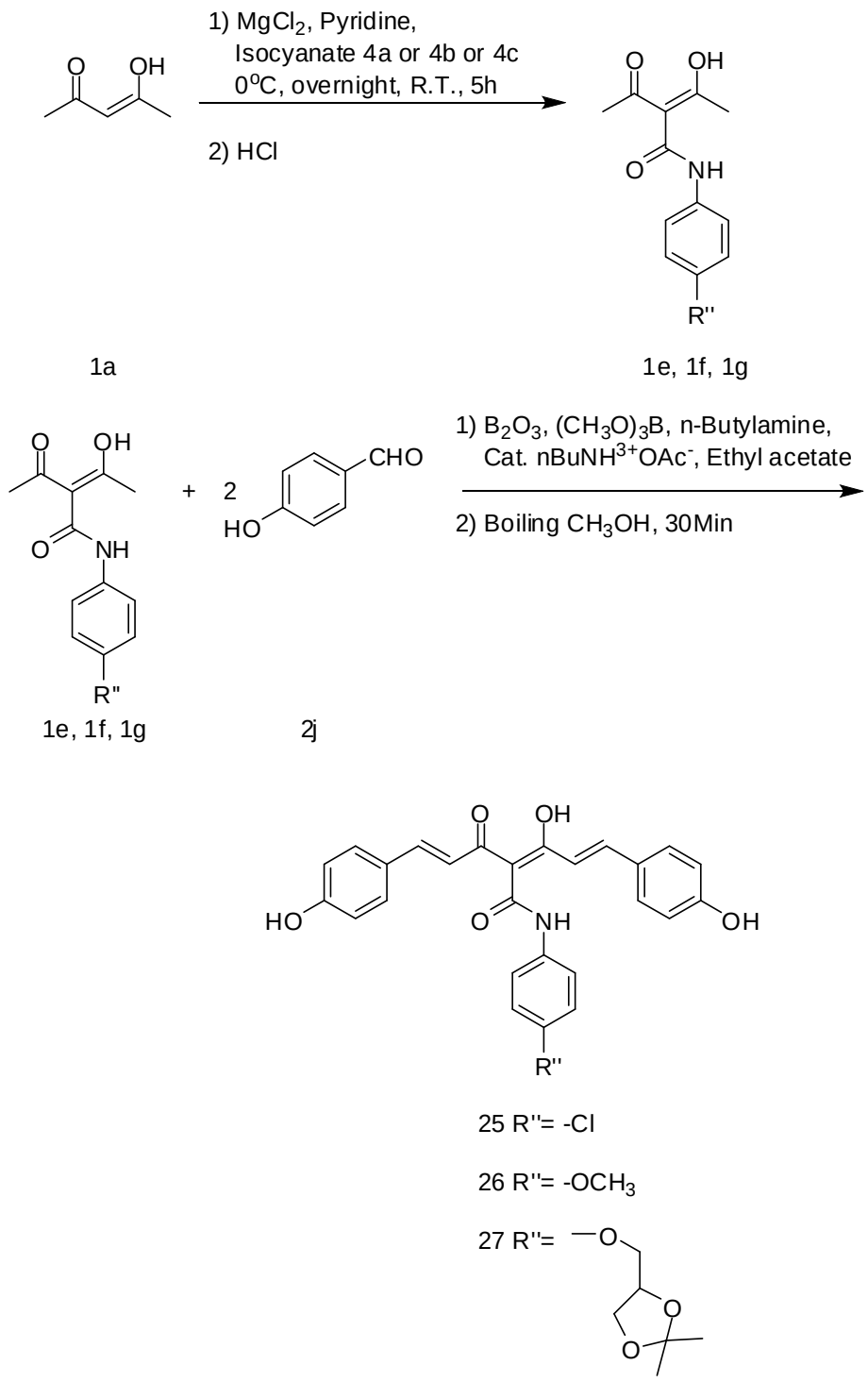
Table 3.3 Symmetric curcumin analogues **22-24**

Compound	Name	Yield (%)	Melting Point (^{o}C)
22	CMC2.22	51.7	208-209
23	CMC2.23	46.6	193-194
24	CMC2.24	46.2	220-221

G. Introduction of a solubilizing group on CMC2.24

Because CMC2.24 (**24**) is tremendously potent in both *in vitro* and *in vivo* studies (data will be shown in the following chapters), modifications were made in an attempt to increase the solubility as well as to maintain the biological efficacy. Firstly, either a chloride or a methoxy group was introduced to the C-4' position in the central phenyl ring of 3-arylaminocarbonyl-2,4-

pentanedione. *In vitro* tests (shown in Chapter 4) revealed that both of these products, namely CMC2.25 (**25**) and CMC2.26 (**26**) are as potent as **24**. In order to obtain compound CMC2.28 (**28**) with potentially increased solubility because of the diol, the corresponding ketal CMC2.27 (**27**) was prepared from 2,4-pentanedione and isocyanate **4c** in the modified Pabon reaction. Isocyanate **4c** was made from the acid **5f** by using diphenylphosphoryl azide which causes a Curtius rearrangement. The product **4c** was used in a condensation with 2,4-pentadione without further purification.[20] Compound **27** was treated with 10% acetic acid in a mixture of methanol and H_2O to give the desired product **28**. However, according to the *in vitro* data in Chapter 4, these two compounds, **27** and **28**, did not show any desirable inhibitory activity against human-derived MMP-9.



Scheme 3.8 Synthesis of compounds **25-27**

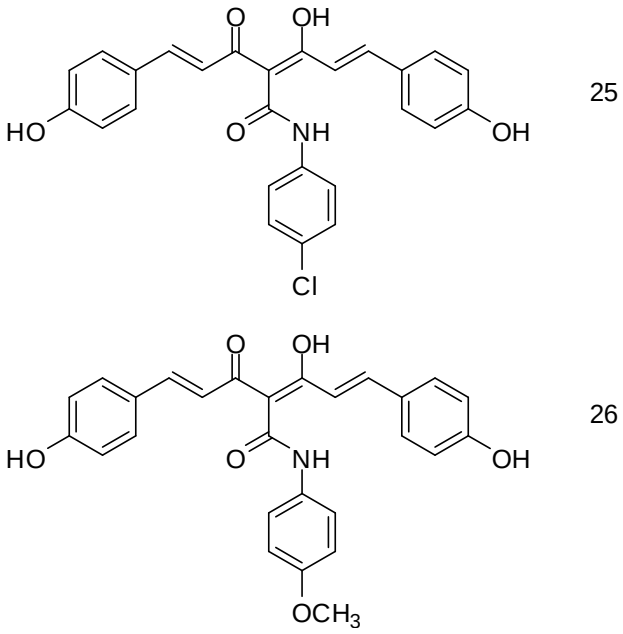
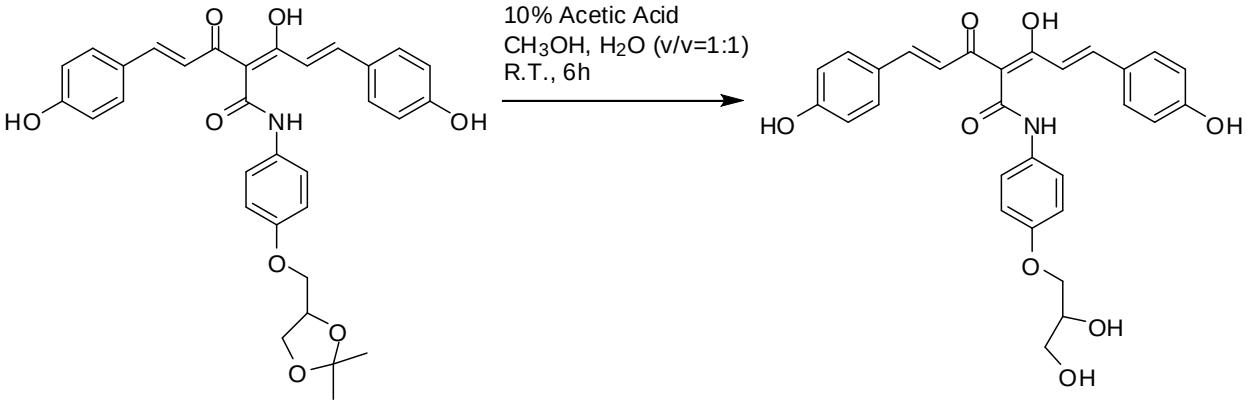
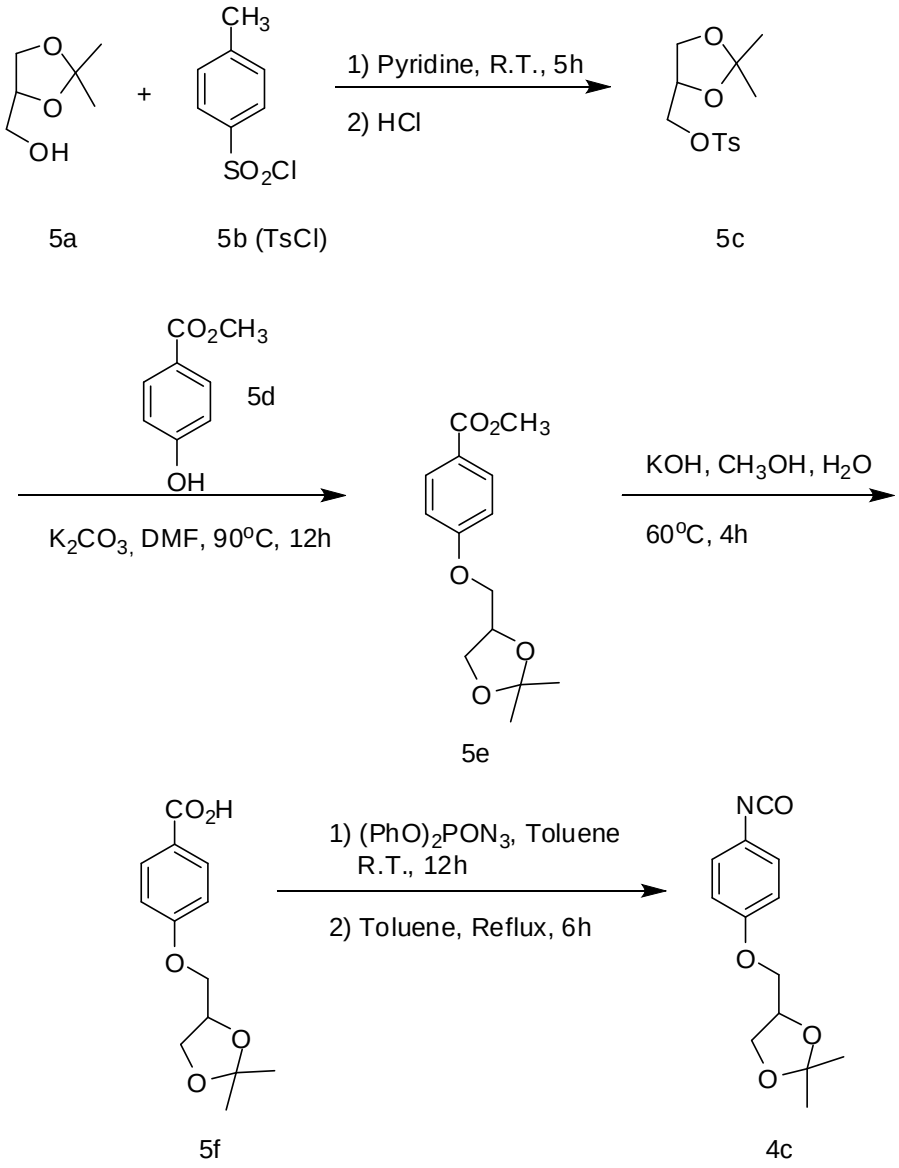


Figure 3.5 Structures of compounds **25**, **26**



Scheme 3.9 Synthesis of compound **28** from compound **27**



Scheme 3.10 Synthesis of compound **4c**

Materials and Methods

All reagents and solvents employed in this experimental work were reagent grade and were used as such unless otherwise specified. Melting points were taken on a Thomas-Hoover open capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Gemini 300 spectrometer either in $CDCl_3$ or DMSO-d_6. Chemical shifts are reported in parts per million (ppm) relative to TMS. Mass spectra were recorded on either a Thermo

Electron DSQ GC/MS equipped with a solid probe inlet and EI ionization or an Agilent 1100LC (API-ES)/MSD-VL(m/z=50-1500) using electrospray ionization. Thin-layer chromatography (TLC) was performed on silica gel sheets (Tiedel-de*Ha ën*, Sleeze, Germany). After appropriate purification all new products showed a single spot on TLC analysis in the following solvent systems: 25% ethyl acetate in hexanes and 10% methanol in dichloromethane. Components were visualized by UV light (l=254 nm). Flash column chromatographic separations were carried out on 60A ˚ (230–400 mesh) silica gel (TSI Chemical Co., Cambridge, MA). All reactants that were moisture or air-sensitive were conducted under dry nitrogen. The starting materials and reagents, unless otherwise specified, were the best grade commercially available (Sigma-Aldrich, Milwaukee, WI or Fluka Chemie GmbH, Sigma-Aldrich, Germany) and were used without further purification.

1. General procedure for the synthesis of 4-substituted-2,4-pentanediones

2,4-Pentanedione (**1a**) (1.00g, 10mmol) was added to a suspension of magnesium chloride (1.35g, 1.2eq) in 20mL methylene chloride, followed by pyridine(2.13mL, 2.5eq), and the mixture was stirred at 0℃ for 1h, then either an alkyl chloroformate or a phenyl isocyanate(1.0eq) was added dropwise at 0 ^{o}C. The mixture was allowed to warm up to room temperature during 8 hours, and then was poured into 3N aqueous HCl solution (10mL) and extracted with methylene chloride (20mL). The organic layer was washed with 20mL brine solution, dried over sodium sulfate and the solvent was removed under vacuo. The product was then either distilled or recrystallized from an appropriate solvent as required. The essential data for each individual compound are given below.

3-Methoxycarbonyl-2,4-pentanedione (1b): Colorless liquid, 68.1% yield. Distilled at 45^{o}C, 0.5mmHg.(lit.[10], bp 75-76^{o}C, 0.3mmHg) ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.00(s, 6H), 3.73(s, 3H), 17.76(s, 1H). ESI (-ve) MS m/z 157.1[M-H]$^{-}$.

3-Ethoxycarbonyl-2,4-pentanedione (1c): Colorless liquid, 60.5% yield. Distilled at 60^{o}C, 1.4mmHg.(lit.[21], bp 88-91^{o}C, 8mmHg) ^{1}H NMR (DMSO-d_6, 300MHz): δ 1.25(t, 3H) , 2.30(s, 6H), 4.20(q, 2H), 17.75(s, 1H). ESI (-ve) MS m/z 171.2[M-H]$^{-}$.

3-Phenylaminocarbonyl-2,4-pentanedione (1d): White solid, 72.0% yield, mp 118-119^{o}C.(lit.[22], mp 117-119^{o}C) ^{1}H NMR (DMSO-d_6, 300MHz): δ 2.15(s, 6H), 7.08(t, 1H), 7.32(t, 2H), 7.64(d, *J*=8.1Hz, 2H), 10.36(s, 1H), 16.46(s, 1H). ESI (-ve) MS m/z 218.1[M-H]$^{-}$.

3-(4-Chloro)-phenylaminocarbonyl-2,4-pentanedione(1e): White solid, 30.5% yield, mp 124-125^{o}C ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.59(s, 3H) , 2.57(s, 3H) , 7.37(d, *J*=8.7Hz, 2H), 7. 55(d, *J*=8.7Hz, 2H), 12.17(s, 1H), 18.45(s, 1H). ESI (-ve) MS m/z 252.7 [M-H]$^{-}$.

3-(4-Methoxy)-phenylaminocarbonyl-2,4-pentanedione(1f): White solid, 34.3% yield, mp 130-131^{o}C. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.49(s, 3H) , 2.51(s, 3H), 7.08(t, 1H), 6.88(d, *J*=9.0Hz, 2H), 7. 41(d, *J*=8.7Hz, 2H), 11.91(s, 1H), 18.65(s, 1H). ESI(-ve) MS m/z 248.2[M-H]$^{-}$.

3-(4-((2,2-Dimethyl-1,3-dioxolan-4-yl)methoxy))-phenylaminocarbonyl-2,4-pentanedione(1g): White solid, 22.3% yield, mp 105-106^{o}C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.29(s, 3H), 1.34(s, 3H), 2.14(s, 6H) , 4.43-4.55(m, 1H), 4.05-4.10(m, 2H), 3.95-3.96(m, 1H), 3.69-3.74(m, 1H), 6.92(d, *J*=9.0Hz, 2H), 7. 54(d, *J*=9.0Hz, 2H), 10.21(s, 1H), 16.42(s, 1H). ESI (-ve) MS m/z 348.1[M-H]$^{-}$.

4-(4-Methylpiperazinyl-1-yl)benzaldehyde (2e): Potassium carbonate (3.50g, 25.3mmol) was added to a solution of 4-fluorobenzaldehyde (2.71mL, 3.14g, 25.3mmol) and *N*-methylpiperazine (2.55mL, 2.30g, 23mmol) in DMSO (23mL) and the mixture was heated under reflux (120^{o}C)

for 5h. The reaction mixture was then poured into water (450mL) and extracted with ether (4×20mL). The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated. The crude material was crystallized from a hexane:ethyl acetate (3:1) to afford **2e** as a pale-yellow crystalline solid, yield 46.0 %, mp 56-57^oC.(lit.[23]) ^1H NMR ($CDCl_3$, 300MHz) δ 2.35(s, 3H), 2.55(t, *J* = 5.1Hz, 4H), 3.42(t, *J* = 5.1Hz, 4H), 6.90-6.94(m, 2H), 7.74-7.77(m, 2H), 9.78(s, 1H). MS (EI) *m/z* (%) 204 ($M^{\cdot+}$, 100), 133(30), 132(35).

(3Z,5E)-6-(4-Dimethylaminophenyl)-4-hydroxyhexa-3,5-dien-2-one (3a): 2,4-Pentanedione (10mmol) and boron oxide (0.49 g, 7mmol, 0.7eq) in a 50 mL flask were heated to 120^oC for 5min to form pale-yellow suspension. Aldehyde (10mmol, 1.0eq) and trimethyl borate (4.16g, 40mmol, 4.0eq) were dissolved in ethyl acetate (10mL) and gradually added to reaction mixture. While stirring, 0.05mL of butylamine and 0.2mL of butylammonium acetate in dimethylformamide solution (0.136g/mL) were added. After 1hour, a red precipitate started to form. The whole reaction was stirred at room temperature for 48hours. The precipitate was filtered and dried, then dissolved in methanol (50mL) and boiled for 30 min at 60oC. Methanol was removed by rotary evaporation and the crude product (3a) was purified by crystallization from dichloromethane (20mL) and methanol (20mL); orange-red crystals; mp 136-137oC; 1H NMR (CDCl3, 300 MHz) δ 2.13(s, 3H), 3.03(s, 6H), 5.60(s, 1H), 6.27(d, J = 15.6 Hz, 1H), 6.65-6.69(m, 2H), 7.41-7.44(m, 2H), 7.56(d, J=15.9 Hz, 1H), 18.46(br s, 1H).

(±)(2,2-Dimethyl-1,3-dioxolan-4-yl)methyl-4-methylbenzenesulfonate(**5c**): 10mmol racemic 2,2-dimethyl-1,3-dioxolane-4-methanol (Soketal, Sigma-Aldrich, Inc., St. Louis, MO) dissolved in Pyridine (20 mL) at room temperature, followed by the addition of 2.1g p-toluenesulfonyl chloride (p-TsCl,11.0 mmol, 1.1eq). The reaction mixture was left at room temperature for 5 h, and then worked up by 30mL 1.0 N HCl. 30mL dicholoromethane was used to extract the

compound, and removed by rotary evaporation to afford compound 5c.[24] Pale yellow viscous liquid, yield 92%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.31(s, 3H), 1.34(s, 3H), 2.45(s, 3H), 3.82-3.85(m, 1H), 3.87-3.94(m, 1H), 3.95-4.00 (m, 2H), 4.28-4.41(m, 1H), 7.36(d, 2H, *J*=8.7Hz), 7.77(d, 2H, *J*=8.7Hz).

(±)4-((2,2-Dimethyl-1,3-dioxolan-4-yl)methoxy)-methyl-benzoate (5e): 2.86g **5c**(10mmol) was dissolved in 80mL DMF, followed by the addtion of 4-Hydroxybenzoate(5d)(1.52g, 10mmol) and potassium carbonate(2.12g, 15mmol) at room temperature. While stirring, the reaction mixture was heated to 90^{o}C for 12h. The reaction mixture was poured into 500mL of H_2O after cooling down to room temperature. 200mL dicholormethane was used for extraction, and removed by rotary evaporation. White solid, 86.5%. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.47(s, 3H), 1.53(s, 3H), 3.97-4.00(m, 1H), 4.04-4.09(m, 1H), 4.14-4.19(m, 1H), 4.22-4.27(m, 1H), 4.52-4.59(m, 1H), 7.00(d, *J*=9.0, 2H), 8.05(d, *J*=9.0, 2H)

(±)4-((2,2-Dimethyl-1,3-dioxolan-4-yl)methoxy)-benzoic acid (5f): 2.66g **5e** (10mmol) was added to 50mL methanol, followed by the addition of 15mL 1N KOH in H_2O. The reaction mixture was left to 60^{o}C for 4h. 20mL 0.5N citric acid was used to neutralize the reaction mixture after cooling down to room temperature. 50mL ethyl acetate was used for extraction, and removed by rotary evaperation.[25] White solid, 95.6% Yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.41(s, 3H), 1.47(s, 3H), 3.88-3.94(m, 1H), 3.99-4.05(m, 1H), 4.09-4.21(m, 2H), 4.47-4.55(m, 1H), 6.96(d, *J*=9.0, 2H), 8.06(d, *J*=9.0, 2H)

2. General method for the synthesis of the curcumin analogues

2,4-Pentanedione or a 3-substituted 2,4-pentanedione (10mmol) and very finely powdered boron oxide (0.49 g, 7mmol, 0.7 eq) were placed in a 50mL flask and heated to 120^{o}C for 5min to form a pale-yellow viscous liquid which was cooled to room temperature. The appropriate aldehyde

(20mmol, 2.0 eq) and trimethyl borate (4.16g, 40mmol, 4.0eq) were dissolved in ethyl acetate (10 mL) and gradually added to reaction mixture over 10 minutes. Thereafter, with stirring, 0.05mL butylamine and 0.2mL butylammonium acetate in dimethylformamide solution (0.136g/mL) was then added. After 1 hour, a red-colored precipitate began to form and stirring was continued at room temperature for 48 hours. The precipitate was removed by filtration, washed with dry ether and air-dried, then dissolved in methanol (50 mL) and boiled for 30 min at 60^{o}C. The methanol was removed by rotary evaporation and the solid crude product was purified by recrystallization from dichloromethane (20 mL) and methanol (20 mL).

1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1E,6E-dien-3,5-dione (1): From 2,4-pentanedione (**1a**) and 4-hydroxy-3-methoxybenzaldehyde (**2a**); orange crystals, yield 77.0%, mp 175-176^{o}C.(lit.4, mp 177-178^{o}C) ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.83(s, 6H), 6.05(s, 1H), 6.75(d, *J*=15.6Hz, 2H), 6.81(d, *J*=7.8Hz, 2H), 7.14(d, *J*=8.4Hz, 1H), 7.15(d, *J*=8.1Hz, 1H), 7.31(d, *J*=2.1Hz, 2H), 7.54(d, *J*=15.9 Hz, 2H), 9.66(s. 2H), 16.40 (s, 1H); ESI (-ve) MS m/z 367.2[M-H]$^{-}$.

1,7-Diphenyl-hepta-1E,6E-dien-3,5-dione (2): From 2,4-pentanedione (**1a**) and benzaldehyde (**2b**); orange crystals, yield 65.1%, mp 141-142^{o}C. (lit.[4] mp 140.5^{o}C) ^{1}H NMR (DMSO-d_6, 300MHz): δ 6.21(s, 1H, =CH), 6.96(d, *J*=15.9Hz, 2H, =CH), 7.41-7.48(m, 6H, Ar-H), 6.65(d, *J*=15.9Hz, 2H, =CH), 7.71-7.75(m, 4H, Ar-H), 16.08(s, 1H, -OH enol). ESI (-ve) MS m/z 275.2 [M-H]$^{-}$.

1,7-Bis(3-pyridyl)-hepta-1E,6E-dien-3,5-dione (3): From 2,4-pentanedione (**1a**) and 3-pyridinecarboxaldehyde (**2c**); yellow solid, yield 62.3%, mp 171-172^{o}C.^{1}H NMR (DMSO-d_6, 300MHz): δ 6.20(s, 1H), 7.12(d, *J*=16.2Hz, 2H), 7.45-7.49(m, 2H), 7.68(d, *J*=15.9Hz, 2H),

8.18(d, *J*=8.4Hz, 2H), 8.57-8.59(m, 2H), 8.90(d, *J*=1.8Hz, 2H), 15.97(s, 1H). ESI (-ve) MS m/z 277.2[M-H]$^-$.

1,7-Bis(4-N,N-dimethylaminophenyl)-hepta-1E,6E-dien-3,5-dione (4): From 2,4-pentanedione (**1a**) and 4-(dimethylamino)benzaldehyde (**2d**); purple crystals, yield 61.1%, mp 212-214^{o}C.(lit. [24] mp 214-215^{o}C) ^{1}H NMR (DMSO-d_6, 300MHz): δ 2.98(s, 12H), 5.95(s, 1H), 6.59(d, *J*=15.9Hz, 2H), 6.72(d, *J*=9.0Hz, 4H), 7.50(d, *J*=15.6Hz, 2H), 7.53(d, *J*=8.7Hz, 4H), 16.62(s, 1H). ESI (-ve) MS m/z 365.1[M-H]$^-$.

1,7-Bis(4-(4-methylpiperazinyl-1-yl))hepta-1E,6E-dien-3,5-dione (5): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-(4-methylpiperazinyl-1-yl)benzaldehyde (**2e**); orange-red crystals, yield 46.4%, mp 239-240^{o}C. ^{1}H NMR ($CDCl_3$, 300MHz) δ 2.36(s, 6H), 2.56(t, *J* = 5.1Hz, 8H), 3.32(t, *J* = 5.0Hz, 8H), 5.74(s, 1H), 6.46(d, *J* = 15.6Hz, 2H), 6.87-6.91(m, 4H), 7.45-7.48(m, 4H), 7.58(d, *J* = 15.6Hz, 2H), 16.20(br s, 1H); ESI (-ve) MS m/z 471.2[M-H]$^-$.

1,7-Bis-phenyl-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (6) : From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and benzaldehyde (**2b**); white solid, yield 52.1%, mp 126-127^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.92(s. 3H), 7.26(d, *J*=15.6Hz, 2H), 7.45-7.47(m, 6H), 7.72-7.75(m, 4H), 7.82(d, *J*=15.6Hz, 2H), 18.07(s, 1H). ESI (-ve) MS m/z 333.2[M-H]$^-$.

1,7-Bis(4-hydroxy-3-methoxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (7): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-hydroxy3-methoxy-benzaldehyde (**2a**); yellow crystals, yield 72.0%, mp 175-176^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.82(s, 3H), 3.91(s, 6H), 6.84(d, *J*=7.2Hz, 2H), 7.03(d, *J*= 15.6Hz, 2H), 7.19(d, *J*=8.1Hz, 1H), 7.20(d, *J*=8.4Hz, 1H), 7.28(d, *J*=1.5Hz, 2H), 7.73(d, *J*=15.6Hz, 2H), 9.81(s. 2H), 18.31(s, 1H); ESI (-ve) MS m/z 425.2[M-H]$^-$.

1,7-Bis(3-hydroxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (8): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 3-hydroxybenzaldehyde (**2f**); yellow crystals, yield 40.2%, mp 188-189^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.91(s, 3H), 6.86(d, *J*=7.8Hz, 2H), 7.08-7.28(m, 8H), 7.73(d, *J*=15.3Hz, 2H), 9.70(s, 2H), 18.11(s, 1H). ESI (-ve) MS m/z 365.2[M-H]$^{-}$.

1,7-Bis(3-pyridyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (9): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 3-pyridinecarboxaldehyde (**2c**); yellow solid, yield 38.7%, mp 195-196^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.92(s, 3H), 7.38(d, *J*= 15.9Hz, 2H), 7.49(t, 2H), 7.86(d, *J*=15.3Hz, 2H), 8.19(d, *J*=7.5Hz, 2H), 8.61(d, *J*=4.2Hz, 2H), 8.90(s, 2H), 17.89(s, 1H). ESI (-ve) MS m/z 335.2[M-H]$^{-}$.

1,7-Bis(2-hydroxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (10): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 2-hydroxybenzaldehyde (**2g**); yellow crystals, yield 46.3%, mp 165-166^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.88(s, 3H), 6.84-6.94(m, 4H), 7.26(d, *J*=15.3Hz, 1H), 7.27(d, *J*=15.3Hz, 1H), 7.29(d, *J*=15.6Hz, 2H), 7.58(d, *J*=7.5Hz,1H), 7.58(d, *J*=8.1Hz, 1H), 8.01(d, *J*=15.6Hz, 2H), 10.42 (s, 2H), 18.11(s, 1H); ESI (-ve) MS m/z 365.2[M-H]$^{-}$.

1,7-Bis(4-N,N-dimethylaminophenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (11) From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-(dimethylamino)benzaldehyde (**2d**); purple crystals, yield 45.1%, mp 224-225^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.01(s, 12H), 3.89(s, 3H), 6.74(d, *J*=8.7Hz, 4H), 6.88(d, *J*=15.3 Hz, 2H), 7.53(d, *J*=9.0 Hz, 4H), 7.70(d, *J*=15.3 Hz, 2H), 18.47(s, 1H); ESI (-ve) MS m/z 419.2[M-H]$^{-}$.

1,7-Bis(3-nitro-4-hydroxy-5-methoxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (12): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-hydroxy-3-methoxy-5-

nitrobenzaldehyde (**2h**); orange solid, yield 26.0%, mp 207-208^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.92(s, 3H), 3.94(d, *J*=1.5Hz, 6H), 6.98(d, *J*=15.9Hz, 1H), 7.21(d, *J*=13.5Hz, 1H), 7.61(d, *J*=15.9Hz, 1H), 7.63(d, *J*=13.5Hz, 2H), 7.77(d, *J*=15.6Hz, 1H), 7.84(d, *J*=9.3Hz, 2H), 11.03(s, 2H), 18.06(s, 1H). ESI (-ve) MS m/z 515.0 $[M-H]^-$.

1,7-Bis(4-hydroxy-3,5-dimethoxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (13): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-hydroxy-3,5-dimethoxybenzaldehyde (**2i**); yellow crystals, yield 77.0%, mp 179-180^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.82(s, 12H), 3.92(s, 3H), 7.03(s, 5H), 7.09(s, 1H), 7.73(d, *J*=15.6Hz, 2H), 9.18(s, 2H), 18.30(s, 1H); ESI (-ve) MS m/z 485.2$[M-H]^-$.

1,7-Bis(4-hydroxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (14): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-hydroxybenzaldehyde (**2j**); yellow crystals, yield 49.2%, mp 214-216^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.90(s, 3H), 6.83(d, *J*=8.4Hz, 4H), 6.99(d, *J*=15.6Hz, 2H), 7.58(d, *J*=8.7Hz, 4H), 7.73(d, *J*=15.6Hz, 2H), 10.17(s, 2H), 18.27(s, 1H); ESI (-ve) MS m/z 365.2$[M-H]^-$.

1,7-Bis(4-acetoxy-3-methoxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (15) : From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-acetoxy-3-methoxybenzaldehyde (**2k**); pale-yellow crystals, yield 46.0%, mp 169-170^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 2.27(s, 6H), 3.84(s, 6H), 3.92(s, 3H), 7.17(d, *J*=8.4Hz, 2H), 7.26(d, *J*=15.9Hz, 2H), 7.36(d, *J*=8.1Hz, 2H), 7. 49(s, 2H), 7.81(d, *J*=15.3 Hz, 2H), 18.04(s, 1H, -OH); ESI (-ve) MS m/z 509.2$[M-H]^-$.

1,7-Bis(4-hydroxy-3-methoxyphenyl)-4-ethoxycarbonylhepta-1E,6E-dien-3,5-dione (16): From 3-ethoxycarbonyl-2,4-pentanedione (**1c**) and 4-hydroxy-3- methoxybenzaldehyde (**2a**); yellow crystals, yield 44.6%. mp 158-159^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 1.32-1.37(t, 3H),

3.82(s, 6H), 4.39(q, 2H), 6.84(d, *J*=8.1Hz, 2H), 7.08(d, *J*=15.3Hz, 2H), 7.17(d, *J*=8.1Hz, 1H), 7.17(d, *J*=8.1Hz, 1H), 7.26(d, *J*=1.5Hz, 2H), 7.72(d, *J*=15.9Hz, 2H), 9.80(s, 2H), 18.30(s, 1H); ESI (-ve) MS m/z 439.2[M-H]$^-$.

1,7-Bis(4-(4-methylpiperazinyl-1-yl)phenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5-dione (17): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 4-(4-methylpiperazinyl-1-yl)benzaldehyde (**2e**); dark-red crystals, 49.2% yield, mp 191-192^{o}C. ^{1}H NMR ($CDCl_3$, 300MHz) δ 3.04(s, 6H), 3.94(s, 3H), 6.65-6.69(m, 4H), 7.00(d, *J* = 14.4Hz, 2H), 7.46-7.49 (m, 4H), 7.78 (d, *J* = 15.3Hz, 2H), 15.69 (br s, 1H). ESI (-ve) MS m/z 529.2[M-H]$^-$.

1,7-Bis(2-hydroxy-3-methoxyphenyl)-4-methoxycarbonylhepta-1E,6E-dien-3,5- dione (18): From 3-methoxycarbonyl-2,4-pentanedione (**1b**) and 2-hydroxy-3-methoxybenzaldehyde (**2l**); yellow crystals, yield 25.8%, mp 201-202^{o}C. ^{1}H NMR (DMSO-d_6, 300MHz): δ 3.82(s, 6H), 3.88(s, 3H), 6.83(t, 2H), 7.04(d, *J*=7.5Hz, 2H), 7.18(d, *J*=7.5Hz, 2H), 7.26(d, *J*=15.9Hz, 2H), 8.04(d, *J*=15.6Hz, 2H), 9.62(s, 2H), 18.13(s, 1H); ESI (-ve) MS m/z 425.2[M-H]$^-$.

1,7-bis(4-hydroxy-3-methoxyphenyl)-4-methoxycarbonylheptane-3,5-dione (19):
7 (0.426g, 1mmol) was added to 10mL ethyl acetate, followed by catalytic amount of 10% Pd/C. The reaction mixture was stirred under the atmosphere of H2 for 2 hours, and then filtered through Celite. The solvent was removed by rotary evaporation, and the solid crude product was purified by recrystallization from dichloromethane (4mL) and methanol (6mL). Pale-yellow solid, yield 52.1%, mp 92-94^{o}C. 1H NMR (DMSO-d6, 300MHz): δ 2.62-2.84(m, 8H), 3.71(s, 3H), 3.72(s, 6H), 6.54(d, J=1.8Hz, 1H), 6.57(d, J=2.1Hz, 1H), 6.63(d, J=2.7Hz, 1H), 6.65(d, J=2.7Hz, 1H), 6.74(d, J=1.5Hz, 2H), 8.73(s, 2H), 17.57(s, 1H); ESI (-ve) MS m/z 429.2[M-H]-.

1-(4-Dimethylaminophenyl)-7-(4-hydroxy-3-methoxyphenyl)-hepta-1E,6E-dien-3,5-dione (20): From 4-hydroxy-3-methoxybenzaldehyde (**2a**) and (3Z,5E)-6-(4-dimethylaminophenyl)-4-

hydroxyhexa-3,5-dien-2-one (**3a**); orange-red crystals; mp 171-172^{o}C; 1H NMR (CDCl3, 300 MHz) δ 3.07(s, 6H), 3.95(s, 3H), 5.77(s, 1H), 5.83(br s, 1H), 6.43(d, J=15.9Hz, 1H), 6.47(d, J=15.9Hz, 1H), 6.66-6.70(m, 2H), 6.93(d, J=9.0Hz, 1H), 7.05(d, J=2.1Hz, 1H), 7.11 (dd, J=8.2, 1.7Hz, 1H), 7.44-7.48(m, 4H), 7.55(d, J=15.9Hz, 1H), 7.63(d, J = 15.9Hz, 1H), 16.20(br s, 1H); MS (EI) m/z (%) 364 (M˙+, 60), 267 (100), 188 (35), 174 (100), 147 (50), 146 (50), 134 (100).

1-(4-Dimethylaminophenyl)-7-((4-N-methylpiperazin-1-yl)phenyl)-hepta-1E,6E-dien-3,5-dione (21): From 4-(4-N-methylpiperazin-1-yl)benzaldehyde (**2e**) and (3Z,5E)-6-(4-dimethylaminophenyl)-4-hydroxyhexa-3,5-dien-2-one (**3a**); orange-red crystals; mp 242-243^{o}C; 1H NMR (CDCl3, 300 MHz) δ 2.36(s, 3H), 2.56(t, J = 5.2Hz, 4H), 3.03(s, 6H), 3.31(t, J = 5.0Hz, 4H), 5.74(s, 1H), 6.43(d, J = 15.6Hz, 1H), 6.46(d, J = 15.6Hz, 1H), 6.66-6.70(m, 2H), 6.87-6.91(m, 2H), 7.44-7.47(m, 4H), 7.57 (d, J = 15.9Hz, 1H), 7.61 (d, J = 15.9Hz, 1H), 16.27 (br s, 1H); MS (EI) m/z (%) 417 (M˙+, 40), 321 (75), 266 (30), 189 (40), 188 (40), 174 (95), 147 (45), 146 (50), 134 (100).

1,7-Bis(4-dimethylaminophenyl)-4-N-phenylaminocarbonylhepta-1E,6E-dien-3,5-dione (22): From 3-phenylaminocarbonyl-2,4-pentanedione (**1c**) and 4-(dimethylamino)benzaldehyde (**2d**); purple crystals, yield 51.7%, mp 208-209^{o}C. 1H NMR (DMSO-d6, 300MHz): δ 2.96(s, 12H), 6.59(d, J=15.6Hz, 2H), 6.69(d, J=8.1Hz, 4H), 7.13(t, 1H), 7.39-7.42(m, 6H), 7.66(d, J=15.3Hz, 2H), 7.73(d, J=8.1Hz, 2H), 10.56(s, 1H), 17.78(s, 1H). ESI (-ve) MS m/z 480.2[M-H]-.

1,7-Bis(4-hydroxy-3-methoxyphenyl)-4-N-phenylaminocarbonylhepta-1E,6E-dien-3,5-dione (23): From 3-phenylaminocarbonyl-2,4-pentanedione (**1c**) and 4-hydroxy-3-methoxybenzaldehyde (**2a**); orange crystals, yield 46.6%, mp 193-194^{o}C. 1H NMR (DMSO-d6, 300 MHz): δ 3.70(s, 6H), 6.73(d, J=15.6 Hz, 2H), 6.79(d, J=8.1Hz, 2H), 7.08-7.15(m, 5H),

7.37(t, 2H), 7.69(d, J=3.9Hz, 2H), 7.72(d, J=3.9Hz, 2H), 9.79(s, 2H), 10.59(s, 1H), 17.56(s, 1H); ESI (-ve) MS m/z 486.2[M-H]-.

1,7-Bis(4-hydroxyphenyl)-4-N-phenylaminocarbonylhepta-1E,6E-dien-3,5-dione (24):

From 3-phenylaminocarbonyl-2,4-pentanedione (**1c**) and 4-hydroxybenzaldehyde (**2j**); yellow crystals, yield 46.2%, mp 220-221^{o}C. 1H NMR (DMSO-d6, 300MHz): δ 6.68(d, J=15.6Hz, 4H), 6.79(d, J=8.7Hz, 4H), 7.13(t, 1H), 7.38(t, 2H), 7.45(d, J=9.0 Hz, 2H), 7.70(d, J=6.3Hz, 2H), 7.72(d, J=9.0Hz, 2H), 10.14(s, 2H), 10.61(s, 1H), 17.56(s, 1H); ESI (-ve) MS m/z 426.2[M-H]-.

1,7-Bis(4-hydroxyphenyl)-4-*N*-(4-chloro)-phenylaminocarbonylhepta-1E,6E-dien-3,5-dione (25): From 3-(4-chloro)-phenylaminocarbonyl-2,4-pentanedione (**1e**) and 4-hydroxybenzaldehyde (**2j**); orange crystals, yield 41.6%, mp 202-203^{o}C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 6.66(d, *J*=15.6Hz, 2H), 6.78(d, 4H, *J*=8.7Hz), 7.41-7.47(t, 6H), 7.71(d, *J*=15.6Hz, 2H), 7.76(d, *J*=8.7 Hz, 2H), 10.16(s. 2H), 10.73(s. 1H), 17.59(s, 1H); ESI (-ve) MS m/z 460.6 [M-H]$^{-}$.

1,7-Bis(4-hydroxyphenyl)-4-*N*-(4-methoxy)-phenylaminocarbonylhepta-1E,6E-dien-3,5-dione (26): From 3-(4-methoxy)-phenylaminocarbonyl-2,4-pentanedione(**1f**) and 4-hydroxybenzaldehyde (**2j**); yellow crystals, yield 52.3%, mp 220-221^{o}C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.75(s, 3H), 6.67(d, *J*=15.6Hz, 2H), 6.79(d, *J*=9.0Hz, 2H), 6.94(d, *J*=9.0Hz, 2H), 7.45(d, *J*=9.0 Hz, 4H), 7.63(d, *J*=9.0Hz, 2H), 7.69(d, *J*=15.3 Hz, 2H), 10.15(s. 2H), 10.46(s. 1H), 17.51(s, 1H); ESI (-ve) MS m/z 456.2 [M-H]$^{-}$.

(±)-1,7-Bis(4-hydroxyphenyl)-4-*N*-(4-((2,2-dimethyl-1,3-dioxolan-4-yl)methoxy)) -phenylaminocarbonylhepta-1E,6E-dien-3,5-dione(27)

From 3-(4-((2,2-dimethyl-1,3-dioxolan-4-yl)methoxy))-phenylaminocarbonyl-2,4-pentanedione(**1g**) and 4-hydroxybenzaldehyde (**2j**); red crystals, yield 17.6%, mp 210-212^{o}C. ^{1}H

NMR (DMSO-d_6, 300 MHz): δ 1.30(s, 3H), 1.36(s, 3H), 3.72-3.77(m, 1H), 3.92-4.12(m, 3H), 4.36-4.46(m, 1H), 6.67(d, *J*=15.3Hz, 2H), 6.79(d, *J*=8.7Hz, 4H), 6.97(d, *J*=9.0Hz, 2H), 7.45(d, *J*=8.7Hz, 4H), 7.63(d, *J*=9.0Hz, 2H), 7.70(d, *J*=15.3Hz, 2H), 10.14(s. 2H), 10.47(s. 1H), 17.52(s, 1H); ESI (-ve) MS m/z 557.2 $[M-H]^-$.

(±)-1,7-Bis(4-hydroxyphenyl)-4-*N*-(4-(2,3-dihydroxypropoxy)phenyl)-phenylaminocarbonylhepta-1E,6E-dien-3,5-dione(28): 200mg of **27** was added to 8mL of methanol and H_2O mixture(v/v=1:1), followed by the addition of 2mL of 10% acetic acid. The reaction was left at room temperature for 6 h until TLC showed no starting material **27**. Then 20mL was used for extraction, and removed by rotary evaporation. Crude product **28** was purified by column chromatography (silica, CH_2Cl_2/MeOH = 90:10).[26] Brown solid, yield 24.3%, mp 234-236^{o}C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 3.42-3.45(m, 2H), 3.72-3.86(m, 3H), 4.62(br, s, 1H), 4.85(br, s, 1H), 6.67(d, *J*=15.9Hz, 2H), 6.75-6.80(m, 4H), 6.94(d, *J*=9.0Hz, 2H), 7.43-7.49(m, 4H), 7.62(d, *J*=9.0Hz, 2H), 7.69(d, *J*=15.3Hz, 2H), 10.15(s. 2H), 10.46(s. 1H), 17.52(s, 1H); ESI (-ve) MS m/z 517.2 $[M-H]^-$.

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Chapter 4. *In vitro* potency of curcumin and chemically-modified curcumins (CMCs)

A. Introduction

The inhibitory concentration at 50% (IC_{50}) is a crucial measure of efficacy of a compound in any functions related to biological or biochemical processes. In this chapter, an *in vitro* enzymology study is described for both curcumin and the CMCs using a variety of MMPs. A preliminary *in vitro* MMP-9 inhibitory assay was first conducted for the first 21 synthesized compounds, by using an HPLC method that was used in previous evaluations of tetracycline.[1] Curcumin, CMC2.5, CMC2.14 showed great potency against human-derived MMP-9 *in vitro*, and were then selected for further study. Two other compounds, CMC2.23 and CMC2.24, where an N-phenylamino carbonyl group had been introduced, were evaluated together with the active compounds from the preliminary study. These five compounds, as well as 1,10-phenanthroline, a known zinc chelator and inhibitor of MMPs, were evaluated in the fluorogenic inhibitory assays against MMP-1, -2, -3, -7, -8, -9, -12, -13 and -14 *in vitro*.[2] Each enzyme individually was incubated with the MMP-susceptible fluorogenic peptide substrate, Mca-K-P-L-G-Dpa-A-R-NH_2. For each compound, the micromolar (μM) concentration of IC_{50} that was responsible for the proteolytic activity of the MMP was determined from a plot of the percentage of the inhibition versus the concentrations of the inhibitor. CMC2.24 showed great potency against most of the MMPs tested in the assay. In addition, four newer CMCs, namely CMC2.25, CMC2.26, CMC2.27 and CMC2.28, were then studied against human-derived MMP-9 *in vitro* by the fluorogenic assay, where CMC2.25 and CMC2.26 showed similar potency to CMC2.24.

B. Preliminary study of curcumin and CMCs in MMP-9 inhibition by an HPLC method

In order to evaluate the *in vitro* potency of curcumin and CMCs as inhibitors of MMP-9, the inhibition assay was carried out by an HPLC method.[1] DNP-octapeptide containing a gly-ileu

susceptible linkage was used as substrate, and 1,10-phenanthroline, a zinc cation binding agent traditionally used to quench *in vitro* MMP activity reactions, was used as the positive control for the experiment. All of the data are the average (Ave.) of three analyses with standard error of deviation (S.D.) and mean (S.E.M.). From the data shown in Table 4.1, the first two chemically-modified curcumins that were prepared had unsubstituted phenyl or pyrid-3-yl rings at the termini of the 7-carbon chain (CMC2.2 and CMC2.3), but these two compounds were found to be inactive against human derived MMP-9. At this point a methoxycarbonyl group was introduced at the carbon-4 position of thirteen related CMCs in an attempt to increase both solubility and zinc-binding efficacy. From the preliminary data, some of these methoxycarbonyl analogues including CMC2.5 and CMC2.14 showed significant potency compared to the analogues with no carbon-4 substitution such as CMC2.2 and CMC2.3, and also the analogues with unsubstituted phenyl side chains such CMC2.4. Some other side chain modifications including the introduction of dimethylamino-, nitro-, methoxy-, 4-methylpiperazinyl were also made, namely CMC2.11, CMC2.12, CMC2.13, CMC2.17. Also a tetrahydrocurcumin analogue of CMC2.5 was prepared, namely CMC2.9. However, this compound didn't show any inhibitory efficacy against MMP-9 *in vitro*. One ethoxycarbonyl curcumin analogue was also prepared, namely CMC2.16, which was no more soluble or potent than the analogue, CMC2.5 having a methoxycarbonyl group at carbon-4. When a 4-*N*-methylpiperazinyl substituent was introduced as in CMC2.17 or CMC2.19**,** in an attempt to increase the solubility, these two compounds were inactive against MMP-9 *in vitro*. Also two unsymmetrical curcumin analogues, namely CMC2.20 and CMC2.21, showed no improved inhibitory activity. Studies were focused on the two significantly active compounds having a 4-methoxycarbonyl, namely CMC2.5, and 4-methoxycarbonyl bis(demethoxy)curcumin (CMC2.14). However, at the time when the N-

phenylamino carbonyl group was introduced at C-4, the substrate, DNP-octapeptide containing gly-ileu susceptible peptide used or the HPLC assay was not available from the supplier. Thus the biological evaluation of the newer compounds was completed by a fluorogenic assay as noted in Section 4.3 of this chapter.

Table 4.1 *In vitro* IC_{50} (μM) of curcumin and the CMCs against MMP-9

Note: An HPLC inhibition assay was used. 1,10-Phenanthroline, the standard zinc chelating agent was used as a positive control. DNP-octapeptide containing gly-ileu susceptible peptide was used as the substrate. Each value represents the mean of at 3 analyses (± S.E.M.).

Compound		IC_{50}(μM)	% Inhibition at 100 μM
	1,10-Phenanthroline	15.3 ±5.5	100
1	Curcumin	18.5 ±6.5	58.1 ±5.7
2	CMC2.2	>100	<50
3	CMC2.3	>100	<50
4	CMC2.7	>100	<50
5	CMC2.19	43.7 ±4.7	63.9 ±4.2
6	CMC2.4	>100	<50
7	CMC2.5	56.3 ±6.9	67.5 ±8.0
8	CMC2.6	72.6 ±5.2	52.3 ±13.1
9	CMC2.8	>100	<50
10	CMC2.10	23.0 ±4.2	73.3 ±11.9
11	CMC2.11	>100	<50
12	CMC2.12	>100	<50
13	CMC2.13	>100	<50

14	CMC2.14	22.6 ±1.8	89.2 ±6.5
15	CMC2.15	61.1 ±2.6	67.5 ±12.4
16	CMC2.16	26.3 ±3.5	76.7 ±9.6
17	CMC2.17	>100	<50
18	CMC2.18	65.3 ±4.9	62.8 ±8.5
19	CMC2.9	>100	<50
20	CMC2.20	>100	<50
21	CMC2.21	>100	<50

C. *In vitro* potency against nine different MMPs by curcumin and the related CMCs

As mentioned in Chapter 2, different MMPs have diverse functions in tissue remodeling and regulation. Herein, curcumin and four CMCs were screened against nine MMPs including MMP-1, -2, -3, -7, -8, -9, -12, -13 and -14 in the *in vitro* fluorogenic MMP inhibition assay.[3,4] MMP-1, collagenase-1, plays an important role in the turnover of various physiological processes such as tissue morphogenesis, wound repair and remodeling of collagen extracellular matrix in various physiological and pathological situations.[5] Curcumin and CMCs are not as potent as 1,10-phenanthroline with respect to the inhibition of this MMP. 1,10-Phenanthroline possesses an IC_{50} of 42.0 µM, in contrast to the 65.0-85.0 µM range of inhibition by curcumin and CMCs. This suggests that both curcumin and CMCs are not strong inhibitors for MMP-1, the constitutive MMP responsible for the regulation of many physiological functions (Figure 4.1, Table 4.2). Gelatinase A (MMP-2), plays an important role in a variety of pathological conditions related to different cancers.[6] Curcumin, CMC2.23 and CMC2.24 are highly active against MMP-2 having IC_{50}s less than 10.0 µM, especially CMC2.24 having an IC_{50} of 4.8 µM in contrast to the positive control 1,10-phenanthroline (IC_{50}=73.8 µM) (Figure 4.2, Table 4.3). Stormelysin-1 (MMP-3), is

capable of degrading a number of structural proteins in the ECM, and is also responsible for the activation of several other MMPs such as Pro-MMP-1, -8, -13 and -9.[7] Also, curcumin, CMC2.23 and CMC2.24 were potent inhibitors of this type of enzyme. CMC2.24 possesses an IC_{50} of 2.9 μM, the most potent one in this assay (Figure 4.3, Table 4.4). Matrilysin (MMP-7), appears to be very active in early-stage tumor development and late-stage invasion of cancer cells.[8] The positive control, 1,10-phenanthroline does not display strong potency against MMP-7. However, CMC2.24 is quite potent as the inhibitor of this enzyme with an IC_{50} of 5.0 μM, about 10 fold more potent than curcumin (Figure 4.4, Table 4.5). Neutrophil collagenase (MMP-8), is very important in the regulation of the inflammatory response induced by carcinogens, and it is involved in different types of pathological conditions related to inflammation and cancer.[9] CMC2.23 was the most potent compound in the *in vitro* inhibition of MMP-8, having an IC_{50} of 2.5 μM. Curcumin and the other CMCs are also more potent than the control 1,10-phenanthroline (Figure 4.5, Table 4.6). Gelatinase B (MMP-9), is crucial for numerous tissue remodeling situations that include wound healing, inflammation and cancer metastasis.[10] Both CMC2.23 and CMC2.24 appear to be very potent against MMP-9 in this assay, having IC_{50}s around 8.0 μM, and they are also better inhibitors than curcumin (Figure 4.6, Table 4.7). Macrophage metalloelastase (MMP-12), has been found to play an essential role in alveolar macrophages of lung diseases, such as acute lung injury and chronic inflammatory airway diseases.[11] Curcumin and CMC2.24 are the most potent inhibitors in the assay with IC_{50}s around 2.0 μM (Figure 4.7, Table 4.8). Collagenase-3 (MMP-13), can cleave a variety of components in basement membranes, and was found to be the most abundant collagenase in rats and mice.[12] Again curcumin, CMC2.23 and CMC2.24 displayed great potency against MMP-13 with an IC_{50} around 3.0 μM (Figure 4.8, Table 4.9). MMP-14 (MT1-MMP), possesses the activities to degrade a variety of substrates in

the ECM, and can activate the Pro-MMPs, -8, -9 and -13.[13] CMC2.24 was found to be the most potent inhibitor of MMP-14, having an IC_{50} around 15.0 μM (Figure 4.9, Table 4.10). In general, when an N-phenylaminocarbonyl group is introduced at the Carbon-4 position, the MMP inhibitory activity was increased very substantially as is evident from the data for CMC2.23 and CMC2.24, but not in the case of CMC2.22. CMC2.24 proved to be significantly more potent than curcumin, specifically against the MMP-7, -9, -12, and -14, all of which are inducible MMPs and which are active in a variety of inflammatory diseases as well as cancer. However, the constitutive collagenase enzyme MMP-1, was much less affected by either curcumin or the CMCs. The N-phenylamino carbonyl group at Carbon-4 also increased the solubility of these compounds, probably associated with the increased acidity (a tricarbonyl methane versus a dicarbonyl methane; pK_a data shown in Chapter 5) versus curcumin and because of its amide character. The data shown below indicate that both curcumin and the 4-oxo-substituted curcumins are better MMP inhibitors, in contrast to the standard zinc chelating agent, 1,10-phenanthroline. Two N-phenylamino carbonyl curcumins, CMC2.23 and CMC2.24 were also tested against several chemokines and cytokines in cell culture, and CMC2.24 was selected as the current lead compound for further wound healing, bone remodeling and cancer studies.

Figure 4.1 MMP-1 inhibition assay

Note: A fluorimetric inhibition assay was used, and 1, 10-phenanthroline was used as the positive control for the experiment. IC_{50}s were measured from the following plots.

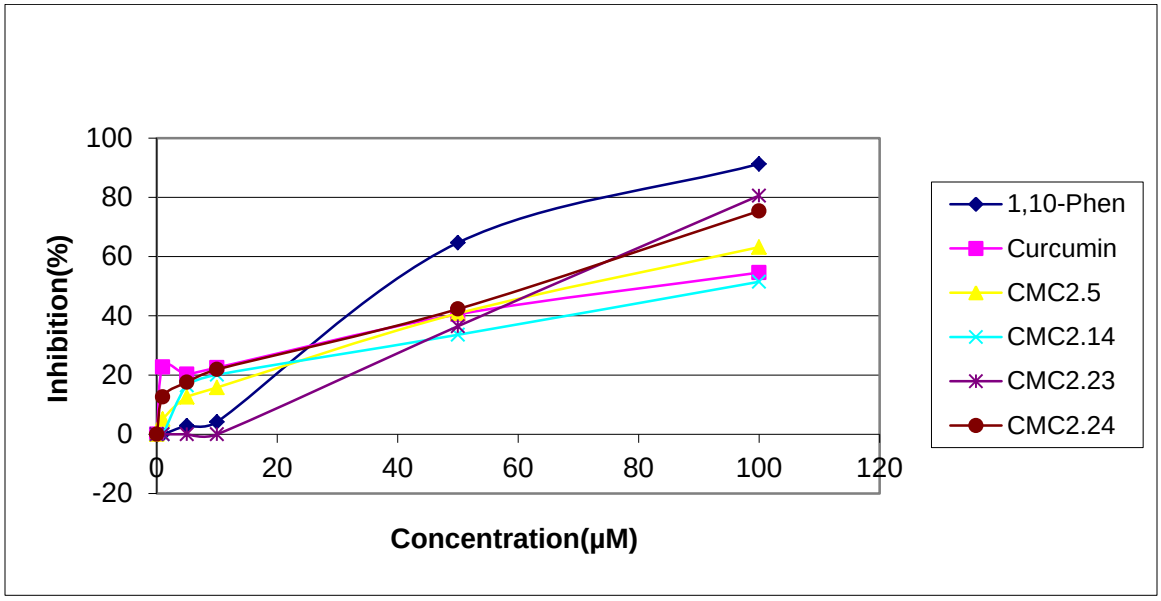

Figure 4.1a Test 1 in MMP-1 inhibition assay

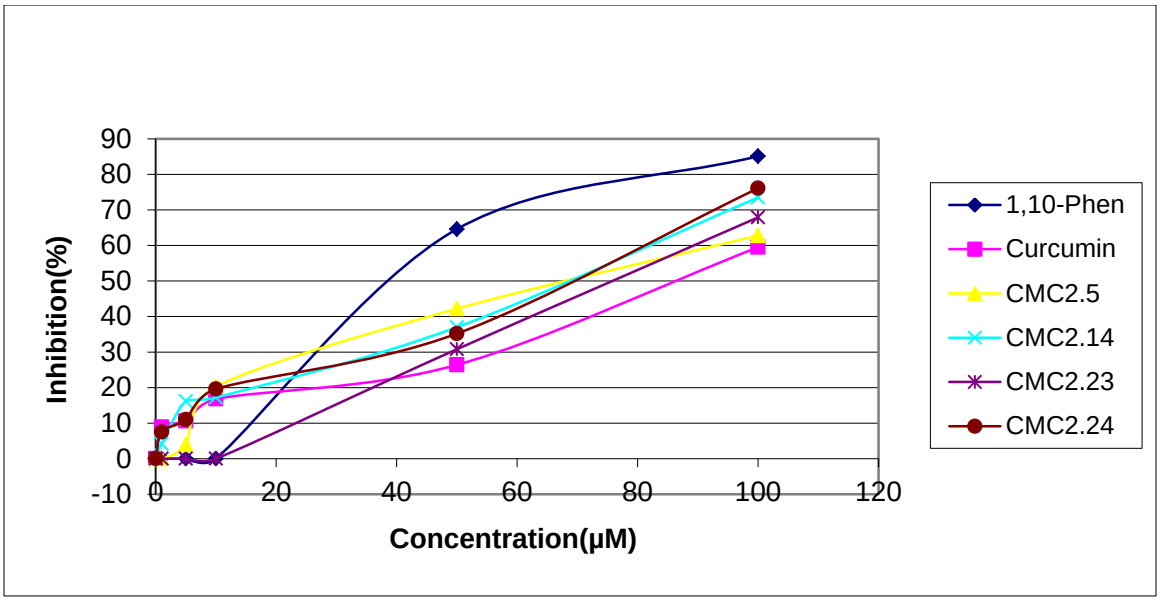

Figure 4.1b Test 2 in MMP-1 inhibition assay

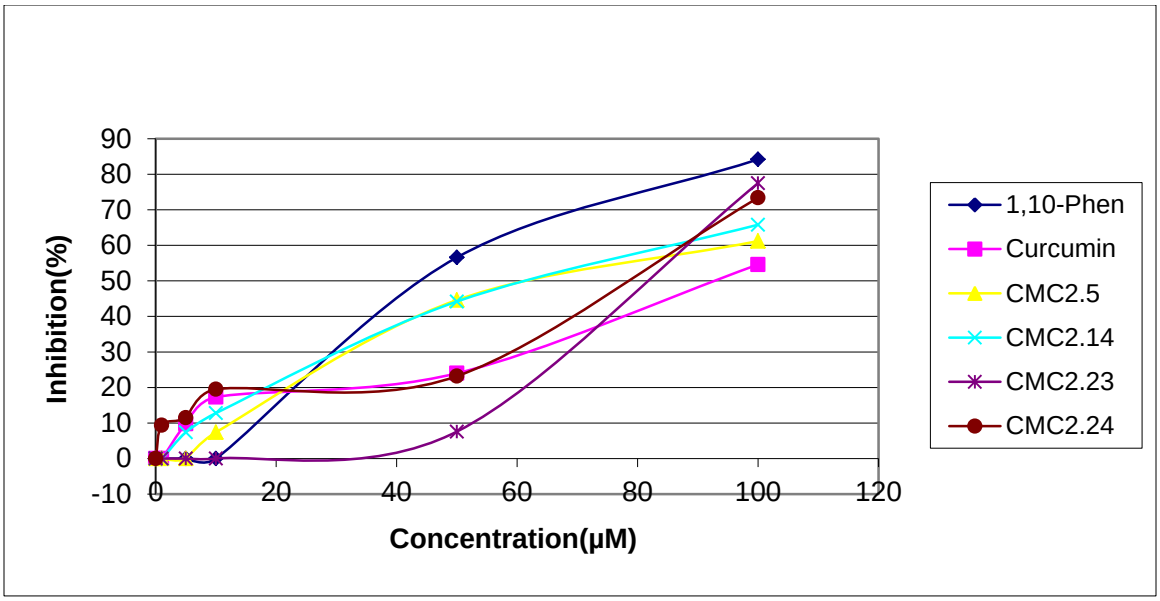

Figure 4.1c Test 3 in MMP-1 inhibition assay

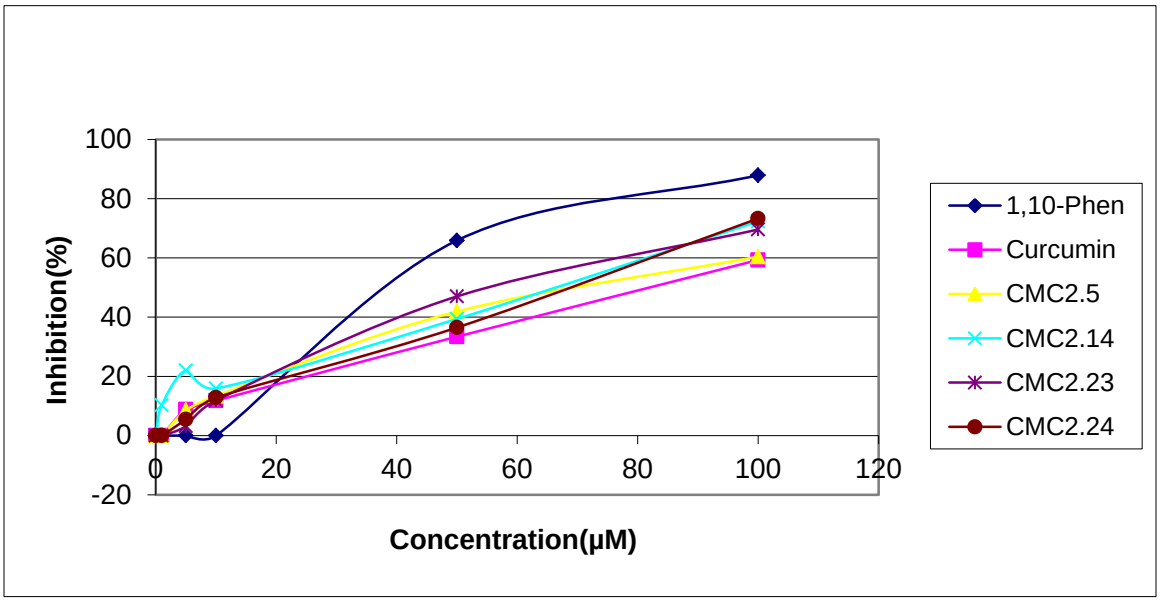

Figure 4.1d Test 4 in MMP-1 inhibition assay

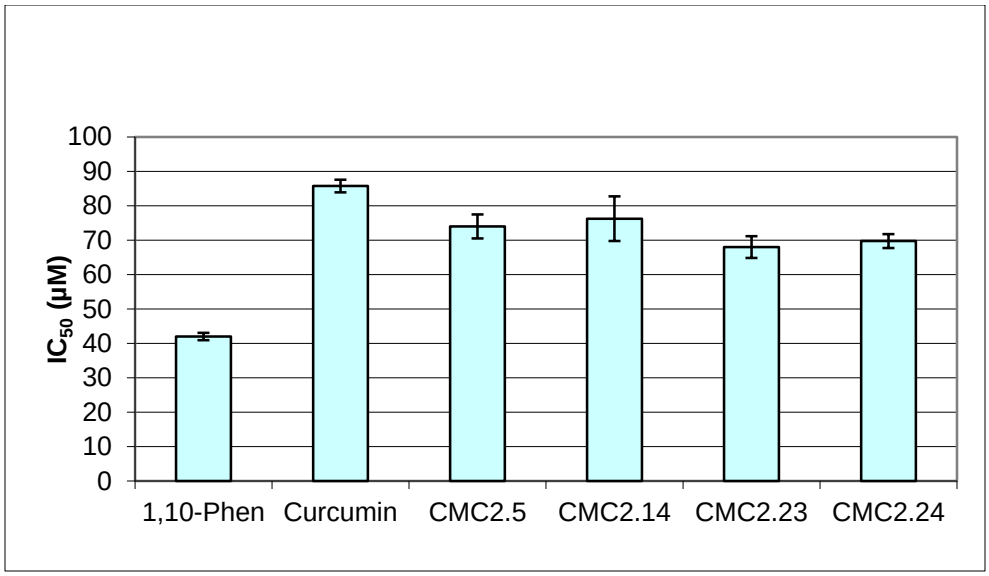

Figure 4.1e IC_{50} of curcumin and selected CMCs against MMP-1

Table 4.2 IC_{50} of curcumin and CMCs against MMP-1

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	42.0	86.0	80.0	93.0	66.0	70.0
Test 2	IC_{50} (µM)	40.0	81.0	79.0	80.0	72.0	64.0
Test 3	IC_{50} (µM)	45.0	90.0	65.0	67.0	74.0	72.0
Test 4	IC_{50} (µM)	41.0	86.0	72.0	65.0	60.0	73.0
	Ave.	42.0	85.8	74.0	76.3	68.0	69.8
	S.D.	2.16	3.68	6.97	12.99	6.32	4.03
	S.E.M.	1.08	1.84	3.49	6.49	3.16	2.01

Figure 4.2 MMP-2 inhibition assay

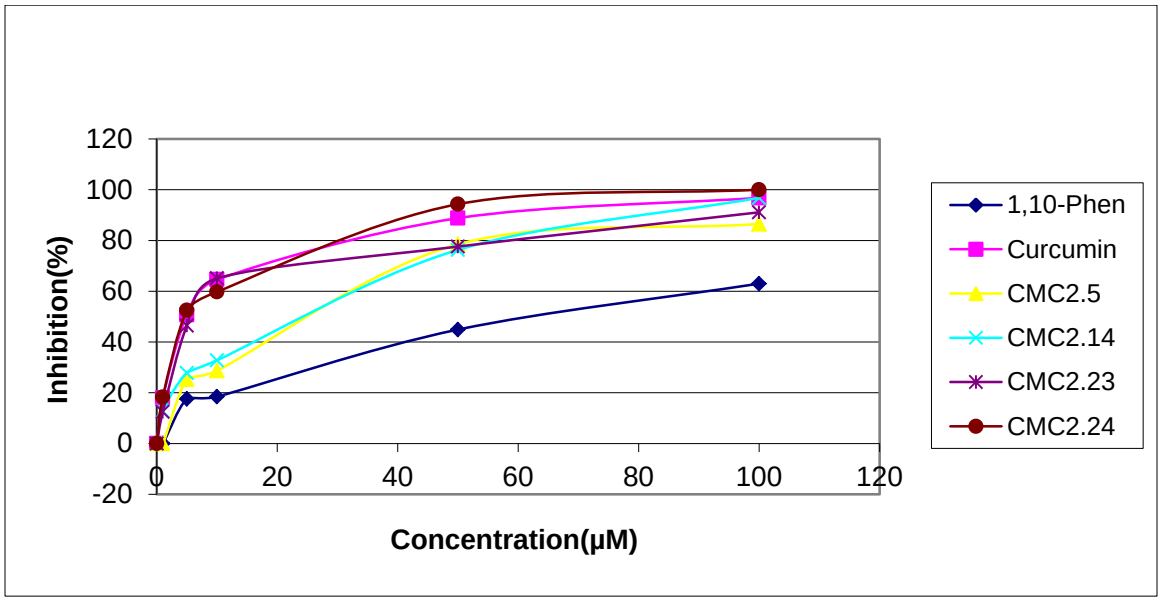

Figure 4.2a Test 1 in MMP-2 inhibition assay

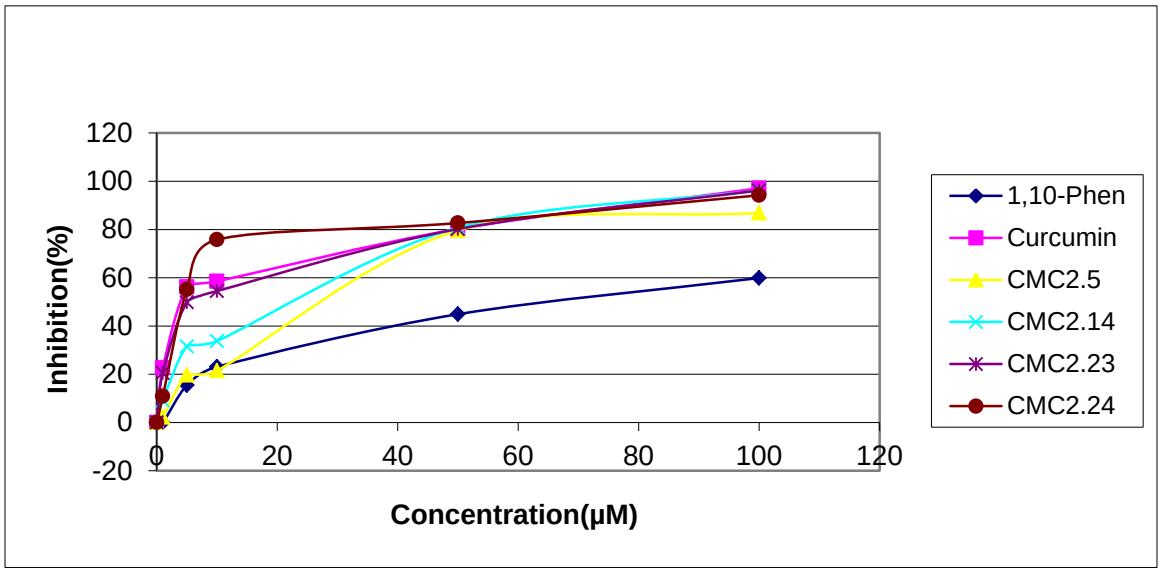

Figure 4.2b Test 2 in MMP-2 inhibition assay

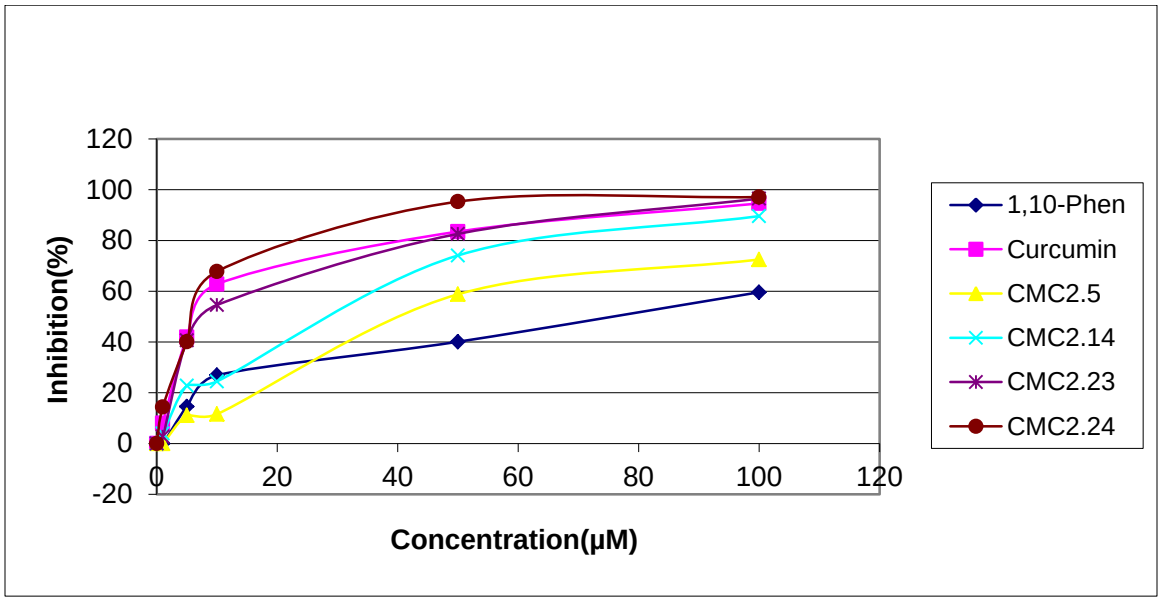

Figure 4.2c Test 3 in MMP-2 inhibition assay

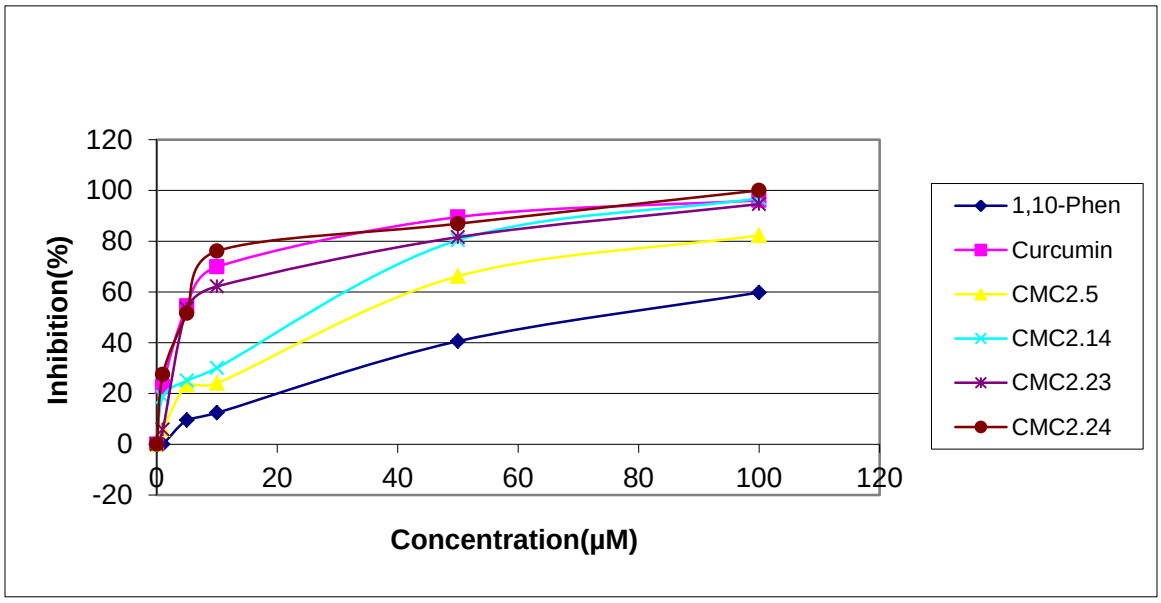

Figure 4.2d Test 4 in MMP-2 inhibition assay

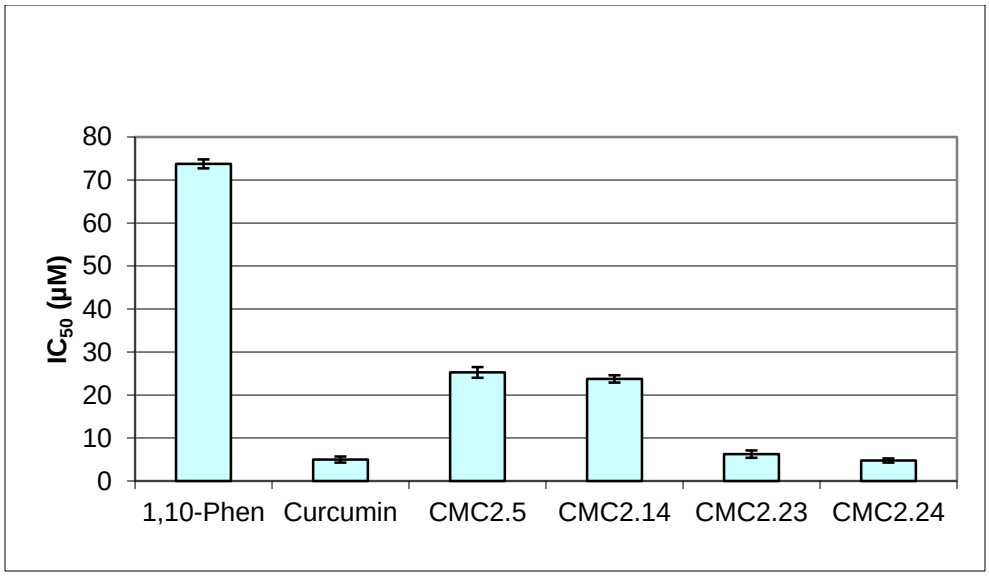

Figure 4.2e IC_{50} of curcumin and selected CMCs against MMP-2

Table 4.3 IC_{50} of curcumin and selected CMCs against MMP-2

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	75.0	5.0	28.0	26.0	6.0	4.0
Test 2	IC_{50} (µM)	72.0	4.0	22.0	24.0	7.0	4.0
Test 3	IC_{50} (µM)	72.0	7.0	25.0	22.0	8.0	6.0
Test 4	IC_{50} (µM)	76.0	4.0	26.0	23.0	4.0	5.0
	Ave.	73.8	5.0	25.3	23.8	6.3	4.8
	S.D.	2.06	1.41	2.50	1.70	1.70	0.96
	S.E.M.	1.03	0.71	1.25	0.85	0.85	0.48

Figure 4.3 MMP-3 inhibition assay

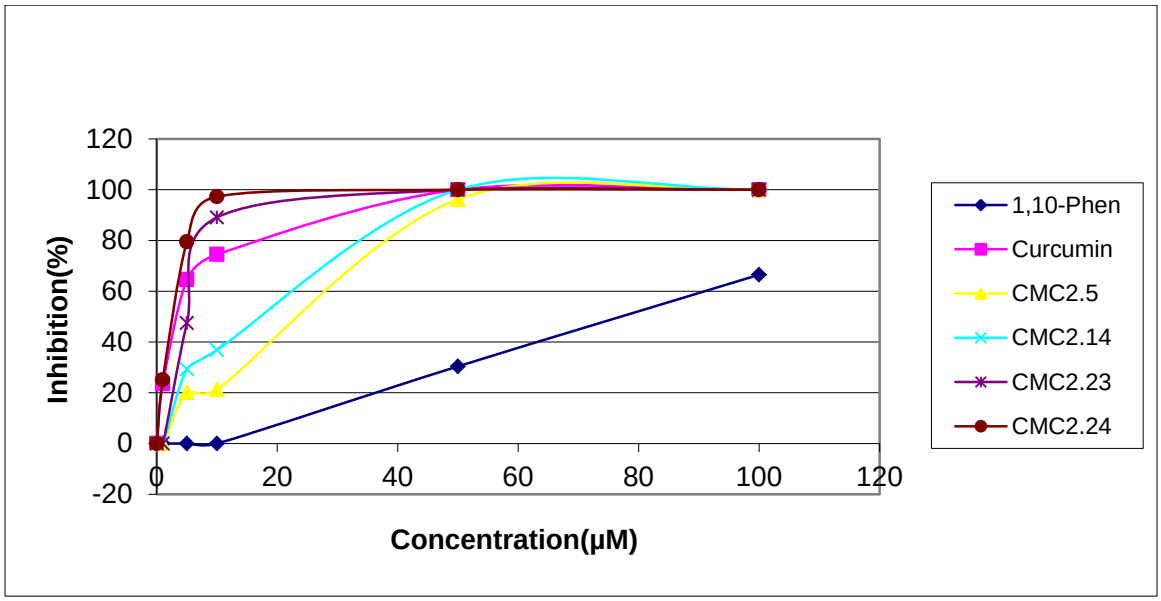

Figure 4.3a Test 1 in MMP-3 inhibition assay

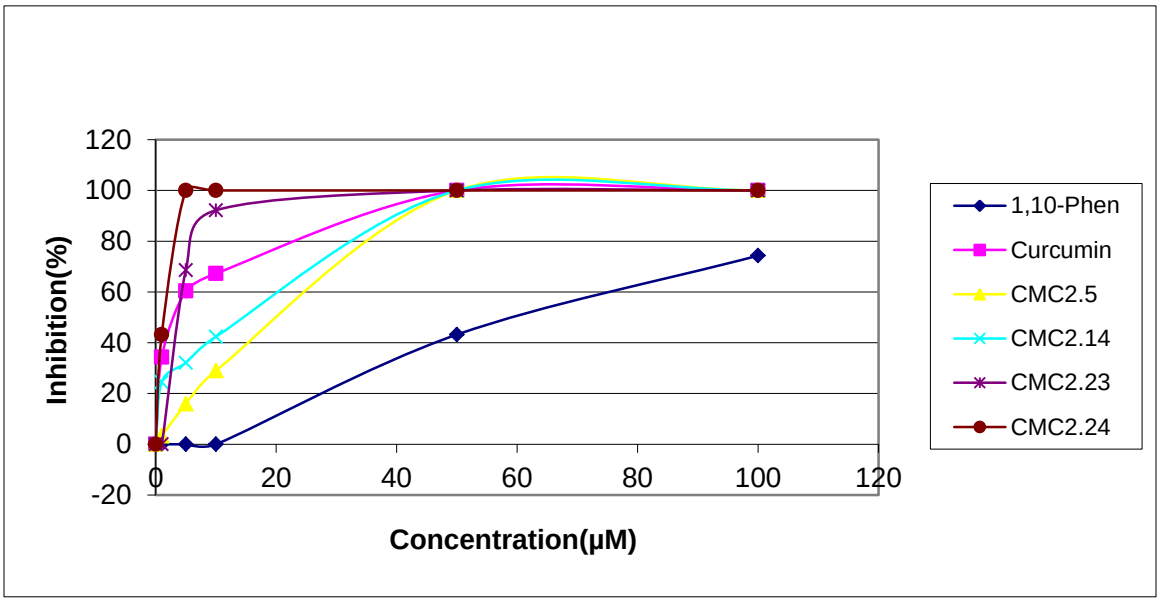

Figure 4.3b Test 2 in MMP-3 inhibition assay

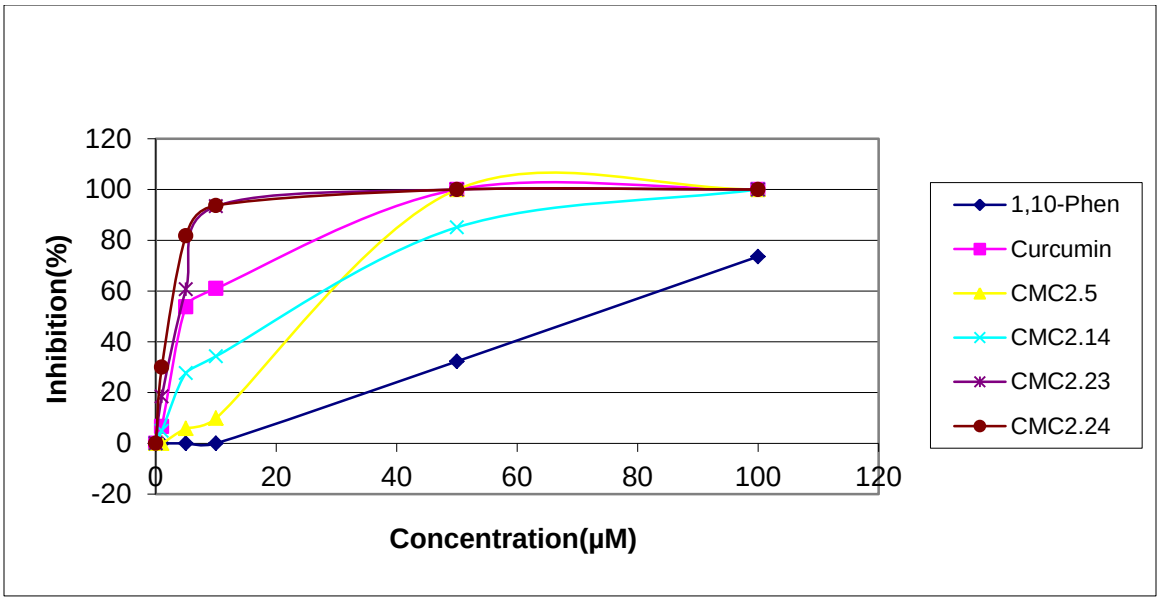

Figure 4.3c Test 3 in MMP-3 inhibition assay

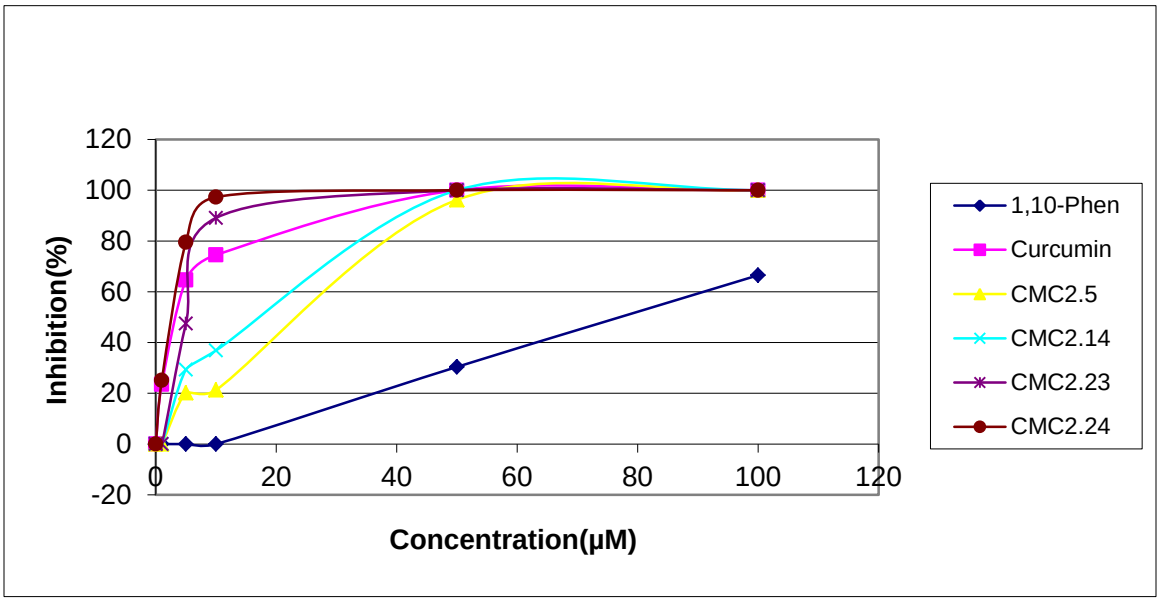

Figure 4.3d Test 4 in MMP-3 inhibition assay

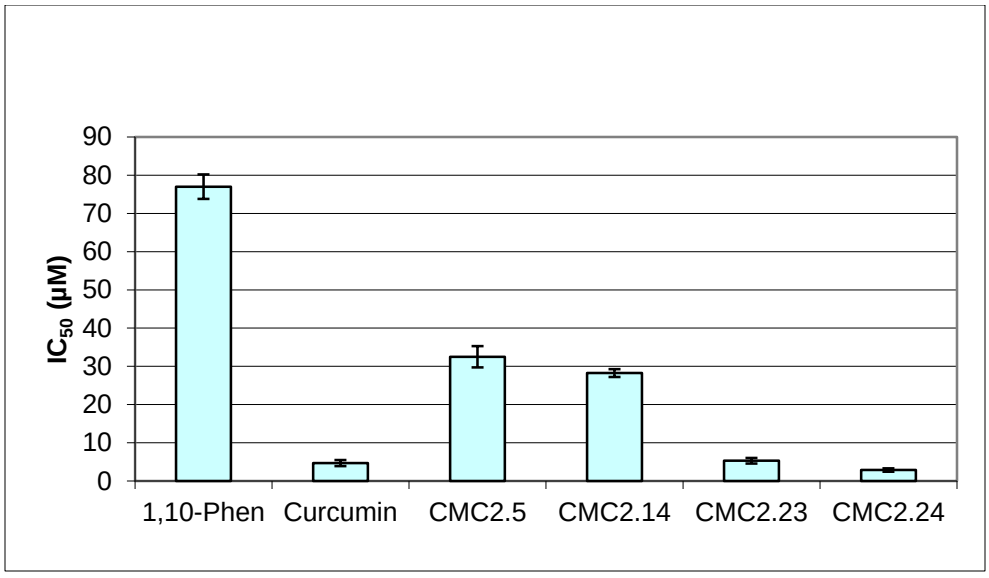

Figure 4.3e IC_{50} of curcumin and selected CMCs against MMP-3

Table 4.4 IC_{50} of curcumin and selected CMCs against MMP-3

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	71.0	3.5	35.0	26.0	4.0	2.0
Test 2	IC_{50} (µM)	75.0	4.0	32.0	30.0	6.0	3.0
Test 3	IC_{50} (µM)	76.0	4.2	38.0	27.0	4.2	2.5
Test 4	IC_{50} (µM)	86.0	7.0	25.0	30.0	7.0	4.0
	Ave.	77.0	4.7	32.5	28.3	5.3	2.9
	S.D.	6.38	1.58	5.57	2.06	1.45	0.85
	S.E.M.	3.19	0.79	2.78	1.03	0.72	0.43

Figure 4.4 MMP-7 inhibition assay

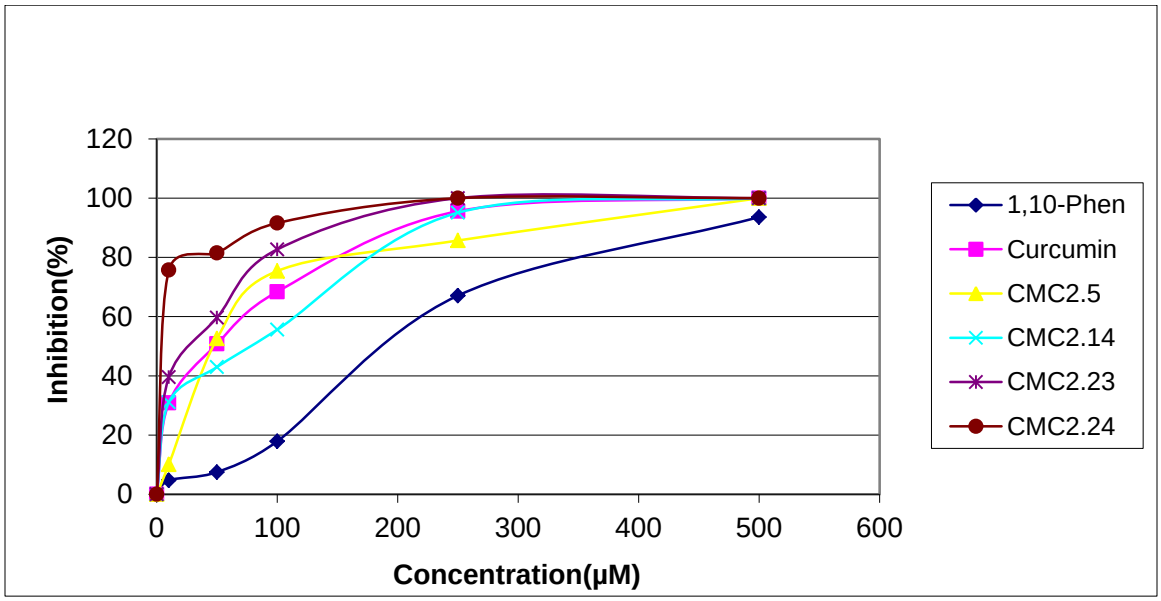

Figure 4.4a Test 1 in MMP-7 inhibition assay

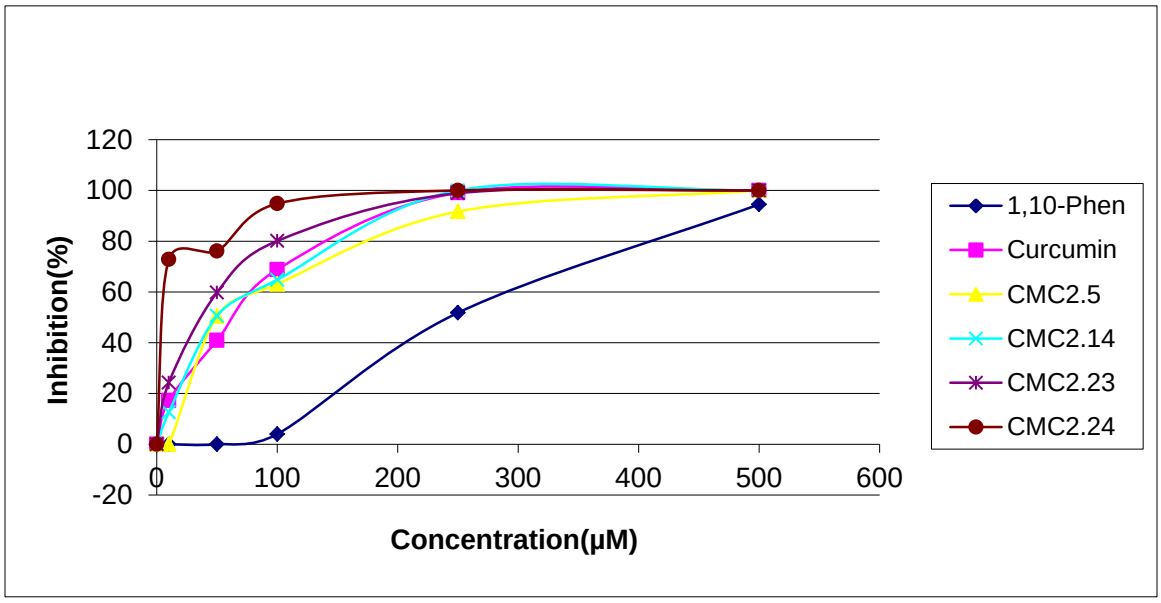

Figure 4.4b Test 2 in MMP-7 inhibition assay

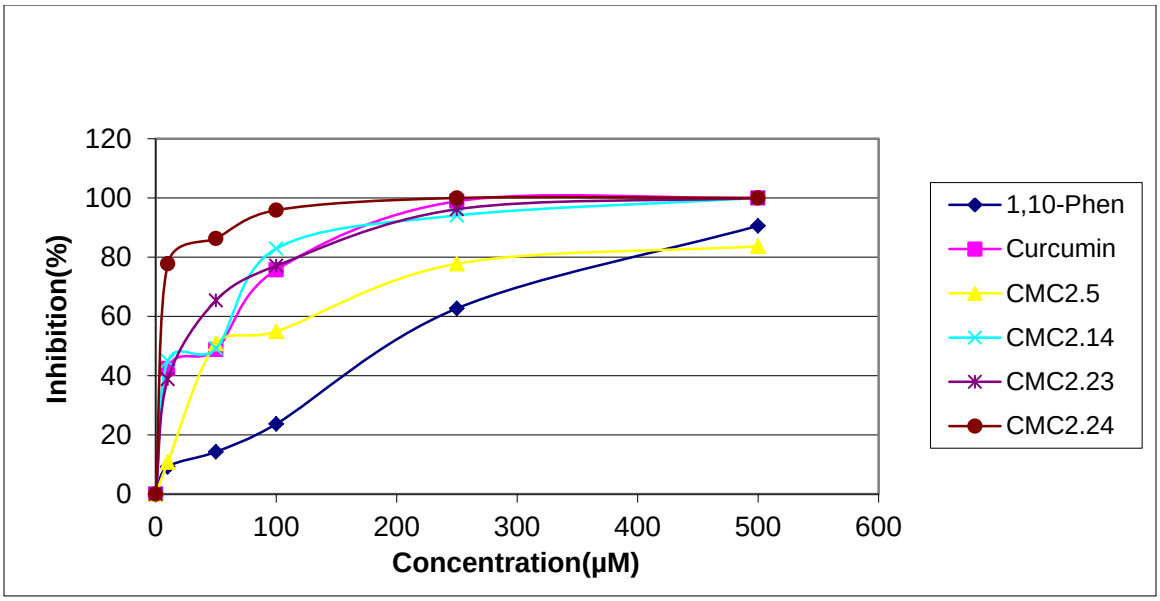

Figure 4.4c Test 3 in MMP-7 inhibition assay

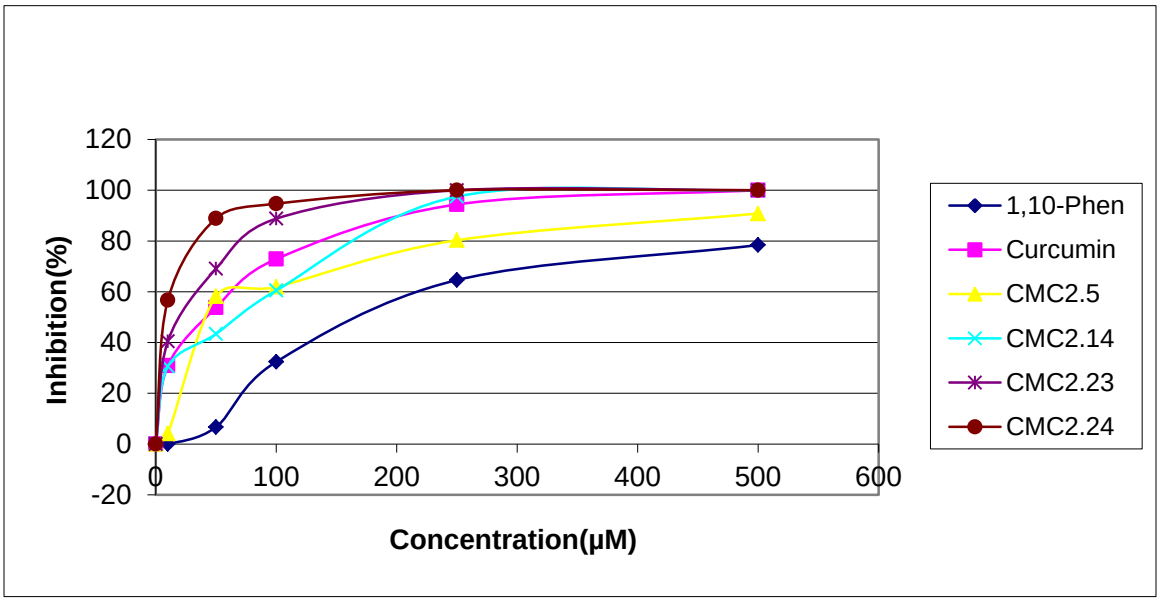

Figure 4.4d Test 4 in MMP-7 inhibition assay

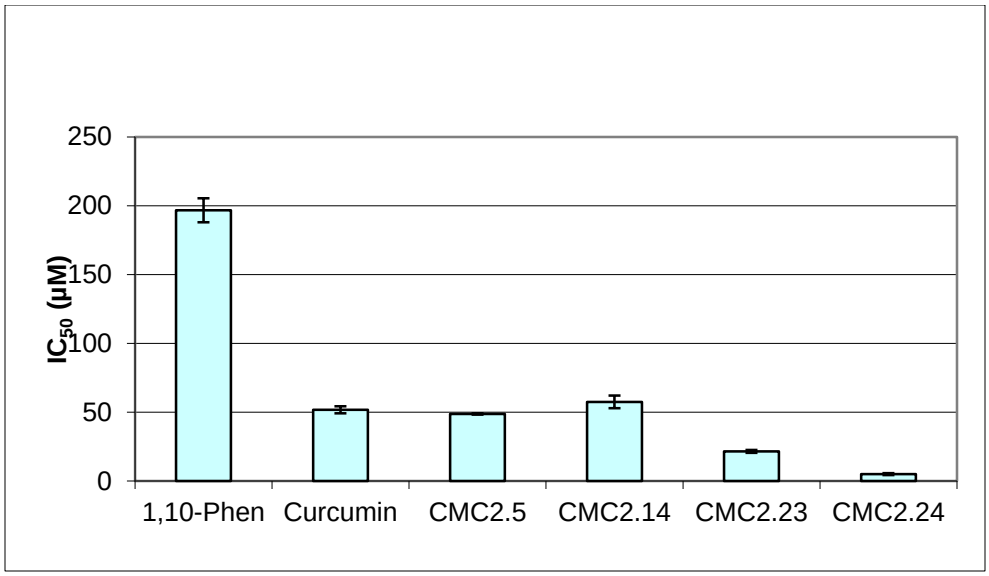

Figure 4.4e IC_{50} of curcumin and selected CMCs against MMP-7

Table 4.5 IC_{50} of curcumin and selected CMCs against MMP-7

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	185.0	49.0	48.0	68.0	19.0	5.0
Test 2	IC_{50} (µM)	218.0	56.0	50.0	48.0	21.0	4.0
Test 3	IC_{50} (µM)	204.0	56.0	49.0	52.0	24.0	4.0
Test 4	IC_{50} (µM)	180.0	46.0	48.0	62.0	22.0	7.0
	Ave.	196.75	51.75	48.75	57.5	21.5	5.0
	S.D.	17.54	5.06	0.96	9.15	2.08	1.41
	S.E.M.	8.77	2.53	0.48	4.57	1.04	0.71

Figure 4.5 MMP-8 inhibition assay

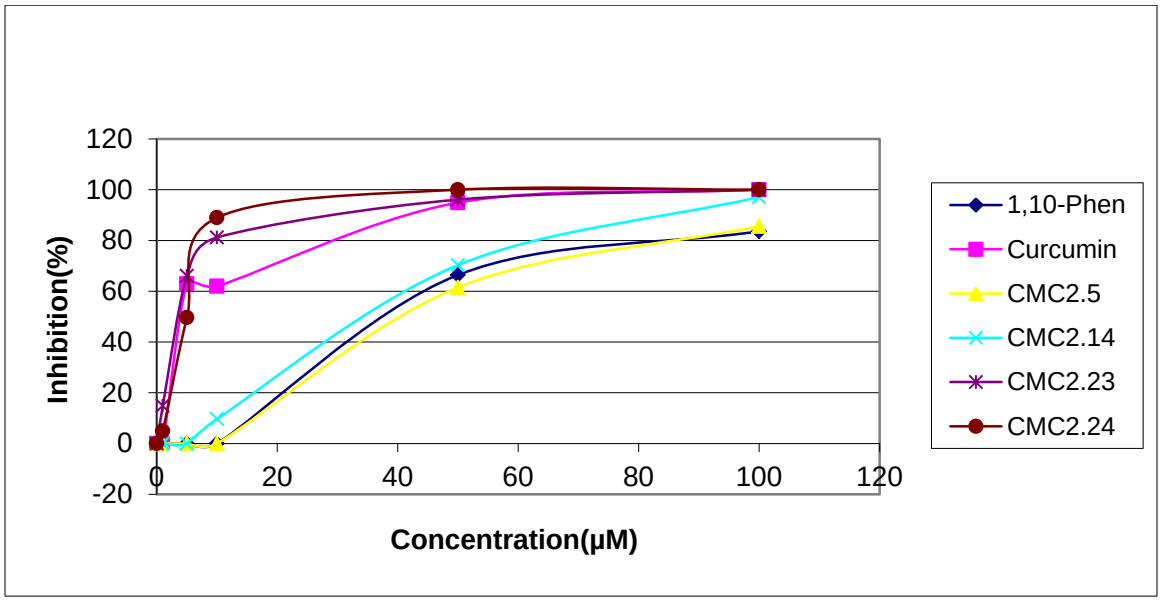

Figure 4.5a Test 1 in MMP-8 inhibition assay

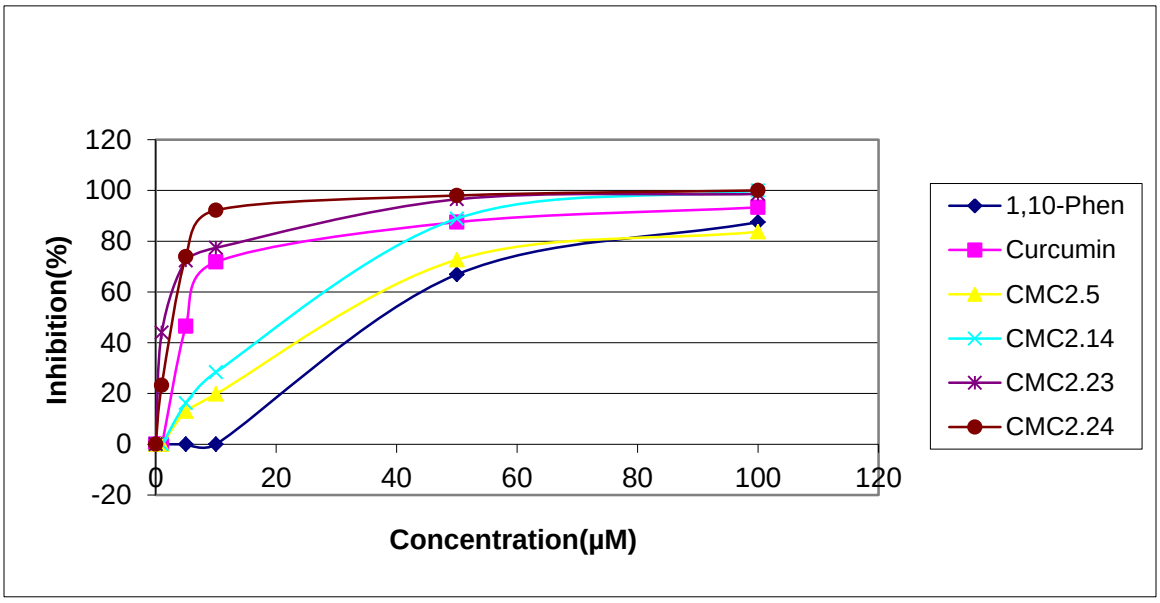

Figure 4.5b Test 2 in MMP-8 inhibition assay

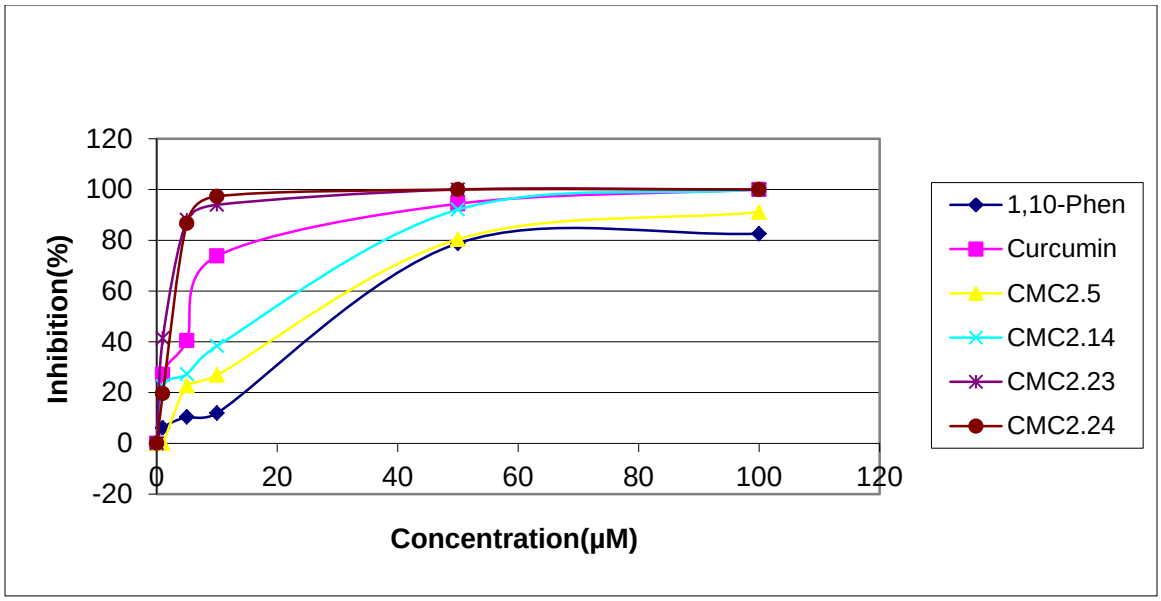

Figure 4.5c Test 3 in MMP-8 inhibition assay

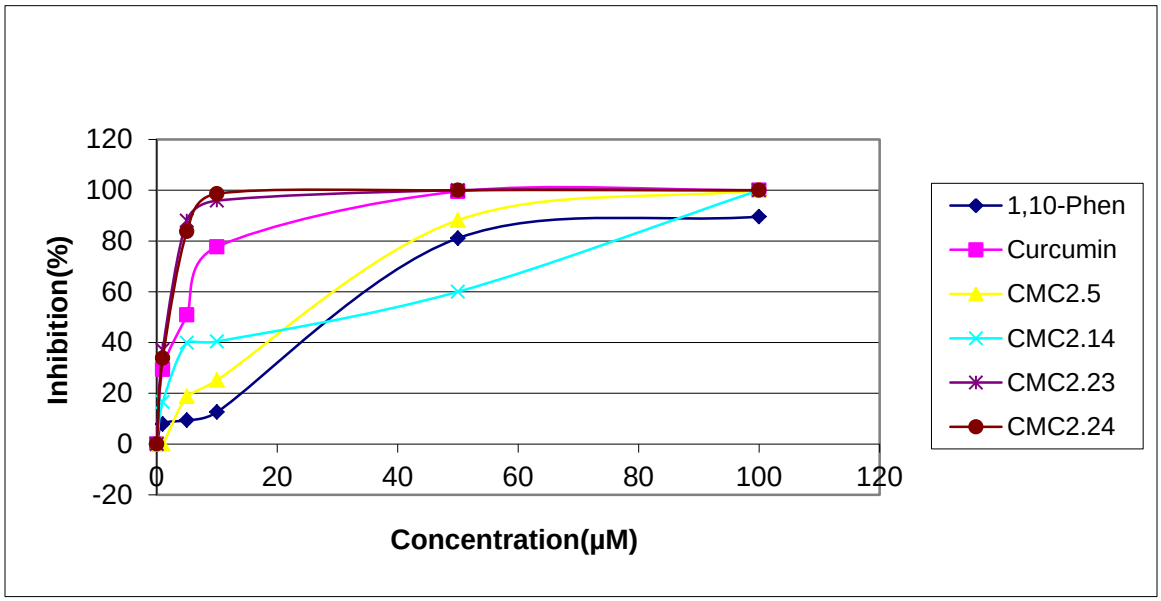

Figure 4.5d Test 4 in MMP-8 inhibition assay

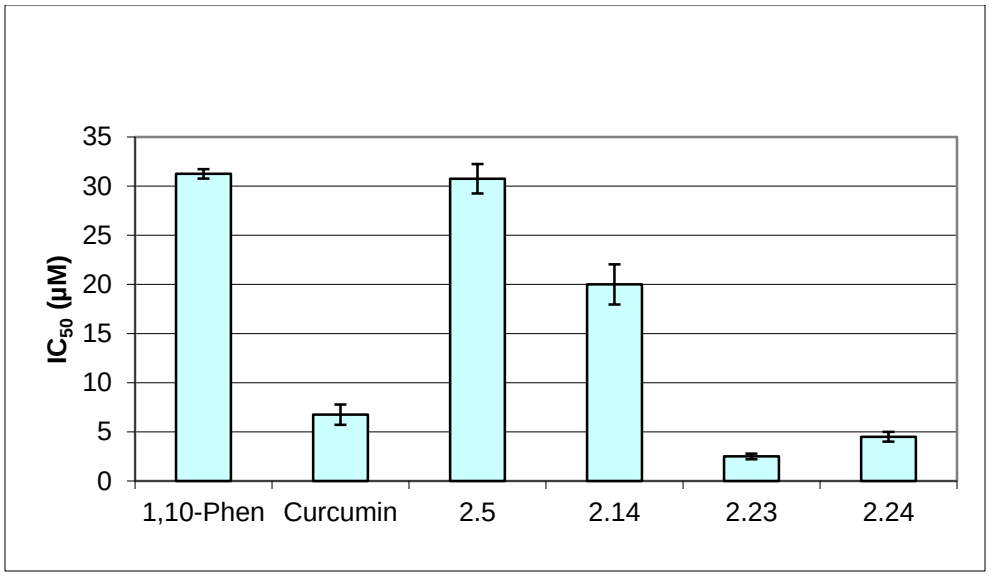

Figure 4.5e IC_{50} of curcumin and selected CMCs against MMP-8

Table 4.6 IC_{50} of curcumin and selected CMCs against MMP-8

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	30.0	5.0	35.0	20.0	3.0	6.0
Test 2	IC_{50} (µM)	32.0	8.0	30.0	25.0	2.0	4.0
Test 3	IC_{50} (µM)	32.0	9.0	28.0	20.0	2.0	4.0
Test 4	IC_{50} (µM)	31.0	5.0	30.0	15.0	3.0	4.0
	Ave.	31.3	6.8	30.8	20.0	2.5	4.5
	S.D.	0.96	2.06	2.99	4.08	0.58	1.00
	S.E.M.	0.48	1.03	1.49	2.04	0.29	0.50

Figure 4.6 MMP-9 inhibition assay

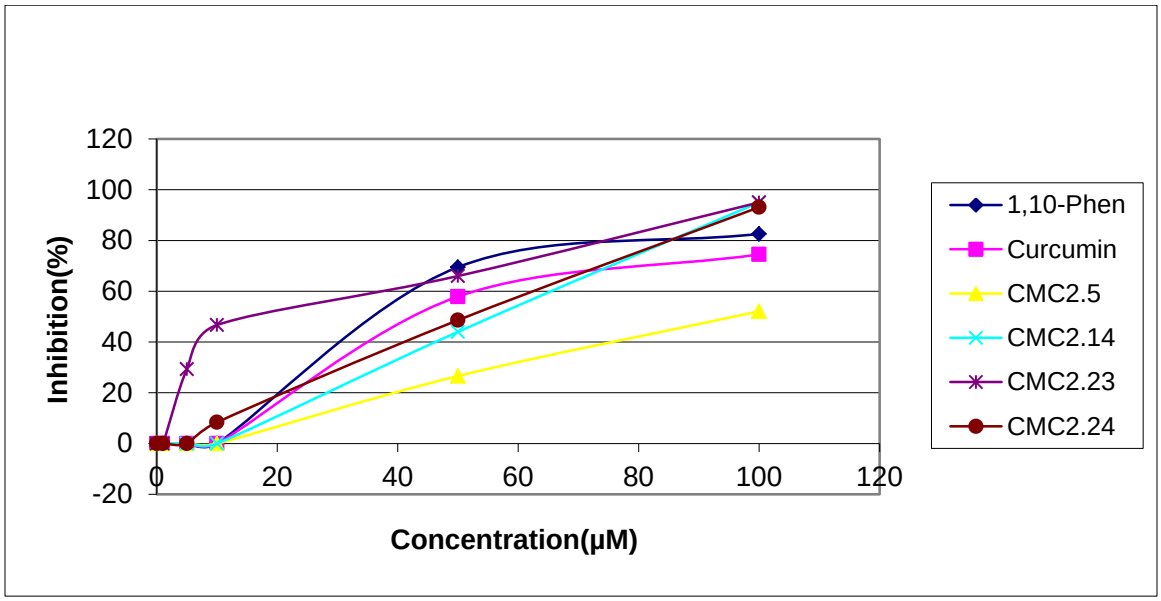

Figure 4.6a Test 1 in MMP-9 inhibition assay

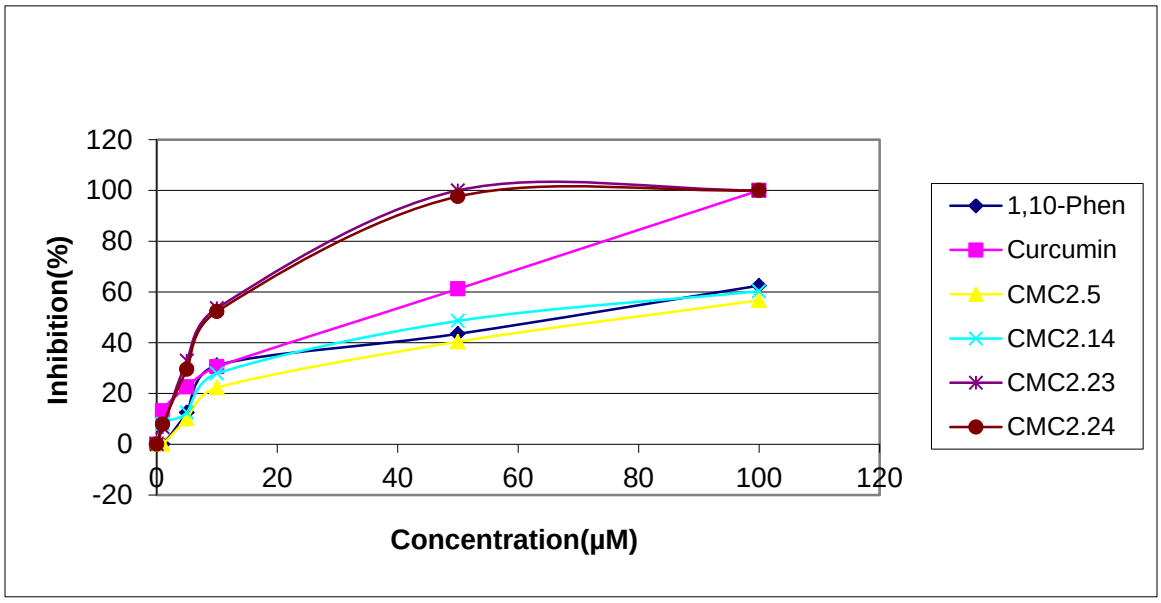

Figure 4.6b Test 2 in MMP-9 inhibition assay

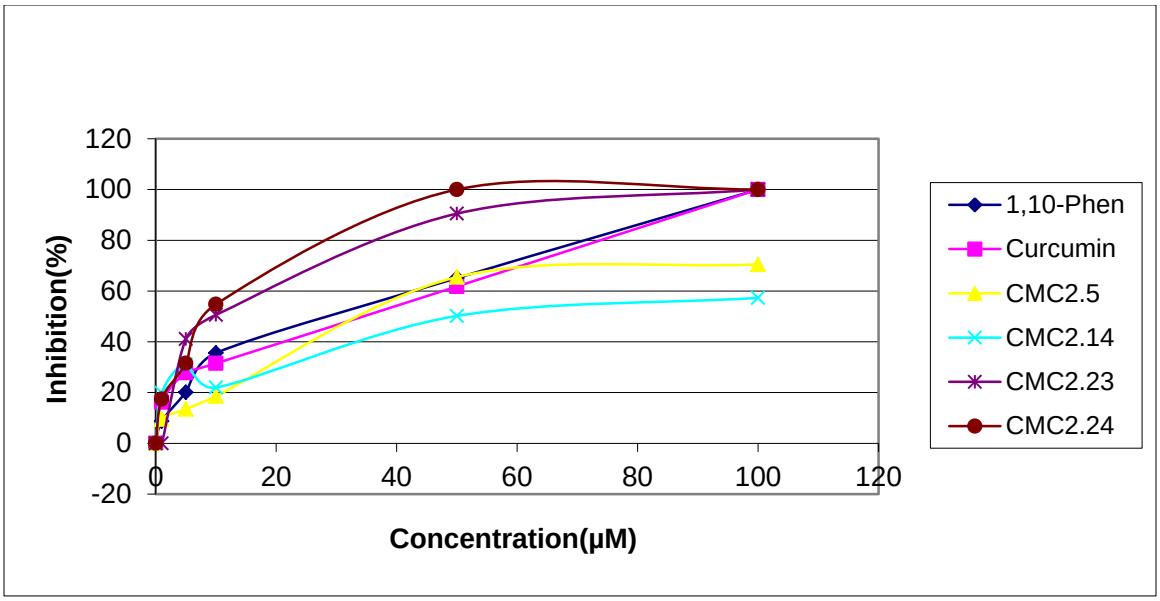

Figure 4.6c Test 3 in MMP-9 inhibition assay

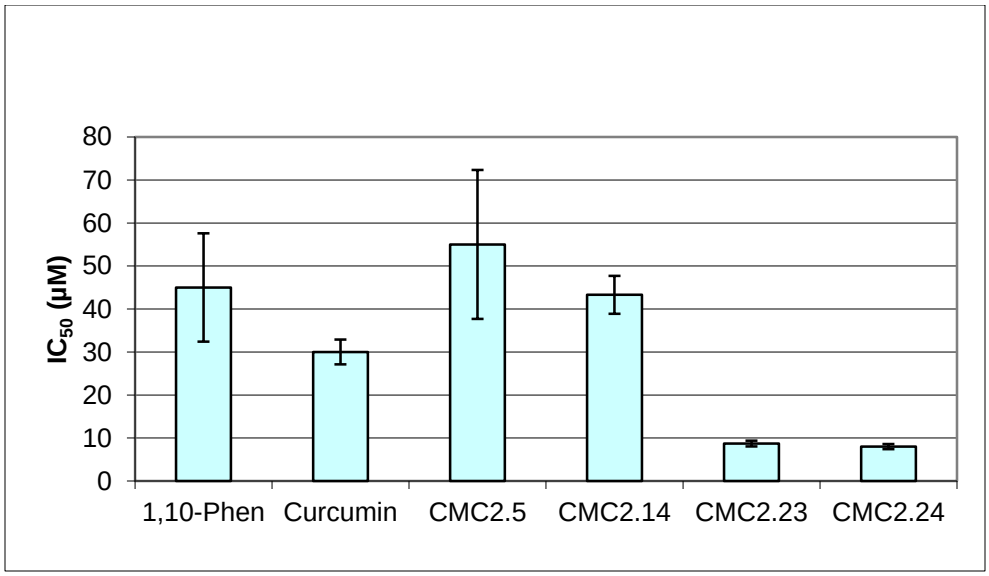

Figure 4.6d IC_{50} of curcumin and selected CMCs against MMP-9

Table 4.7 IC_{50} of curcumin and selected CMCs against MMP-9

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	30.0	35.0	25.0	50.0	10.0	8.0
Test 2	IC_{50} (µM)	70.0	30.0	85.0	45.0	8.0	9.0
Test 3	IC_{50} (µM)	35.0	25.0	55.0	35.0	8.0	7.0
	Ave.	45.0	30.0	55.0	43.3	8.7	8.0
	S.D.	21.79	5.00	30.00	7.64	1.15	1.00
	S.E.M.	12.58	2.89	17.32	4.41	0.67	0.58

Figure 4.7 MMP-12 inhibition assay

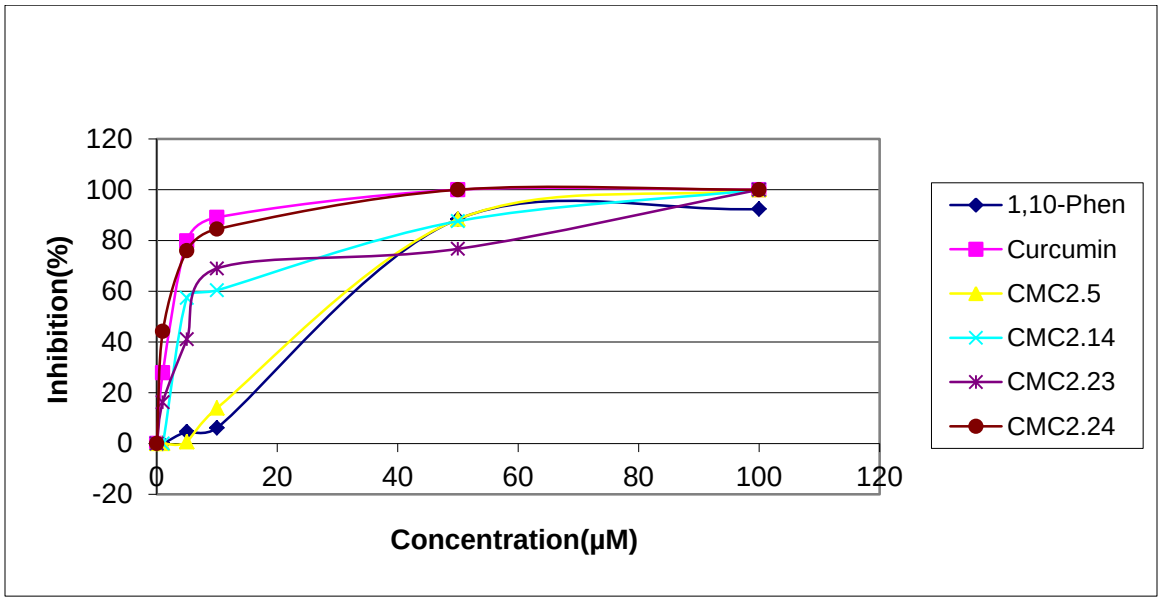

Figure 4.7a Test 1 in MMP-12 inhibition assay

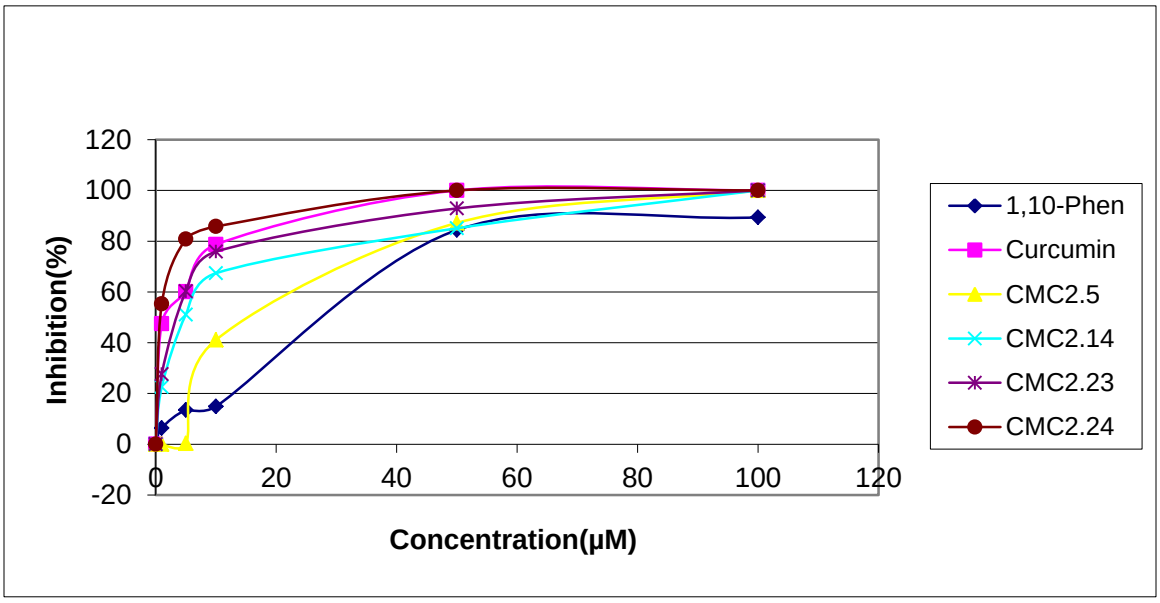

Figure 4.7b Test 2 in MMP-12 inhibition assay

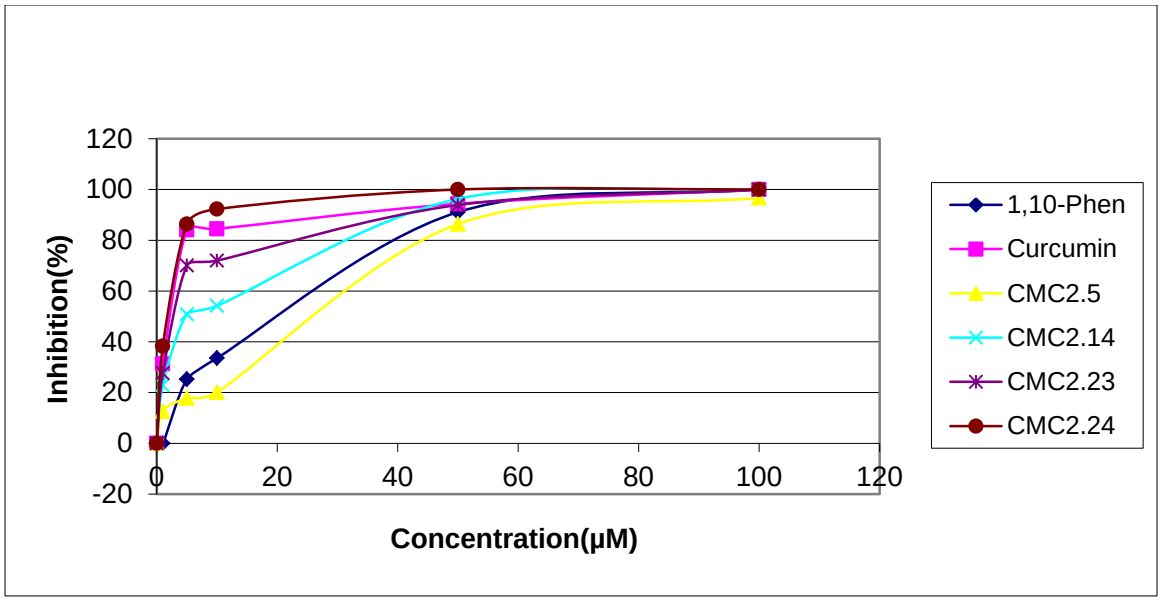

Figure 4.7c Test 3 in MMP-12 inhibition assay

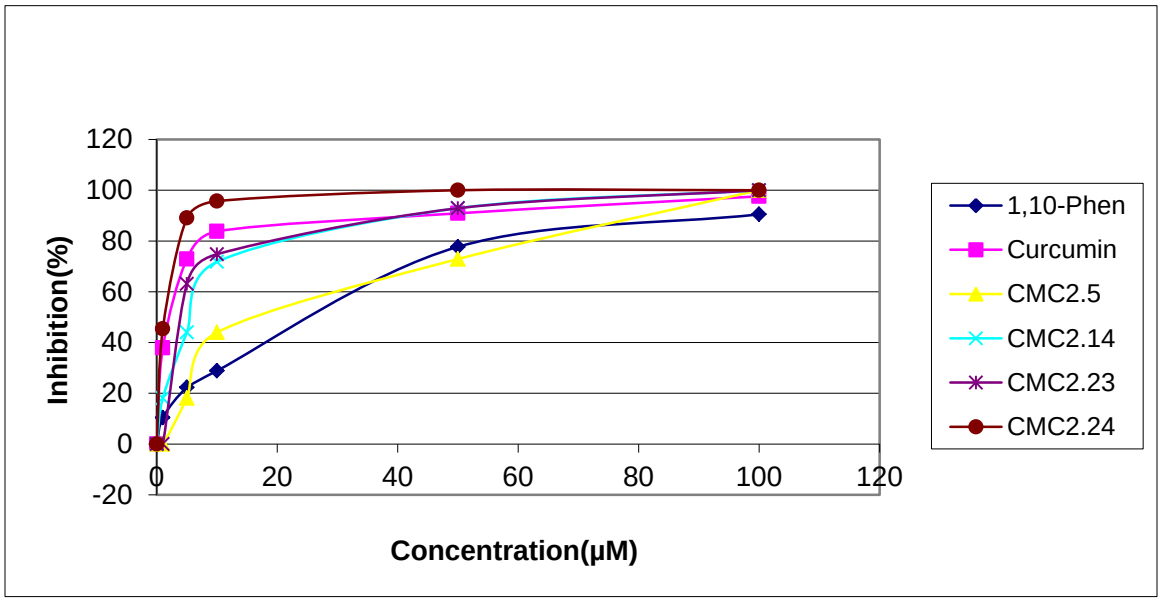

Figure 4.7d Test 4 in MMP-12 inhibition assay

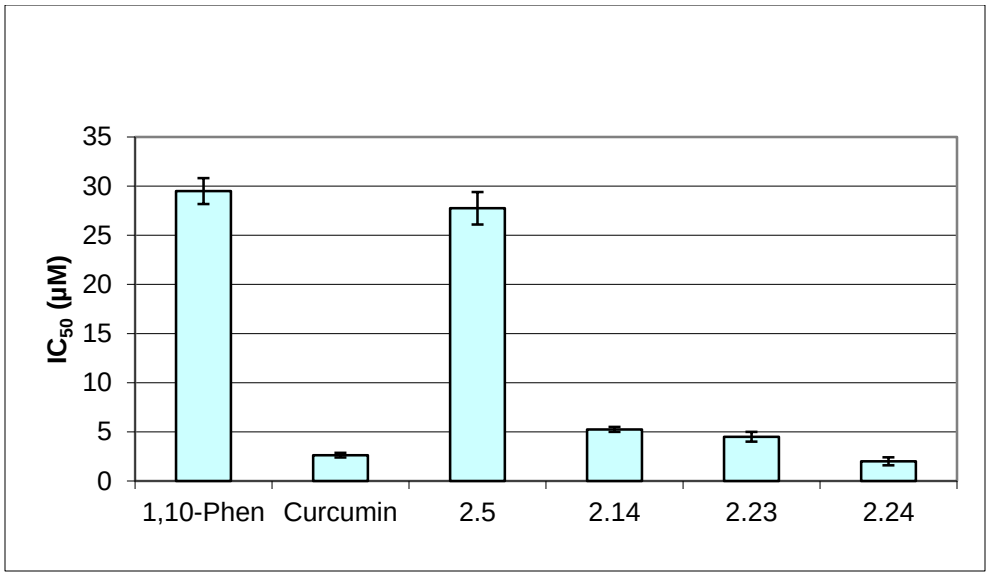

Figure 4.7e IC_{50} of curcumin and selected CMCs against MMP-12

Table 4.8 IC_{50} of curcumin and selected CMCs against MMP-12

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	33.0	3.0	31.0	5.0	6.0	2.0
Test 2	IC_{50} (µM)	30.0	3.0	26.0	5.0	4.0	1.0
Test 3	IC_{50} (µM)	27.0	2.5	30.0	5.0	4.0	2.0
Test 4	IC_{50} (µM)	28.0	2.0	24.0	6.0	4.0	3.0
	Ave.	29.5	2.6	27.8	5.3	4.5	2.0
	S.D.	2.65	0.48	3.30	0.50	1.00	0.82
	S.E.M.	1.32	0.24	1.65	0.25	0.50	0.41

Figure 4.8 MMP-13 inhibition assay

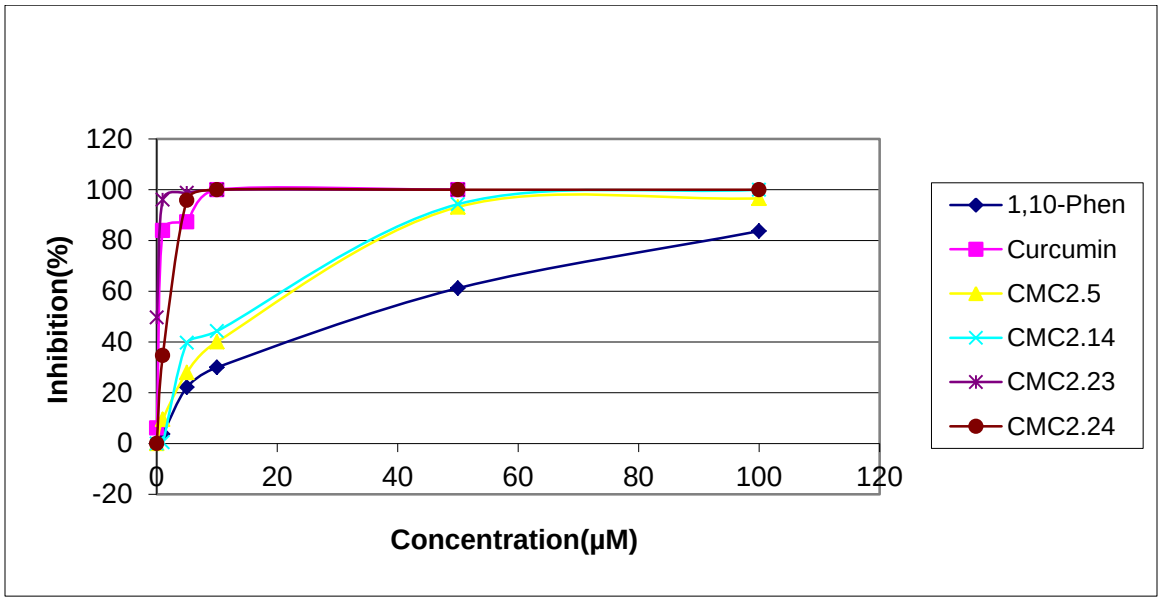

Figure 4.8a Test 1 in MMP-13 inhibition assay

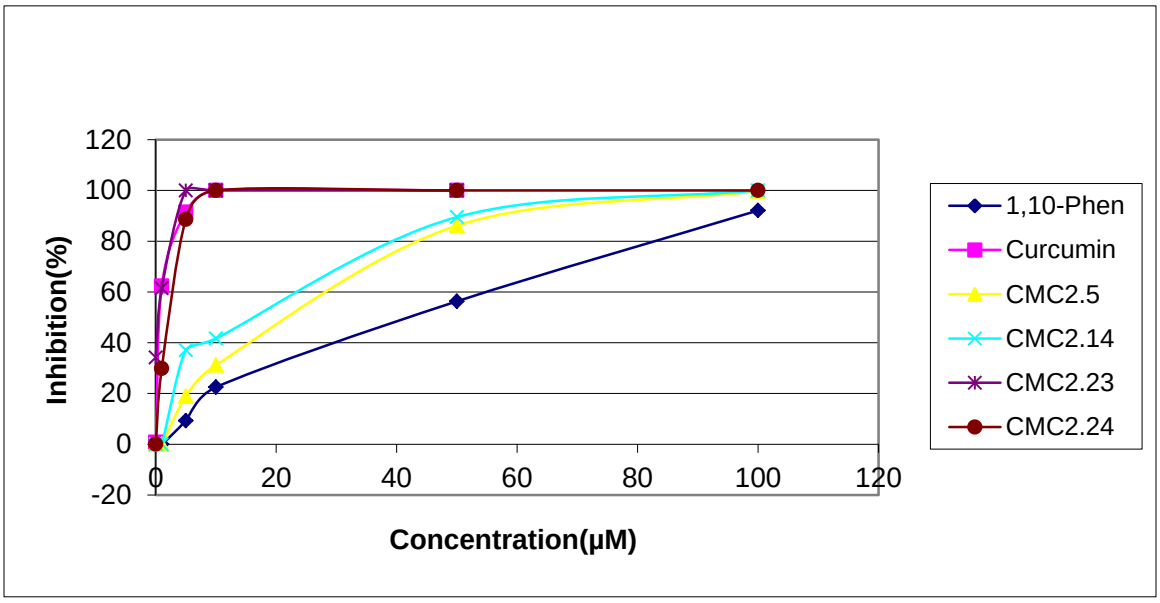

Figure 4.8b Test 2 in MMP-13 inhibition assay

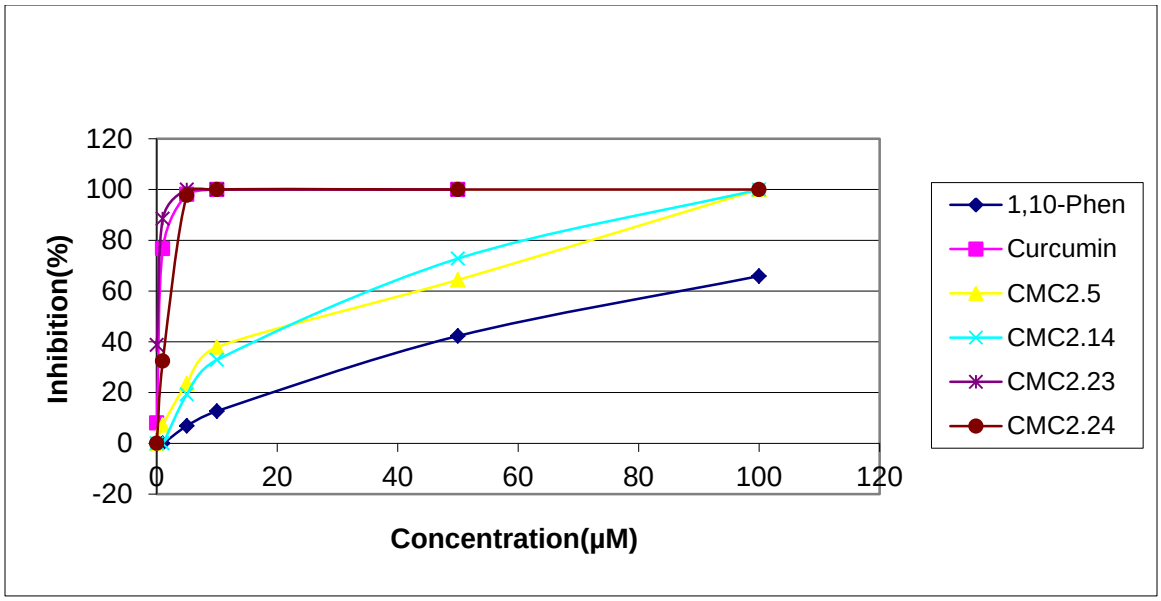

Figure 4.8c Test 3 in MMP-13 inhibition assay

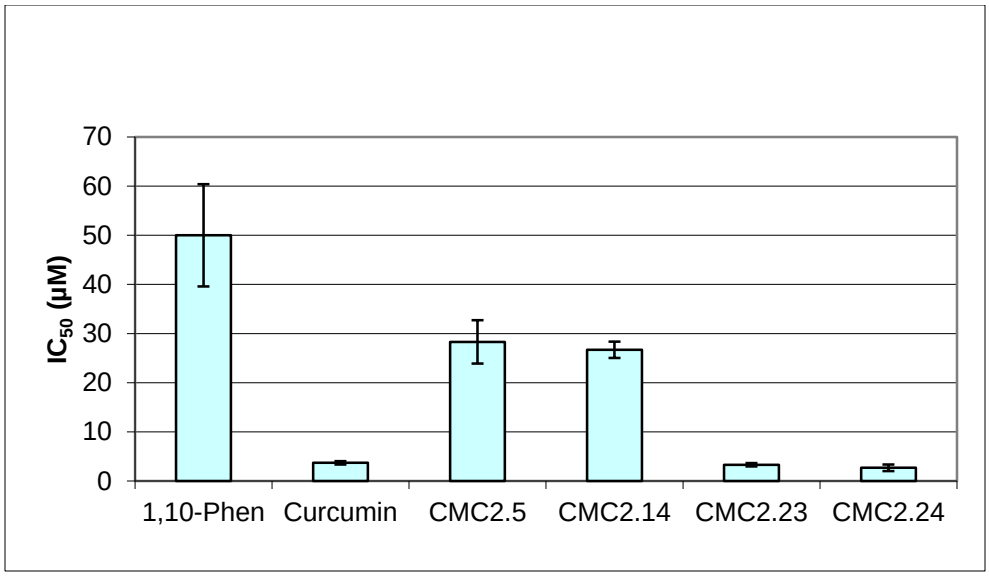

Figure 4.8d IC_{50} of curcumin and selected CMCs against MMP-13

Table 4.9 IC_{50} of curcumin and selected CMCs against MMP-13

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (uM)	35.0	4.0	20.0	25.0	3.0	4.0
Test 2	IC_{50} (uM)	45.0	4.0	30.0	25.0	4.0	2.0
Test 3	IC_{50} (uM)	70.0	3.0	35.0	30.0	3.0	2.0
	Ave.	50.0	3.7	28.3	26.7	3.3	2.7
	S.D.	18.03	0.58	7.64	2.89	0.58	1.15
	S.E.M.	10.41	0.33	4.41	1.67	0.33	0.67

Figure 4.9 MMP-14 inhibition assay

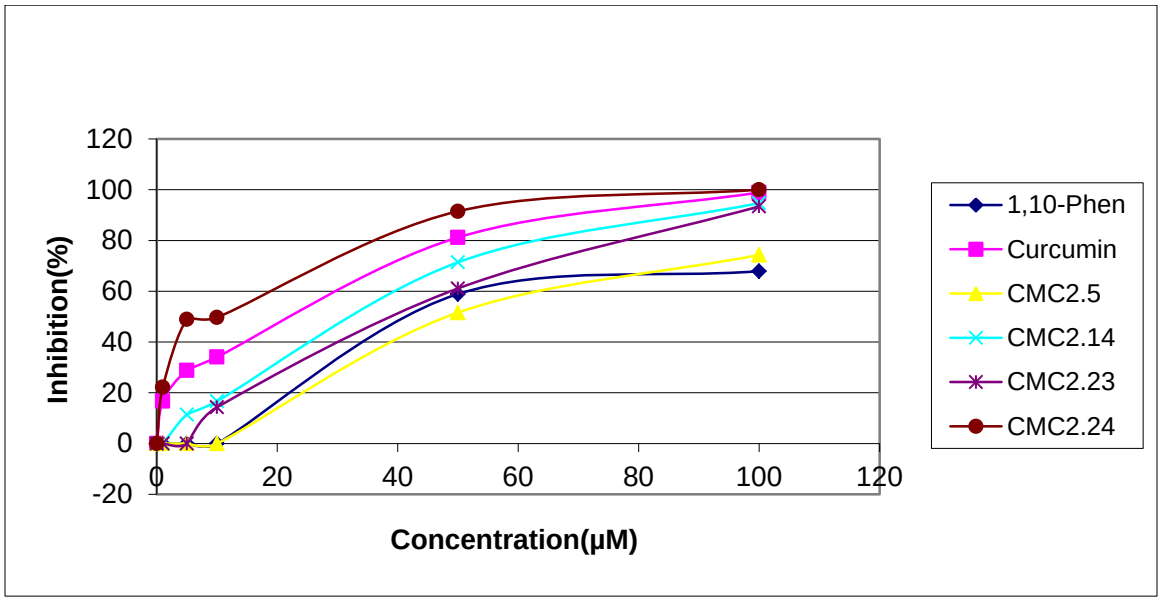

Figure 4.9a Test 1 in MMP-14 inhibition assay

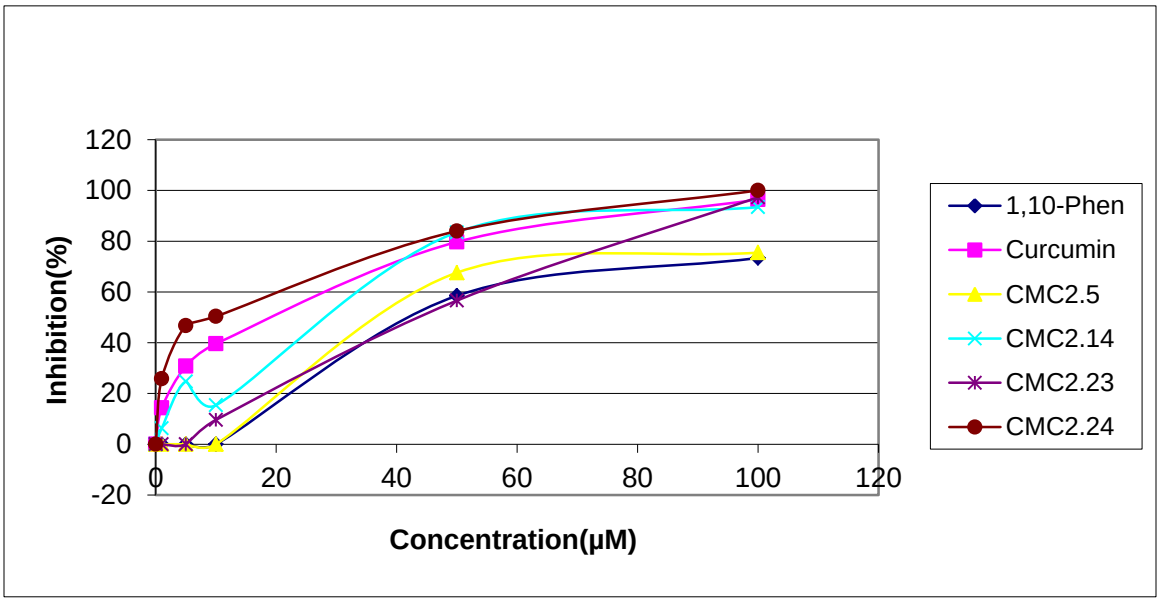

Figure 4.9b Test 2 in MMP-14 inhibition assay

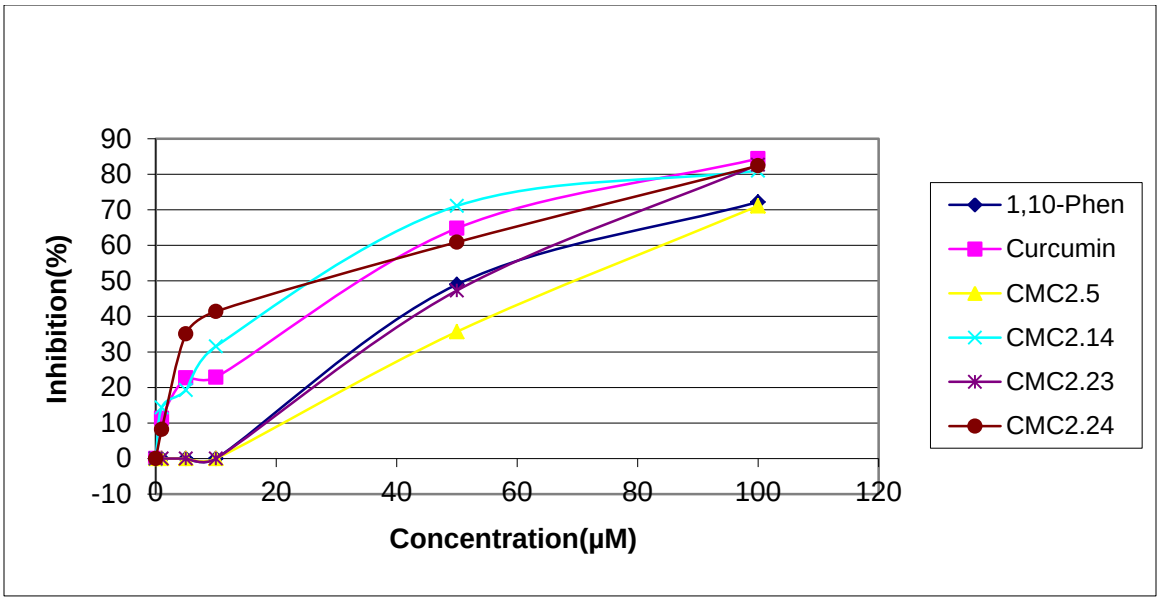

Figure 4.9c Test 3 in MMP-14 inhibition assay

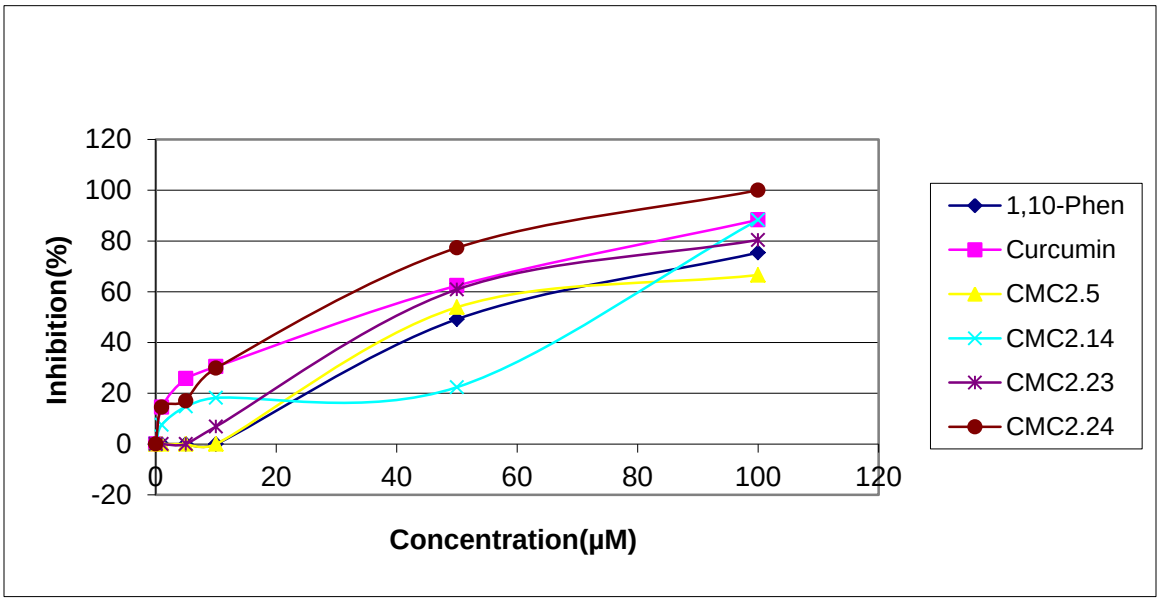

Figure 4.9d Test 4 in MMP-14 inhibition assay

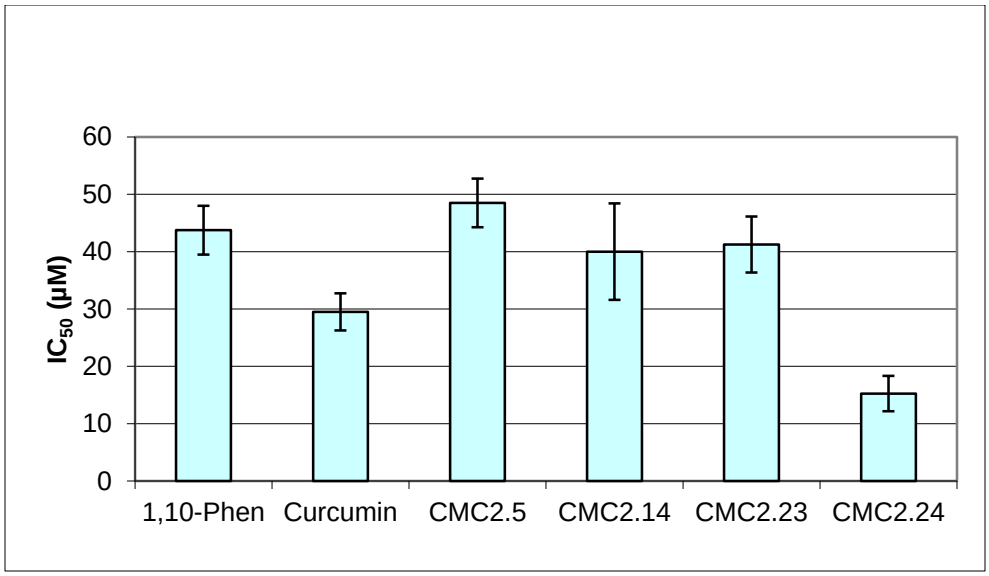

Figure 4.9e IC_{50} of curcumin and selected CMCs against MMP-14

Table 4.10 IC_{50} of curcumin and selected CMCs against MMP-14

		1,10-Phen	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Test 1	IC_{50} (µM)	35.0	25.0	46.0	30.0	32.0	12.0
Test 2	IC_{50} (µM)	38.0	24.0	42.0	30.0	38.0	10.0
Test 3	IC_{50} (µM)	52.0	31.0	61.0	35.0	55.0	15.0
Test 4	IC_{50} (µM)	50.0	38.0	45.0	65.0	40.0	24.0
	Ave.	43.8	29.5	48.5	40.0	41.3	15.3
	S.D.	8.50	6.45	8.50	16.83	9.78	6.18
	S.E.M.	4.25	3.23	4.25	8.42	4.89	3.09

Table 4.11 *In vitro* potency of curcumin and selected chemically-modified curcumins (CMCs)

Note: 1,10-Phenanthroline, was used as the positive control for the fluorogenic MMP inhibitory assay. Curcumin and CMCs were evaluated as inhibitors of nine different mammalian (human-derived) MMPs. IC_{50}s (in μM concentration) were measured using a synthetic fluorescent peptide substrate (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2) for MMPs and which has a cleavage site between Gly and Leu. Each value represents the mean of at 3 or 4 analyses (± S.E.M.).

Compounds / MMPs		1,10-Phenanthroline	Curcumin	CMC2.5	CMC2.14	CMC2.23	CMC2.24
Collagenases	MMP-1	42.0 ±1.1	85.8 ±1.8	74.0 ±3.5	76.3 ±6.5	68.0 ±3.2	69.8 ±2.0
	MMP-8	31.3 ±0.5	6.8 ±1.0	30.8 ±1.5	20.0 ±2.0	2.5 ±0.3	4.5 ±0.5
	MMP-13	50.0 ±10.4	3.7 ±0.3	28.3 ±4.4	26.7 ±1.7	3.3 ±0.3	2.7 ±0.7
Gelatinases	MMP-2	73.8 ±1.0	5.0 ±0.7	25.3 ±1.3	23.8 ±0.9	6.3 ±0.9	4.8 ±0.5
	MMP-9	45.0 ±12.6	30.0 ±2.9	55.0 ±17.3	43.3 ±4.4	8.7 ±0.7	8.0 ±0.6
Others	MMP-3	77.0 ±3.2	4.7 ±0.8	32.5 ±2.8	28.3 ±1.0	5.3 ±0.7	2.9 ±0.4
	MMP-7	196.8 ±8.8	51.8 ±2.5	48.8 ±0.5	57.5 ±4.6	21.5 ±1.0	5.0 ±0.7
	MMP-12	29.5 ±1.3	2.6 ±0.2	27.8 ±1.7	5.3 ±0.3	4.5 ±0.5	2.0 ±0.4
	MMP-14	43.8 ±4.2	29.5 ±3.2	48.5 ±4.3	40.0 ±8.4	41.3 ±4.9	15.3 ±3.1

D. Inhibition of MMP-9 by newer curcumin analogues

Four newer chemically-modified curcumins, namely CMC2.25, CMC2.26, CMC2.27 and CMC2.28 were synthesized based on the lead compound CMC2.24 in order to increase the solubility. These four compounds were evaluated in the MMP-9 *in vitro* assay first, and the data reveal that CMC2.25 (the introduction of chloride on the middle phenyl ring) and CMC2.26 (the introduction of methoxy group on the middle phenyl ring) were as potent as CMC2.24. However, CMC2.27 (the introduction of ketal group on the middle phenyl ring) and CMC2.28 (the introducion of adjacent diol on the middle phenyl ring) didn't show great activity in the inhibition assay against MMP-9 (Table 4.12, Figure 4.10).

Table 4.12 IC_{50} of newer CMCs against MMP-9

		1,10-Phen	CMC2.24	CMC2.25	CMC2.26	CMC2.27	CMC2.28
Test 1	IC_{50} (μM)	37.0	7.0	5.0	4.0	46.0	63.0
Test 2	IC_{50} (μM)	42.0	4.0	6.0	5.0	52.0	61.0
Test 3	IC_{50} (μM)	41.0	7.0	8.0	5.0	56.0	68.0
	Ave.	40.0	6.0	6.3	4.7	51.3	64.0
	S.D.	2.64	1.73	1.52	0.57	5.03	3.6
	S.E.M.	1.32	0.86	0.76	0.28	2.51	1.8

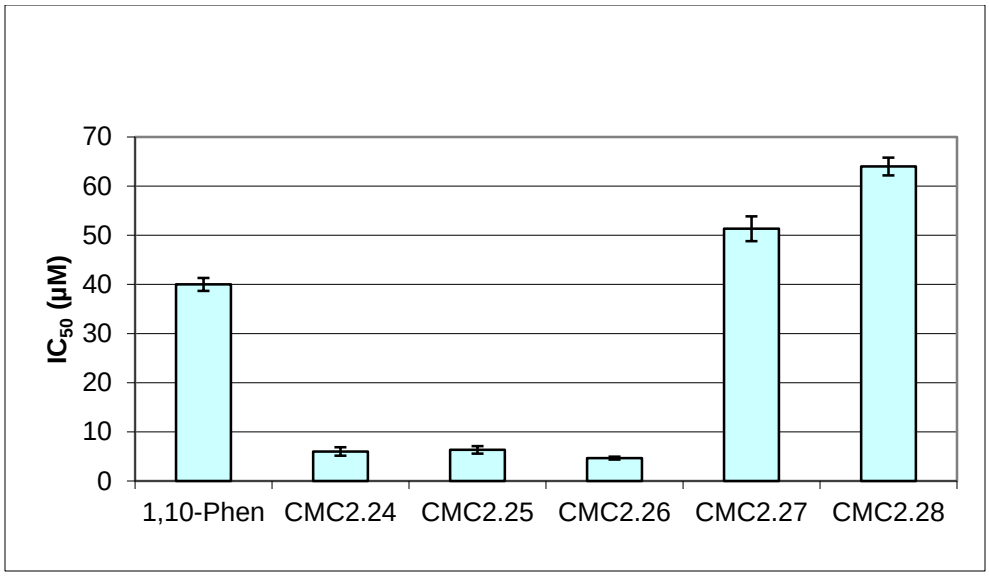

Figure 4.10 IC_{50} of newer CMCs against MMP-9

Materials and Methods

1. HPLC MMP inhibition assay

MMP-9 human neutrophil monomer was purchased from Calbiochem, EMD Biosciences, Inc. (La Jolla, CA) and activated by adding *p*-aminophenylmercuric acetate (APMA) purchased from Sigma-Aldrich (St. Louis, MO) to achieve a final concentration of 1mM in DMSO purchased from Sigma-Aldrich (St. Louis, MO). Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH, purchased from BACHEM (Torrance, CA) was used as substrate. Stock solutions of 1,10-phenanthroline, curcumin and curcumin analogues were prepared in DMSO at a final concentrations of 1, 5, 10, 25, 50, 100, 250 and 500 µM in the reaction mixture. Both the substrate and the tripeptide breakdown product absorb at 380nm with the same extinction coefficient. 90 µL of 5mM $CaCl_2$, 0.2M NaCl and 50mM Tris/HCl pH=7.6 buffer, 1 µL of MMP-9 producing 10-15% lysis of the synthetic peptide substrate, 1 µL of stock solutions of 1,10-phenanthroline, curcumin or curcumin analogues prepared in DMSO and 10 µL of substrate were added and then incubated at 37^{o}C for

4h. The reaction mixture was stopped by adding 100 µL of the stop solution (30% acetonitrile in 2M acetic acid, containing 4mM 1,10-phenonthroline); samples were vortexed and centrifuged for 10min at 10,000rpm. An aliquot of 120 µL was injected into HPLC (Waters Alliance 2695 System) for analysis (HPLC developing system: 20% Acetonitrile, 80% H_2O), and the percentage of lysis was calculated and converted to the inhibition percentage.

2. Fluorogenic MMP inhibition assay

MMP-1, -2, -3, -7, -8, -12, -13 and -14 were all recombinant human enzymes and were purchased from R&D Systems, Inc. (Minneapolis, MN), whereas MMP-9 human neutrophil monomer was purchased from Calbiochem, EMD Biosciences, Inc. (La Jolla, CA). MMP-1, -2, -3, -7, -8, -9, -12 and -13 were activated by adding *p*-aminophenylmercuric acetate (APMA) purchased from Sigma-Aldrich (St. Louis, MO) to achieve a final concentration of 1mM in DMSO purchased from Sigma-Aldrich (St. Louis, MO). MMP-14 was activated by recombinant human furin, purchased from R&D Systems, Inc. (Minneapolis, MN). The substrate for MMP-1, -2, -7, -8, -9, -12, -13 and -14 was Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 fluorogenic peptide substrate IX, purchased from R&D Systems, Inc. (Minneapolis, MN). [Mca: (7-Methoxycoumarin-4-yl) acetyl, Dpa: N-3-(2, 4-Dinitrophenyl)-L-2,3-diaminopropionyl]. The substrate for MMP-3 was Mca-Arg-Pro-Lys-Pro-Val-Glu-Nval-Trp-Arg-Lys(Dnp)-NH_2 fluorogenic peptide substrate II, purchased from R&D Systems, Inc. (Minneapolis, MN). [Mca: (7-Methoxycoumarin-4-yl)acetyl, Nval: Norvaline, Dnp: 2, 4-Dinitrophenyl]. Cleavage of the fluorogenic substrate was measured by the differences in intensities of the excitation and emission wavelengths at 320nm and 405nm, respectively. Stock solutions of 1,10-phenanthroline, curcumin and curcumin analogues were prepared in DMSO at concentrations of 1, 5, 10, 25, 50, 100, 250 and 500 µM. 100 µL solution containing 80 µL of 1mM $CaCl_2$, 0.2M

NaCl and 50mM Tris/HCl (pH=7.6) buffer and 10 μL of enzyme, were added first to the 96 well plate, followed by the addition of different concentrations of inhibitor (1 μL) and substrate (10 μL) to a final reaction volume now containing 1% DMSO. The whole mixture was incubated at 37^{o}C for 4h, and Fluoro Count (Packard Instrument Co., CT) was used to measure the increase in fluorescence when the substrate was cleaved. By comparison with the uninhibited degradation from enzyme and substrate, the percentage of inhibition was determined by subtracting the degradation of the enzyme, substrate and inhibitor mixture. The IC_{50} for each compound was obtained from a plot of the percentage of inhibition versus the inhibitor concentration.

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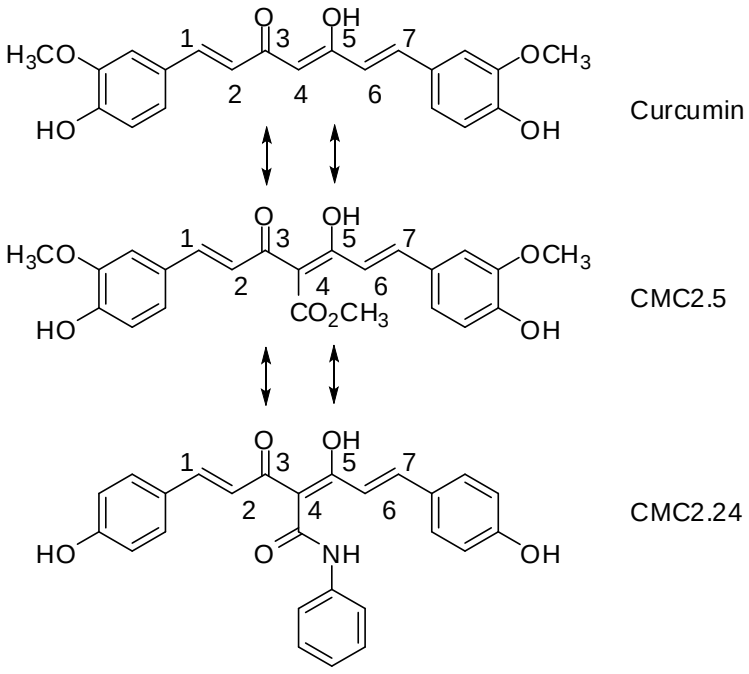
Chapter 5. pK_a, zinc- and bovine serum albumin binding, and antimicrobial studies on curcumin, CMC2.5 and CMC2.24

A. Introduction

The acid dissociation constant, K_a is used to quantitatively describe the acidity of a compound in a particular solution. Normally, the logarithmic form pK_a ($-\log_{10}K_a$) is used to compare the acidities of different compounds. Values of the pK_as of the three acidic groups of curcumin have been reported by Bernabé-Pineda *et al*.[1] As mentioned in Chapters 1 and 2, the poor aqueous solubility of curcumin limits its therapeutic use. Two factors might affect the bioavailability: charge type (charging aqueous solubility at physiological pH) and enhanced solubility in the blood. We therefore determined the pK_as of the three acidic groups in the compounds: curcumin, CMC2.5 and CMC2.24. It has also been established that some MMP inhibitors appear to act by binding to the enzymic Zn^{2+} through a β-diketone assembly.[2-4] We hope that the modified compounds might bind to Zn^{2+} more strongly than curcumin does, because of the electron-withdrawing power of the 4-substitutions. We therefore studied Zn^{2+} binding to these compounds. A bovine serum albumin assay was also carried out for curcumin and CMC2.24 to determine their binding to the major plasma transport protein, serum albumin.[5-6] Because FDA only permits the use of subantimicrobial doses of antibiotics for other diseases than infections, therefore an antimicrobial assay was carried out for curcumin, CMC2.5 and CMC2.24 accordingly, for both minimum inhibitory concentration (MIC) test and β-galactosidase inhibition, whereas the known antibiotic, doxycycline was used as a positive control.



Scheme 5.1 Structures of curcumin, CMC2.5 and CMC2.24

B. pK_a measurement

The pK_as of curcumin have been reported by Bernabé-Pineda *et al.*, and were found to be pK_a1=8.38 for $H_3D = H_2D^- + H^+$, pK_a2=9.88 for $H_2D^- = HD^{2-} + H^+$, and pK_a3=10.51 for $HD^{2-} = D^{3-} + H^+$ (D is designated here as any deprotonated form of curcumin).[1] In our studies on CMC2.5 and CMC2.24, we obtained the spectra seen in Figure 5.1, which shows the raw spectra as a function of pH. In Figure 5.1 at certain wavelengths, 3 or 4 species were found and correlated with the progressive forms of deprotonation products of curcumin, CMC2.5 and CMC2.24. Figure 5.2 shows the fitted curves extracted from these data, displayed at wavelengths chosen to maximize the contrasts. Figure 5.3 shows the derived spectra of the various species for these three compounds. It was noted that CMC2.5 and CMC2.24 are more acidic with regard to the enolic proton, which correlates with an introduction of the electron-withdrawing group, either methoxycarbonyl or phenylaminocarbonyl group at carbon-4, whereas the two phenolic protons

on either CMC2.5 or CMC2.24 are likewise more acidic than those of curcumin. (Table 5.1) However, for CMC2.5, a third deprotonation was not observed. The standard error for all the pK_a measurements for curcumin, CMC2.5 and CMC2.24 was less than 0.1 in all cases.

Table 5.1 pK_a of curcumin, CMC2.5 and CMC2.24

Compound	pK_a
Curcumin	pK_a1= 8.41, pK_a2=9.94, pK_a3=11.12
CMC2.5	pK_a1= 6.50, pK_a2= pK_a3=8.82
CMC2.24	pK_a1= 6.98, pK_a2=8.40, pK_a3=9.80

For curcumin, the data correlated surprisingly well with the reported pK_a values.[1] One intermediate species was found after graph fitting, which suggests a tautomeric form of curcumin. An isosbestic point (448.5nm) was found for curcumin at pH 8.9, and then the curve shifted to the lower wavelength. The rate of transformation was remarkably slow when the pH was raised to 11.0. As for CMC2.5, the third pK_a was not observed because of 1) the striking isobestic point above pH 9.0; 2) the dramatic change in the range of pH 6.5. Two distinct processes were found after the fitting for CMC2.24, in which the rate of transformation became slow around pH 11.0. A time dependence was observed for all three compounds, and the deprotonation process was slow for the protic acids. (Figure 5.4) As had Bernabé-Pineda *et al.*[1] earlier, we observed that the spectra of all three species varied with time, and so we determined the pK_a values extrapolated to zero. For curcumin, the time-dependence was such that we could determine the spectra for an intermediate compound.

Figure 5.1 Spectra for pH titration

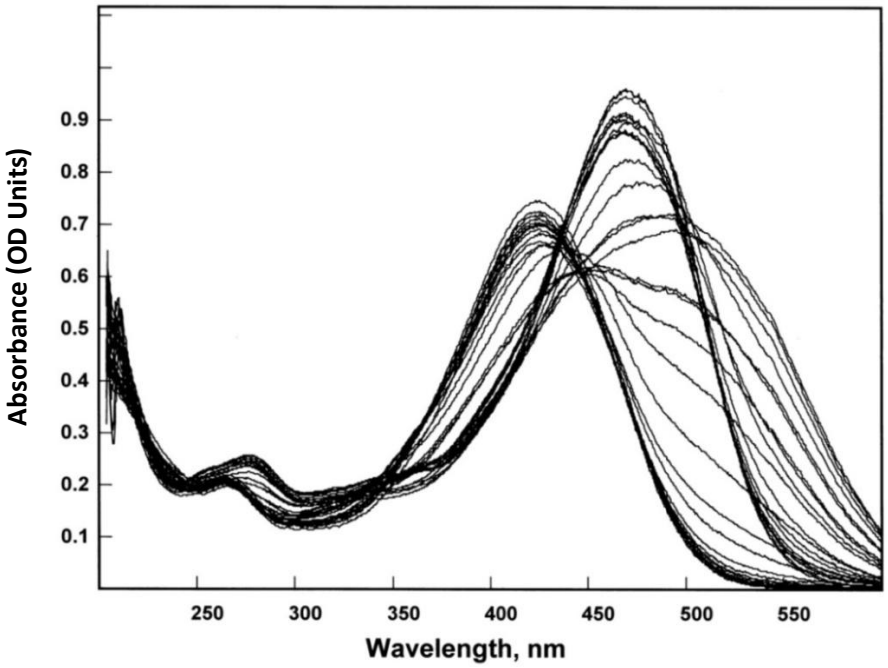


Figure 5.1a Spectra for pH titration of curcumin

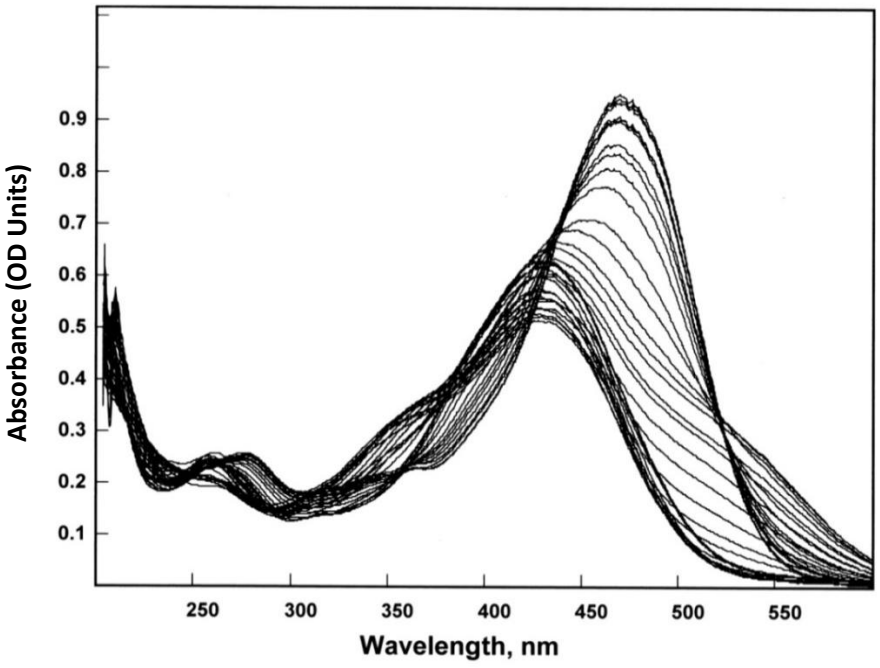


Figure 5.1b Spectra for pH titration of curcumin intermediate

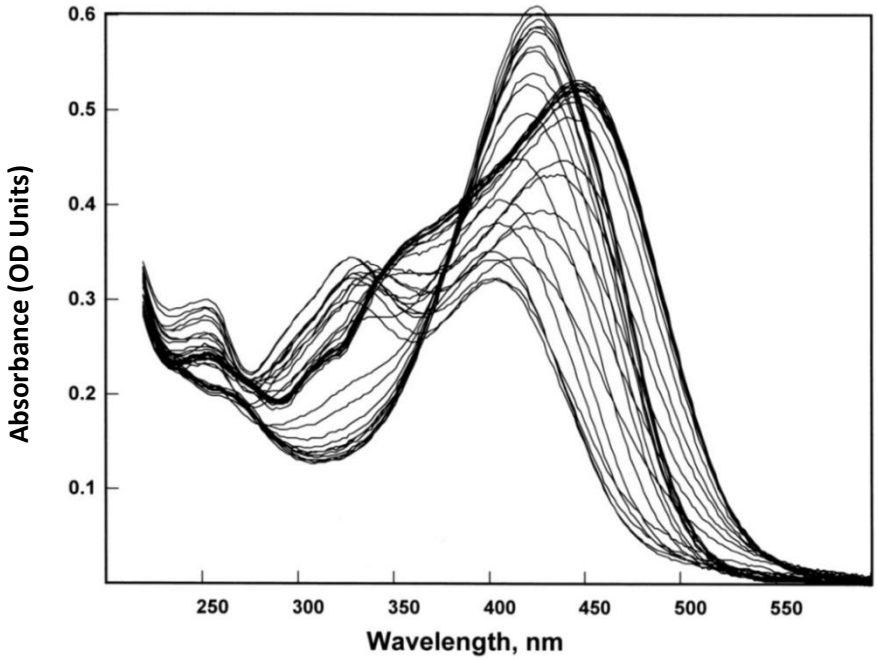


Figure 5.1c Spectra for pH titration of CMC2.5

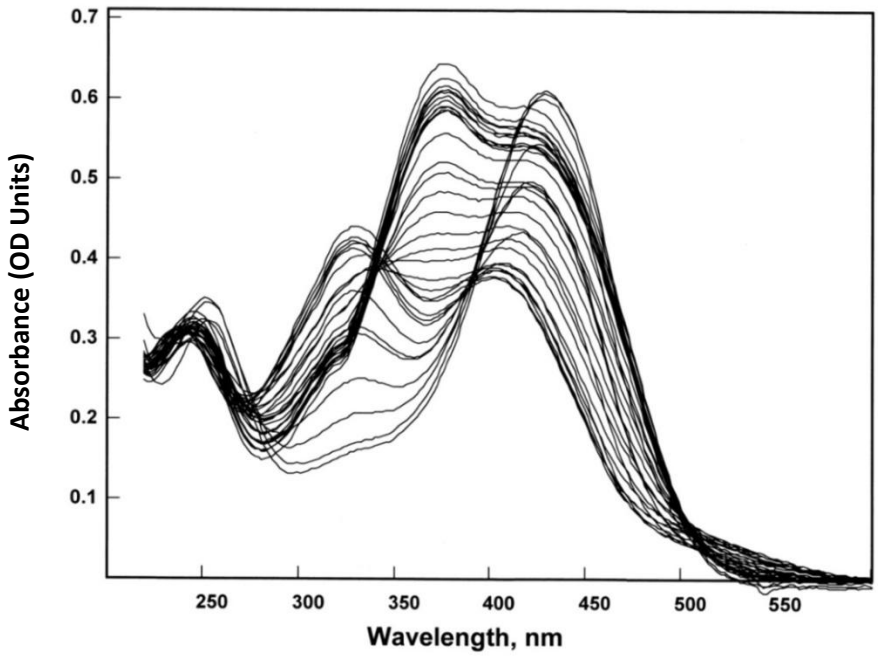


Figure 5.1d Spectra for pH titration of CMC2.24

Figure 5.2 Fittings at selected wavelengths for curcumin, CMC2.5 and CMC2.24

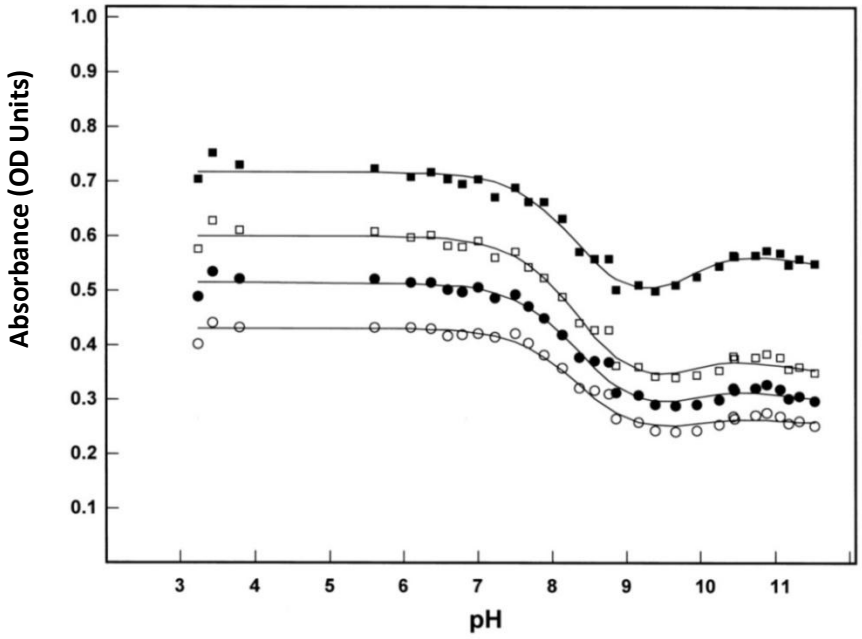


Figure 5.2a Fitting of curcumin, reading up at pH 7.2: 380, 390, 400, 425nm

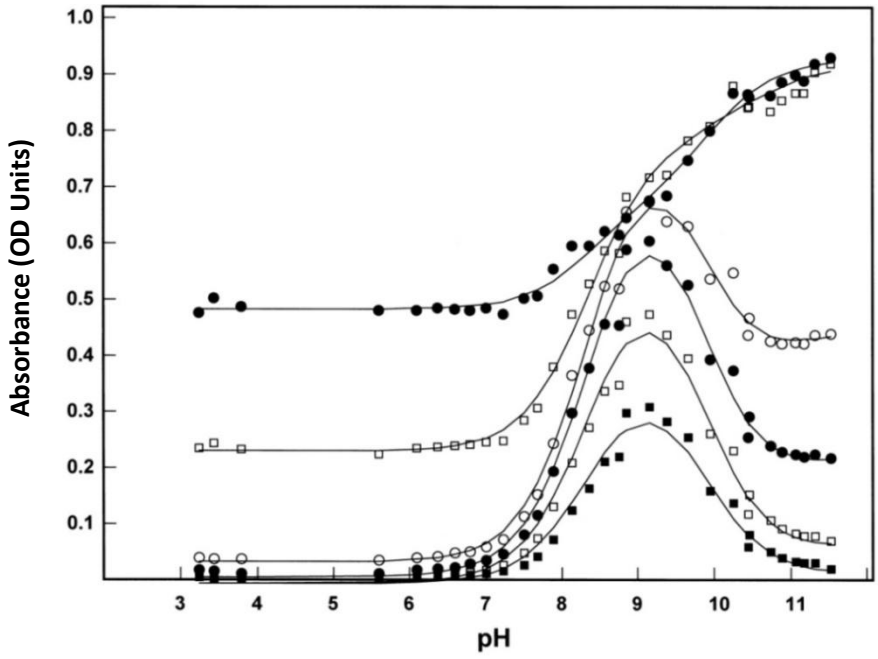


Figure 5.2b Fitting of curcumin, reading down at pH 11.0: 460, 482, 495, 515, 530, 550, 570nm

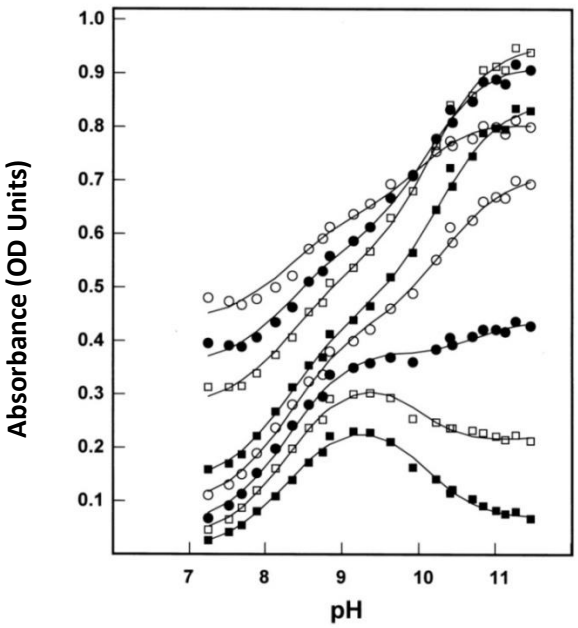


Figure 5.2c Fitting of curcumin intermediate, reading down at pH 7.2: 448,460, 470, 490, 500, 515, 530, 550nm

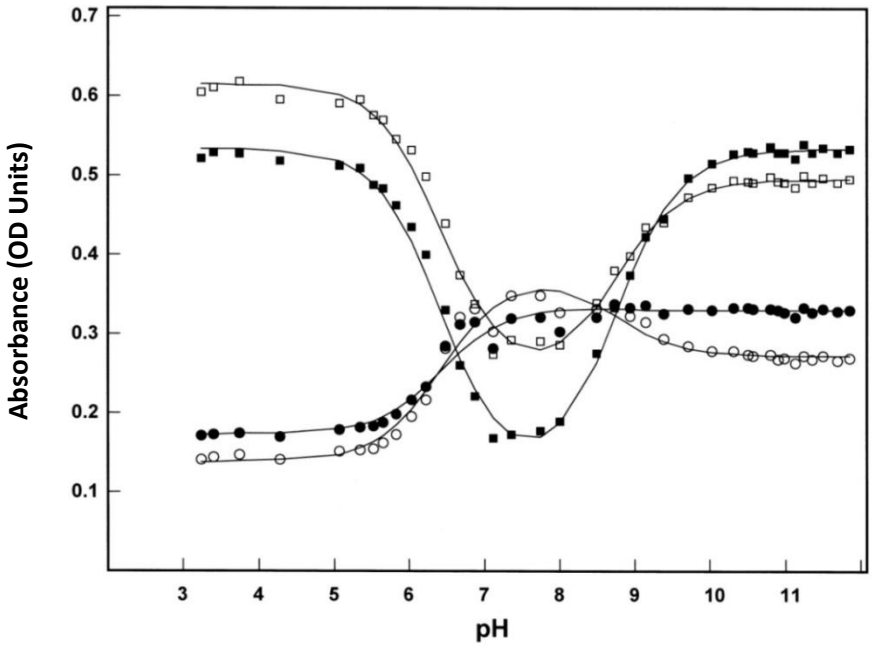


Figure 5.2d Fitting of CMC2.5: reading up at pH 3.0: 329, 344, 446nm

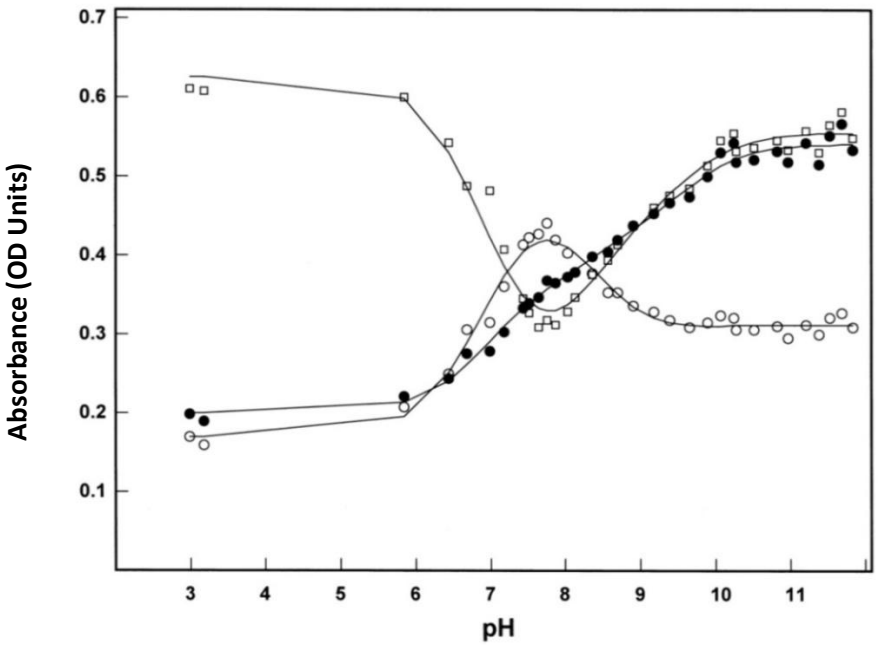


Figure 5.2e Fitting of CMC2.24, open circle, 329nm, filled circle, 356nm; open square, 428nm

Figure 5.3 Calculated spectra for curcumin, curcumin intermediate, CMC2.5 and CMC2.24

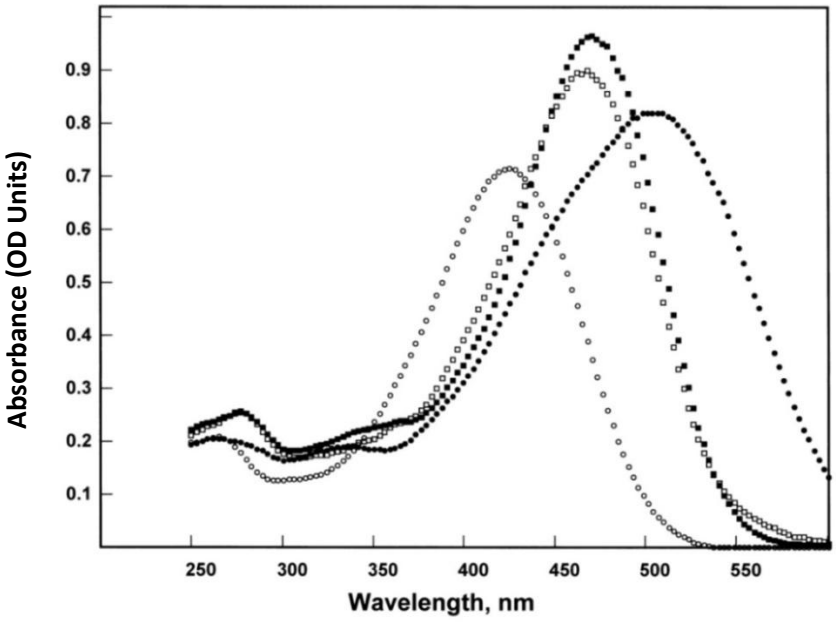


Figure 5.3a Calculated spectra for curcumin, reading down at 470nm: D^{3-}, HD^{2-}, H_2D^{-}, H_3D

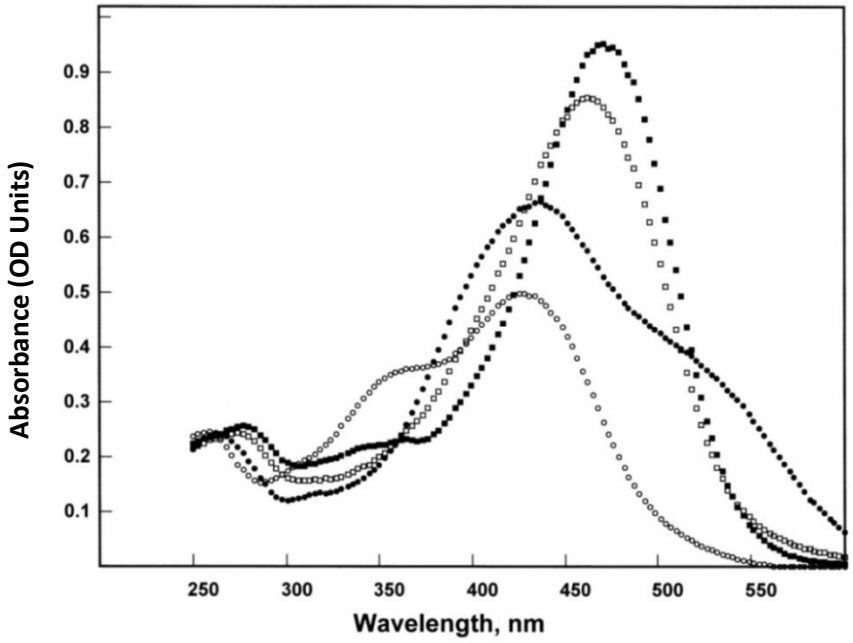


Figure 5.3b Calculated spectra for curcumin intermediate, reading down at 470nm: D^{3-}, HD^{2-}, H_2D^{-}, H_3D

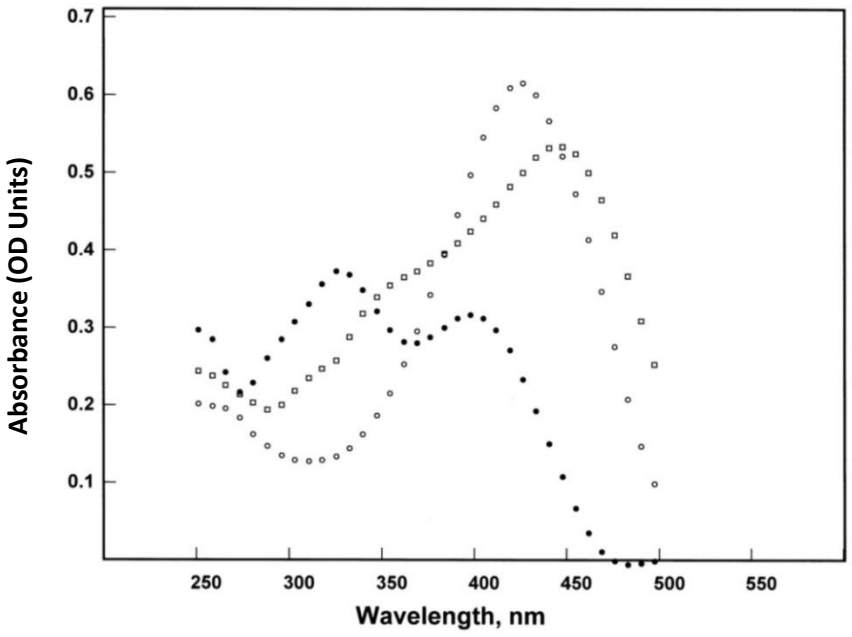


Figure 5.3c Calculated spectra for CMC2.5, reading down at 440nm: H_3D, HD^{2-}, D^{3-}

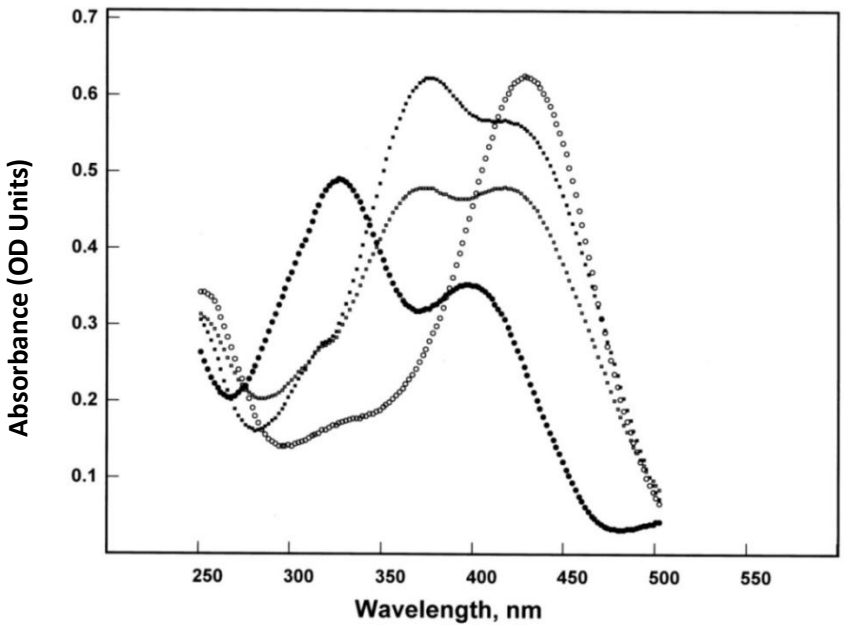


Figure 5.3d Calculated spectra for CMC2.24, reading down at 440 nm: H_3D, H_2D^-, HD^{2-}, D^{3-}

Figure 5.4 Time-dependent spectra for curcumin and CMC2.5

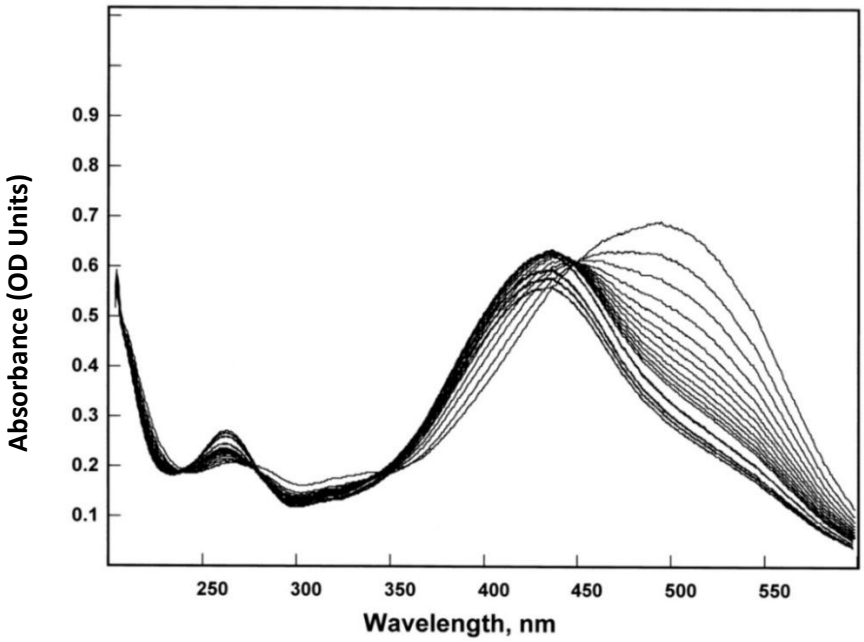


Figure 5.4a Spectra of curcumin, pH 8.94, through the isosbestic point at 448.71nm, 2sec intervals to 24sec; darker, displaced lines, 40, 60, 80, 100sec

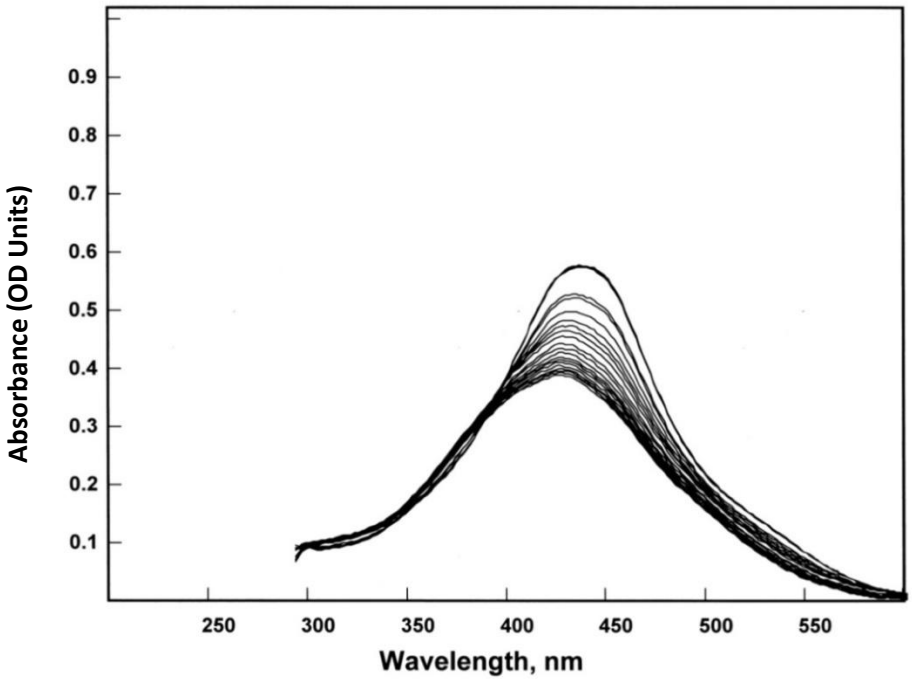


Figure 5.4b Spectra of curcumin in 88 μM BSA, 1h intervals

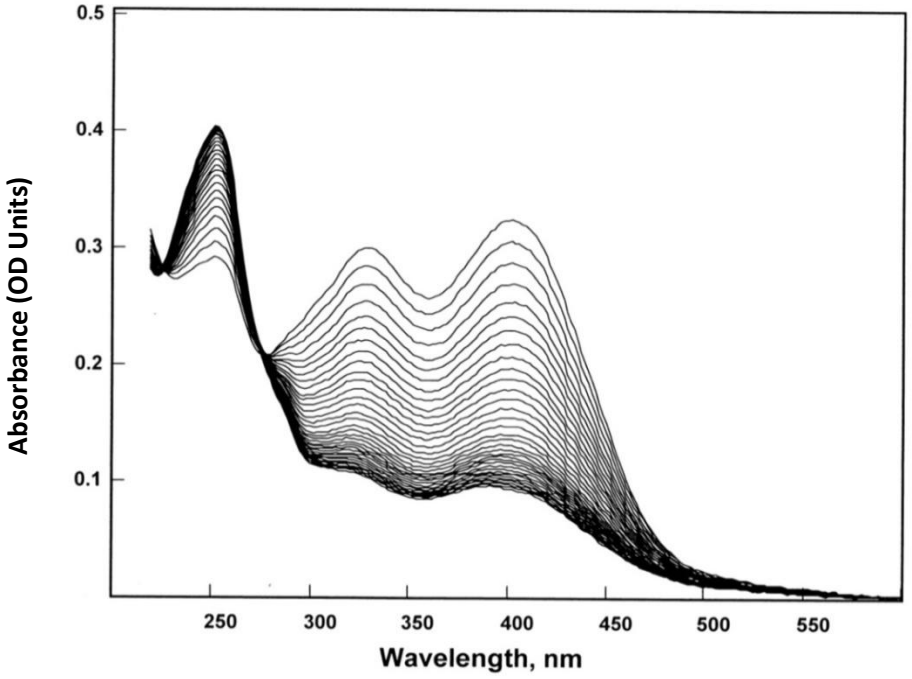


Figure 5.4c Spectra of CMC 2.5, pH 7.00, 5sec intervals

C. Zinc binding study

Because the MMPs are zinc-dependent enzymes, the binding to zinc ions is a probable contributor to the inhibition, as first shown for doxycycline.[2-4] Therefore zinc binding studies were carried out for curcumin, CMC2.5 and CMC2.24. Figure 5.5 shows the spectra of curcumin, CMC2.5 and CMC2.24 at different concentrations of zinc ions. These data were fitted at multiple wavelengths to extract the derived spectra. Figure 5.6 shows the fitted spectra for these compounds. Several wavelengths were plotted and calculated for the dissociation constant: $K = (cb - cx)(ca - cx)/cx$, where *cb* is the total concentration of zinc ion, *ca* is the concentration of curcumin or a CMC and *cx* is the concentration of the complex formed by the zinc with curcumin or a CMC. When *cb* >>*cx*, K_D reduced to $cb(ca - cx)/cx$. The dissociation constants found were 1385 ±89 µM for curcumin, 1880 ±68 µM for CMC2.5 and 765 ±20 µM for CMC2.24. CMC2.24 shows slightly stronger binding to zinc ion compared to both curcumin and CMC2.5. It should be noted that the interaction of CMC2.24 with zinc ion would be strong enough to form a complex with the zinc ion in the catalytic domain of MMPs, thus it is responsible for its inhibitory activity. The free energy of binding for CMC2.24, is deduced from $\Delta G = -RTln\,K$, and is approximately -28kJ, which would enable the enolate form to bind to the zinc ion strongly.

Figure 5.5 Spectra for zinc binding study for curcumin, CMC2.5 and CMC2.24

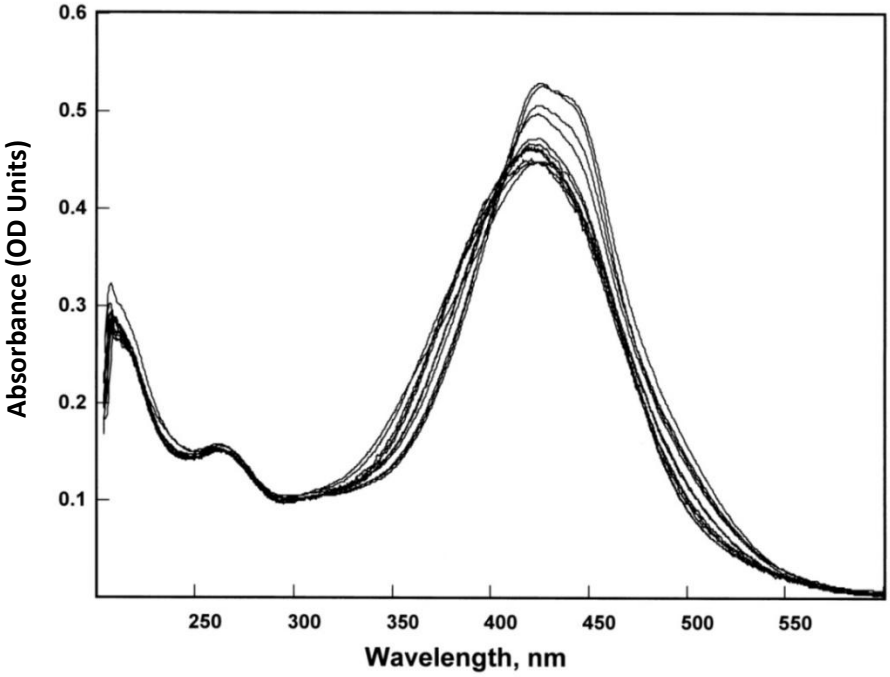


Figure 5.5a Zinc binding of curcumin

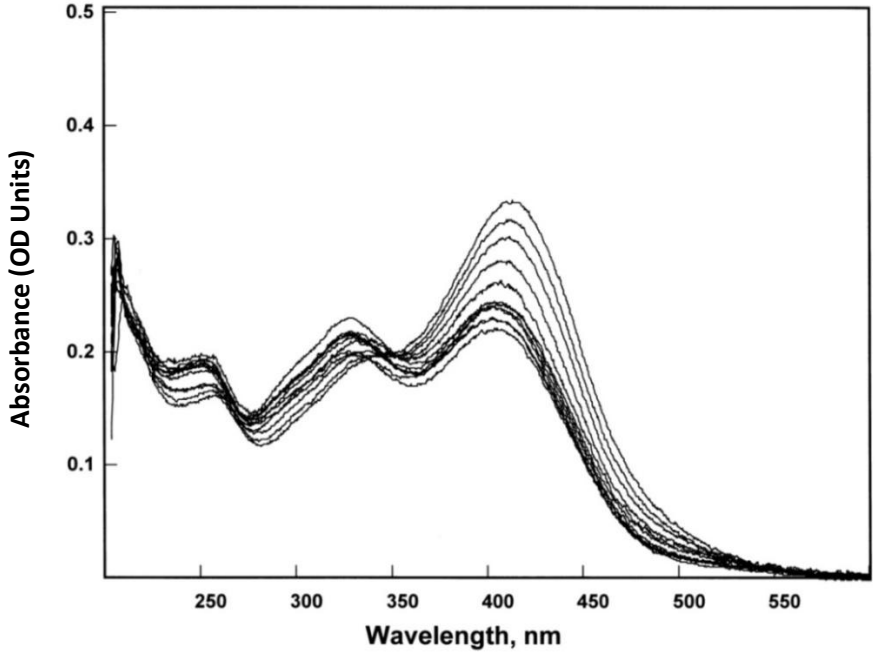


Figure 5.5b Zinc binding of CMC2.5

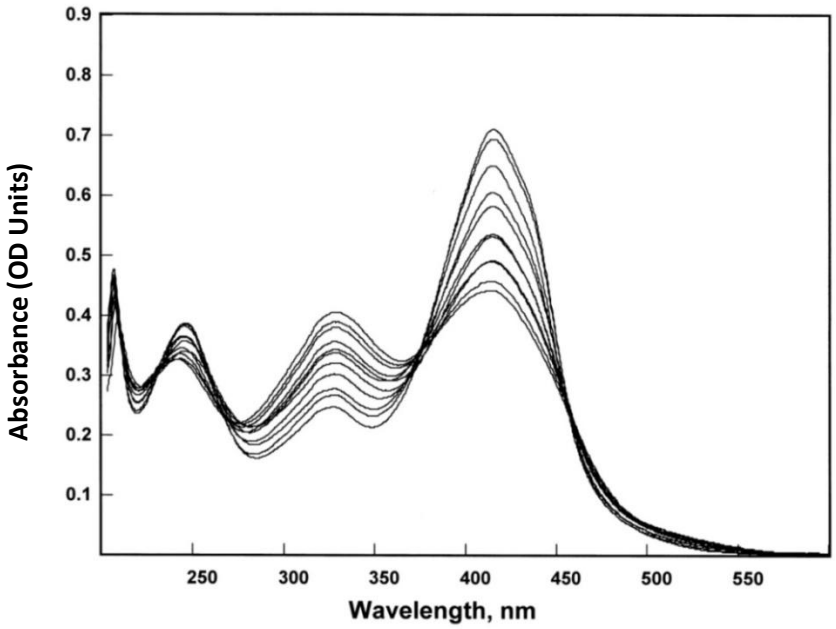


Figure 5.5c Zinc binding of CMC2.24

Figure 5.6 Fitted spectra for zinc binding for CMC2.5 and CMC2.24 at selected wavelengths

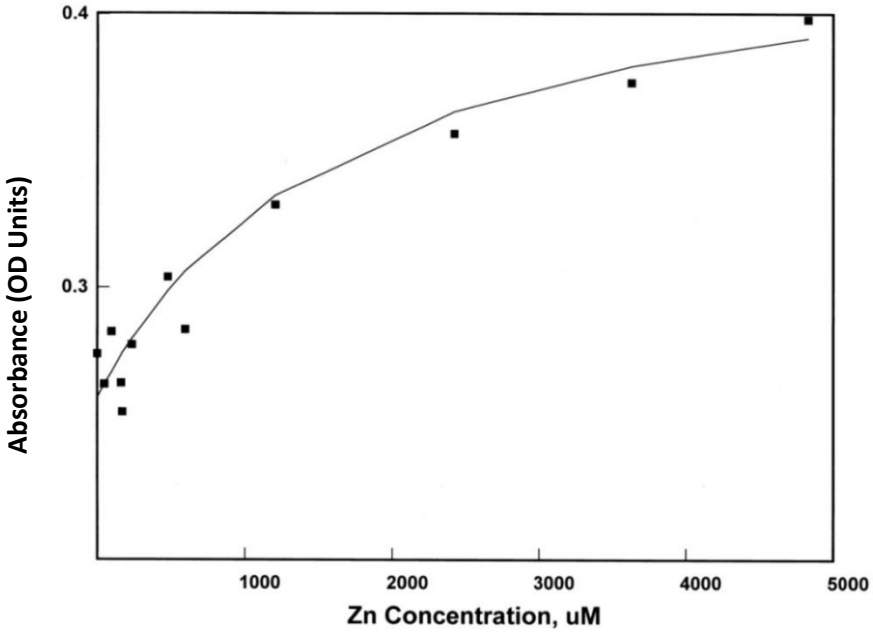


Figure 5.6a Fitted zinc binding of CMC2.5 at 413nm

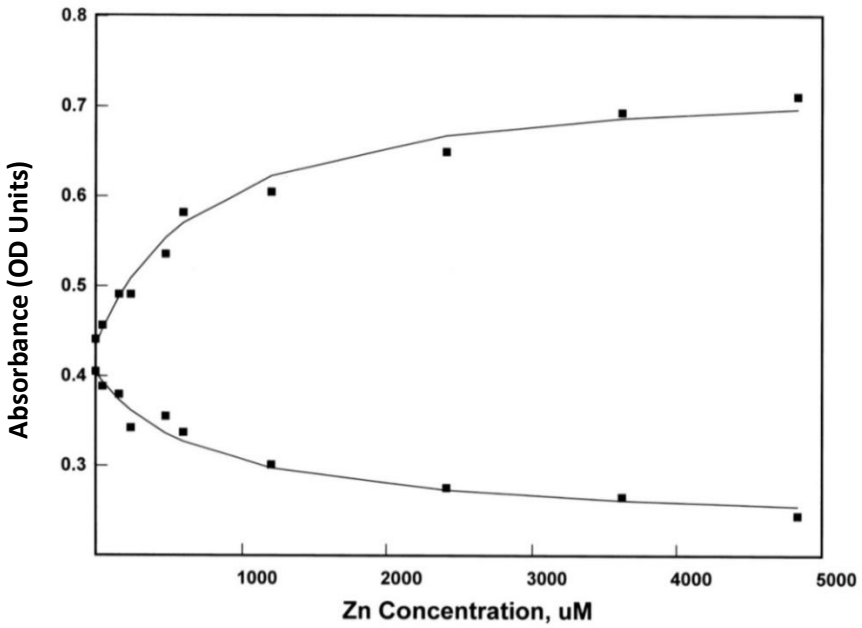


Figure 5.6b Fitted zinc binding of CMC2.24 at 329nm, open; 415nm, filled

D. Bovine Serum Albumin (BSA) binding study

Given that curcumin and CMCs show relative time-dependence of spectral transformation in various pH buffers, and they are neither soluble enough nor stable enough in salt solutions, it would be crucial to study the binding of these compounds to serum albumin, which resembles the conditions in plasma or extracellular fluid to maintain useful therapeutic concentrations.[5,6] Accordingly, a serum albumin binding assay was carried out. Serum albumin is the major plasma proteins in mammals. Bovine serum albumin (BSA) was used in the assays of serum binding studies because of structural similarity with human serum albumin (HSA). BSA contains 581 residues and has multiple binding sites with specificities. The binding of BSA to curcumin was studied by Bourassa *et al.*, who reported a dissociation constant K_D of 30 μM using an indirect fluorescence spectroscopic method at a BSA concentration of 20mg/mL.[7] In the current study, the UV-Visible technique which was used for the pK_a determination and the zinc binding study was utilized to evaluate the binding to serum by two compounds, namely curcumin and CMC2.24. The observed spectra are shown in Figure 5.7, and the calculated figures are shown in Figure 5.8. The dissociation constants are: K_d=1.32 ±0.17 μM for curcumin and K_d=0.56 ±0.08 μM for CMC2.24. The dissociation constant for curcumin was lower than the one determined by Bourassa *et al.* by an order of magnitude. In human plasma, the concentration of serum albumin is about 700 μM, which strongly suggests that approximately 99.8% of CMC2.24 is bound to the albumin.

Figure 5.7 Spectra for bovine serum albumin binding study

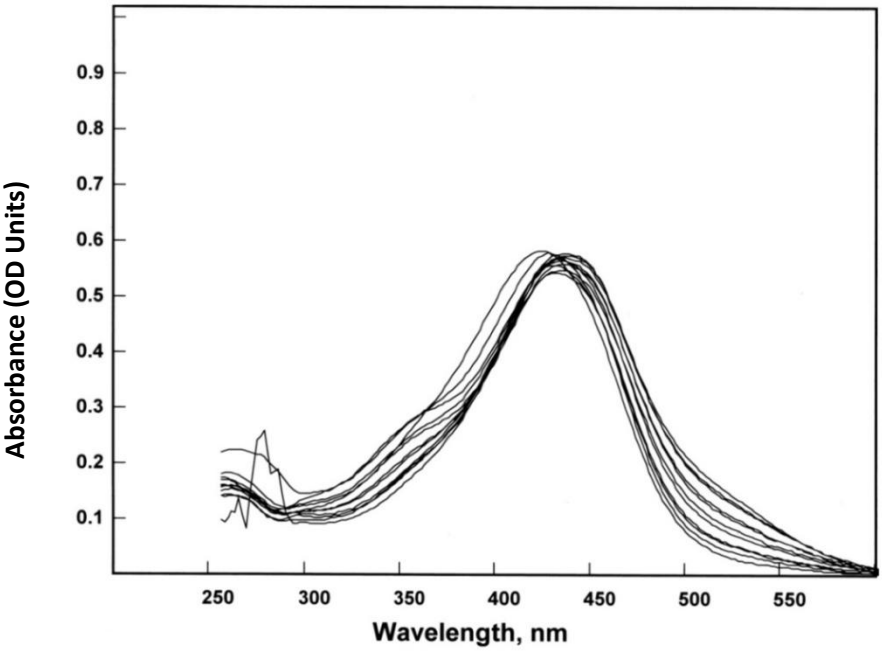


Figure 5.7a Bovine serum albumin binding of curcumin

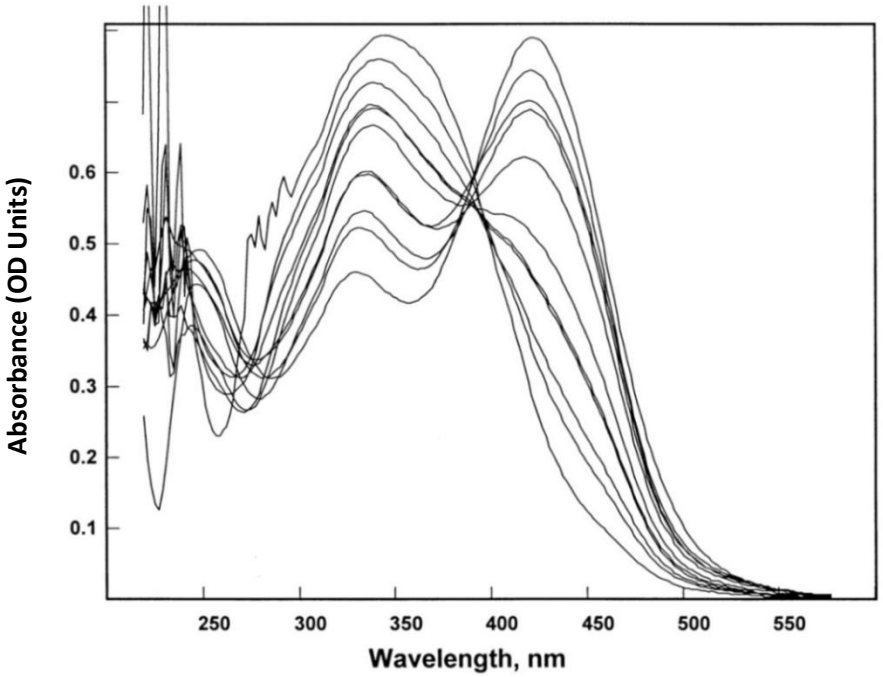


Figure 5.7b Bovine serum albumin binding of CMC2.24 (14.5mM)

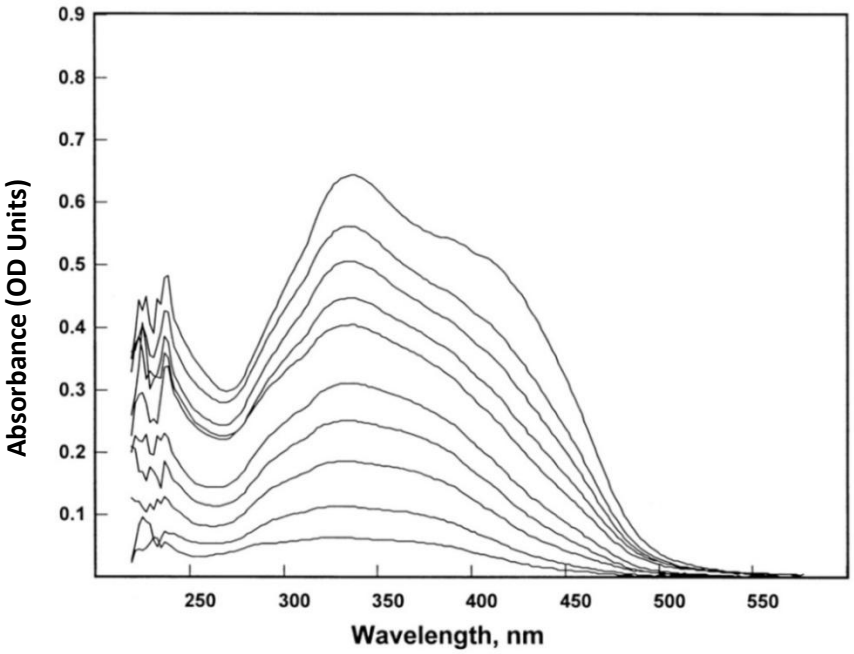


Figure 5.7c Bovine serum albumin (13.0 μM) binding of CMC2.24

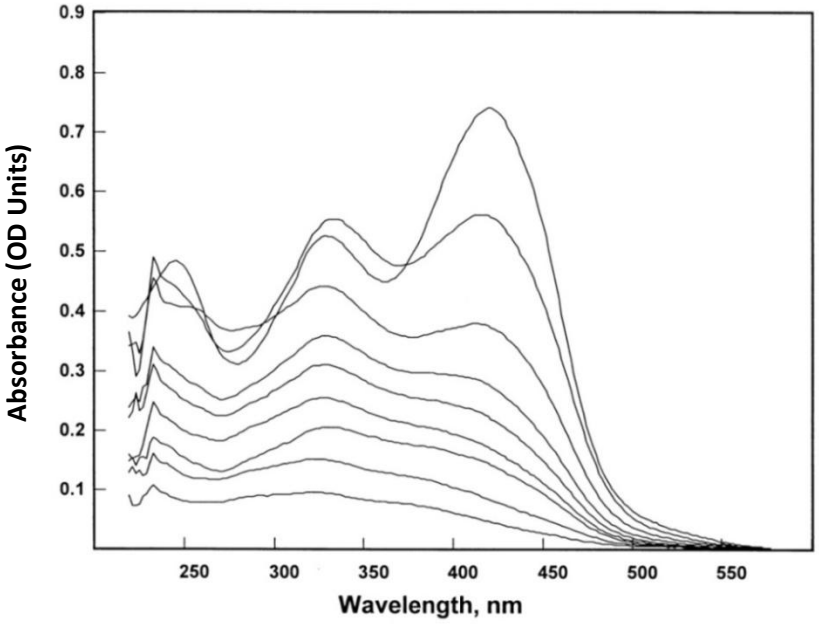


Figure 5.7d Bovine serum albumin (6.0 μM) binding of CMC2.24

Figure 5.8 Fitted spectra for BSA binding at selected wavelengths

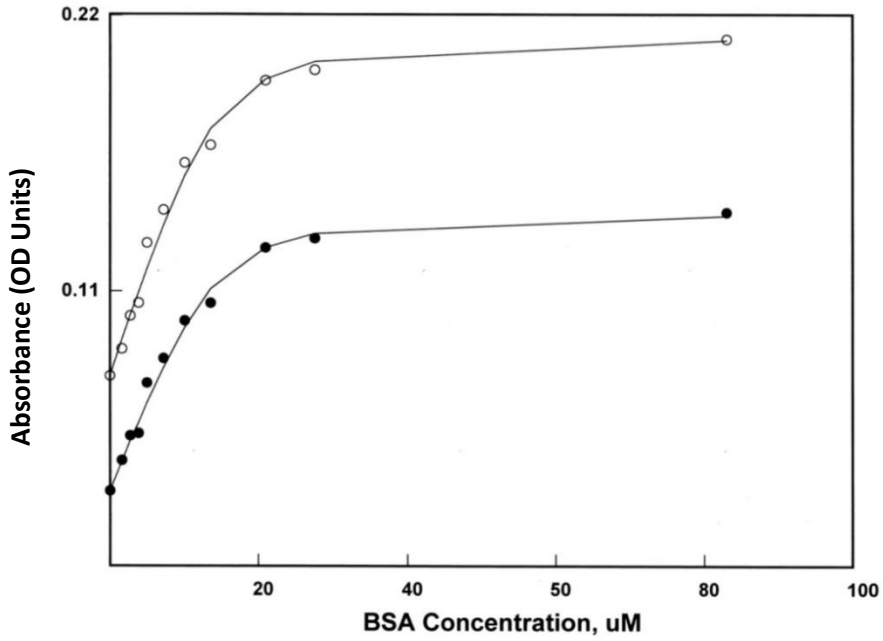


Figure 5.8a Fitted spectra for BSA binding of curcumin at 500nm, 525nm

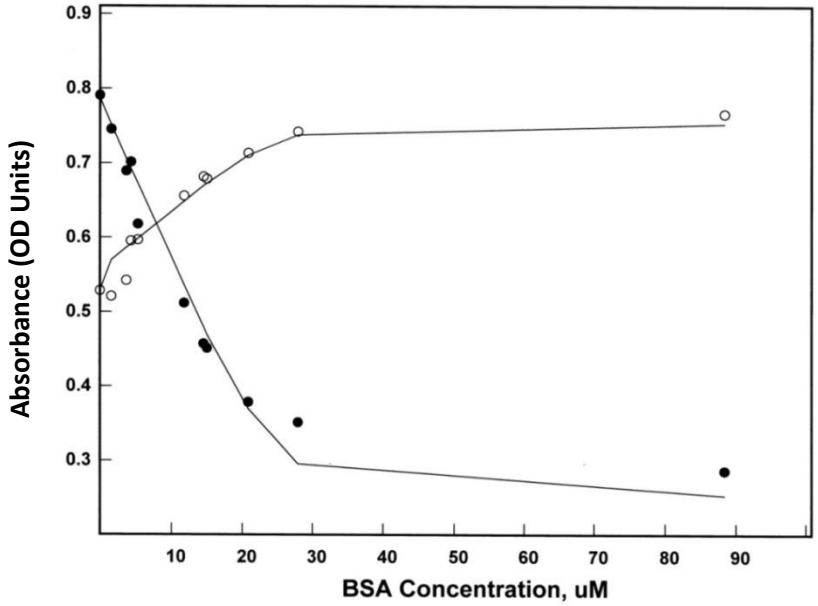


Figure 5.8b Fitted spectra for BSA binding of CMC2.24, 330nm, 421nm

E. *In vitro* antimicrobial activity of curcumin, CMC2.5 and CMC2.24

1. Minimum Inhibitory Concentration (MIC)

An ntimicrobial (antibiotic) compound is defined as an agent that kills or slows down the growth of bacteria, and is used to treat infectious diseases. They are either bactericidal or bacteriostatic. Tetracyclines are a class of antibiotics, and are inhibitors of protein synthesis. Doxycycline is one of these. In 1983, Golub *et al.* found that tetracyclines could inhibit MMPs via a mechanism quite distinct from their antibiotic activity.[8.9] Even those tetracyclines having no antibiotic activity (lacking a dimethylamino group), still possess the ability to inhibit the MMPs. Because curcumin analogues contain the same binding site that is responsible for the MMP inhibitory activity as tetracyclines, namely the β-diketone assembly, it appeared important to determine if these novel chemically-modified curcumins possessed any antibiotic activity. Curcumin has been reported to lack anti-microbial activity in previous studies[10-12], whereas the novel curcumin analogues CMC2.5 and CMC2.24 which show great inhibitory activity against human-derived MMPs *in vitro* and *in vivo* have never been tested. *E. coli* representing a Gram negative bacterium and *S. aureus* representing a Gram positive bacterium were selected as representative organisms to evaluate the minimum inhibition concentrations (MIC). The MIC is defined as the lowest serial dilution of the antimicrobial agents for which no growth of bacteria can be observed after overnight incubation. From the results obtained, doxycycline showed strong inhibition of bacterial growth with MIC=1.0 μg/mL against *E.coli* and MIC=2.0 μg/mL against *S. aureus*. However, curcumin and CMC2.5 did not show any observed inhibitory activity against these two bacterial species, whereas for CMC2.24, an MIC of 62.5 μg/mL was revealed against *S. aureus* but there was no inhibition of *E.coli* at all the concentrations tested. Although CMC2.24 showed some activity against *S. aureus*, the effect is small.

Table 5.2 Minimum inhibitory concentrations (MIC) in μg/mL of curcumin and CMCs

Note: Doxycycline was used as a positive control.

Strains	Doxycycline (control)	Curcumin	CMC2.5	CMC2.24
E.coli(G-)	1.0	>500	>500	>500
S. aureus(G+)	2.0	>500	>500	62.5

2. β-galactosidase inhibition

β-Galactosidase is the enzyme that catalyzes the hydrolysis of β-D-galactose residues into monosaccharides. Kinetic studies carried out by Gasteiger *et al.* suggested that the onset inhibition of β-galactosidase synthesis inhibits protein synthesis at the initiation of translation.[13] β-Galactosidase was produced *in vitro* by an *E. coli* transcription / translation system.[14-16] The synthesis of β-galactosidase is used to determine the inhibition of ribosome protein synthesis.[17] The *E.coli* S30 extract system (Promega) utilizes a circular plasmid containing the β-galactosidase gene. β-Galactosidase activity assay (Promega) was conducted after the protein synthesis *in vitro* in the presence of various concentrations (1 μM, 5 μM and 10 μM) of potential protein inhibitors (doxycycline, curcumin and CMC2.24), whereas doxycycline, the known antibiotic, was used as a positive control.[18,19] The reaction mixture was maintained at 37^{o}C for 1 hour, and the UV absorbance was measured in order to determine the residual activity of β-galactosidase. From the result of Table 5.2, doxycycline inhibits 6.4% production of β-galactosidase at 1 μM, 12.2% at 5 μM and 30.7% at 10 μM in contrast to curcumin and CMC2.24, both of which show the inhibition of β-galactosidase production at less than 13.5% at 10 μM (the highest concentration in the assay). The result strongly suggests that CMC2.24 is comparable to curcumin, regarding the inhibition of β-galactosidase production, and both of these compounds

exhibit much less inhibitory activities than doxycycline (the positive control), which is antibiotic at higher doses. Given the fact that curcumin is known to be safe for oral use as a natural product in the food ingredient curry powder, neither curcumin nor CMC2.24 shows any significant antibiotic activity. *P-value*, the probability in statistical significance for each two groups, was also calculated for the β-galactosidase inhibition. At 10 μM, there is statistically significant difference between doxycycline and curcumin ($p<0.01$), and no statistical difference between curcumin and CMC2.24 ($p>0.05$). At 1 μM and 5 μM, no significant difference was observed between curcumin and CMC2.24. As for doxycycline, 1 μM and 5 μM are not statistically different ($p<0.05$), while both are significantly different at 10 μM. The result strongly suggests that doxycycline exhibits inhibitory activities (30%) at 10 μM as an antibiotic agent in comparison to its lower concentrations at 1 μM or 5 μM. For both curcumin and CMC2.24 at 1 μM, 5 μM or 10 μM, no antimicrobial activity was observed.

Table 5.3 β-galactosidase inhibition of curcumin and CMC2.24

	Doxycycline			Curcumin			CMC2.24		
	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM	1 μM	5 μM	10 μM
Inhibition %	0.3	6.7	26.8	3.6	6.1	10.1	2.1	4.3	9.1
Inhibition %	7.0	10.1	30.2	4.5	9.4	12.2	1.2	8.8	13.1
Inhibition %	6.4	12.2	34.1	0	0.9	14	0	8.2	17.1
AVE	4.6	9.7	30.7	2.7	5.5	12.1	1.1	7.1	13.1
S.D.	3.7	2.8	3.7	2.4	4.3	1.9	1.1	2.4	4.0
S.E.M.	2.14	1.60	2.11	1.37	2.47	1.13	0.61	1.41	2.31

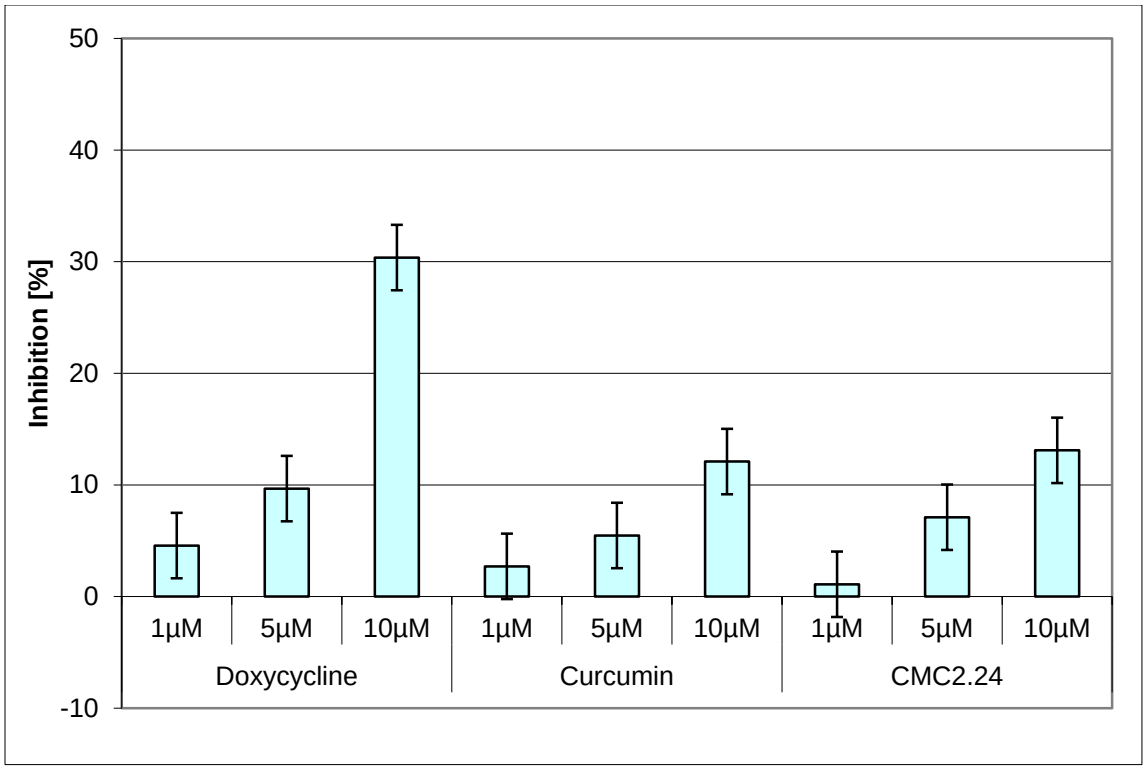


Figure 5.9 β-galactosidase inhibition of curcumin and CMC2.24

Materials and Methods

1. pH equilibria

Buffers containing 0.006 M KH_2PO_4, 0.001 M HCl, 0.093 M KCl or 0.003 M KH_2PO_4, K_2HPO_4, 0.004 M $KHCO_3$ and 0.090 M KCl, ionic strength 0.10 M (prepared by analytic techniques), were adjusted with 0.10 M KOH to span the range pH 3.0-12.0. Aliquots of 3.00 mL at each pH were delivered to the thermostatted dry cuvette, and the baseline re-zeroed. At zero time, 0.1000 mL fresh stock CMC 2.24, CMC 2.5, or curcumin, typically 450 μM in acetonitrile, was added; efficient (99.9%) mixing was accomplished in 1 sec with a single mixer stroke. The UV-visible spectra sets were collected with an OceanOptics USB 2000 spectrophotometer, operated by SpectraSuite computer software. Acidity (pH) was measured with a Thermo/Orion Model 420+

meter using an MI-410 combination microelectrode from Microelectrodes, Inc. True pH values were calculated from the millivolt readings after linear correction for small drifts in the intervening standard pH buffer readings (commercial pH 4.00, 7.00 and 10.00 buffers from Fisher or J.T. Baker, standardized 0.00872 M KOH - 0.0900 M KCl for pH 11.82). Temperature was monitored to $\pm 0.1^{o}$C using a home-made thermistor bridge. Thirty-five to forty pH sets were obtained for each compound.

2. Zn^{2+} and BSA binding

Crystalline bovine serum albumin from Pentex, shown to be fatty-acid-free was used without further treatment. Stock solutions of KCl-tris (hydroxymethyl)aminomethane (for Zn^{2+}, to avoid zinc phosphate precipitation) or KCl-phosphate (for BSA) with or without zinc acetate or BSA, all adjusted to pH 7.4, were prepared. Samples (5 mL) of varying zinc acetate or BSA concentration were prepared by analytical-balance weight-weight dilution. For two BSA series w/w dilution samples of CMC 2.24 in acetonitrile were prepared. Typically, 2.991 $\pm$0.005 mL aliquots were added to the dry cuvette, and spectra were taken (primarily to establish the BSA concentration), after which the baseline was rezeroed to eliminate the BSA absorbance peak (the slight effect of further dilution with the 0.0981 mL acetonitrile aliquot is dealt with during post-processing). As before, data collection was initiated and the aliquot of compound in acetonitrile was added with one-second mixing, thus establishing "zero time" with an uncertainty smaller than the data interval. The final BSA concentration was determined from the corrected spectra using an absorptivity ε_{279} at 279 nm of 43,824 $M^{-1}cm^{-1}$. The absorptivity of CMC 2.24 at 418.0 nm, in acetonitrile, was determined here as $(4.19 \pm 0.15) \times 10^{4} M^{-1}cm^{-1}$.

3. Data processing

The spectra for each pH, Zn^{2+}, or BSA data set are fitted with a multiwavelength non-linear least- squares algorithm originally used to analyze the HgI_n^{2-n} equilibria and later the thermorubin pK_a equilibria.[20,21] The operative equations are Beer's Law and conservation of mass:

(1) $A_{\lambda,j}=d \sum_i \varepsilon_{\lambda,j} c_{i,j}$ for M samples *j*, N species *i*. $c_j^0 = \sum_i c_{i,j}$

where the $c_{i,j}$ are the concentrations of absorbing species *i* and the $\varepsilon_{\lambda,i}$ their intrinsic *absorptivities* at wavelength λ. The light path length *d* is 1.000 cm. The absorptivities $\varepsilon_{\lambda,i}$ and the equilibrium constants (pK_a or K_D) that determine the $c_{i,j}$ through the equilibrium expressions are adjusted to convergence by the algorithm, which minimizes the sum of squares of absorbance deviations ($A_{\lambda,j}^{obs} - A_{\lambda,j}^{calc}$). Typically, 12 wavelengths, spaced to select those maximizing contrast among species, were used. With the pK_a values determined by the fitting then established, the entire spectral range for all samples was fitted with only the remaining absoptivities as fitting parameters. In many instances only two of the species (or internally-equilibrating populations, in kinetics cases), say A and B, that are interconverting, have non-negligible concentrations in the range of some data subset. Beer's Law reduces to

(2) $A_{\lambda,j}=\varepsilon_{\lambda,A} c_{A,j+} \varepsilon_{\lambda,B} c_{B,j}=\varepsilon_{\lambda,A} c_j^0 + (\varepsilon_{\lambda,B} - \varepsilon_{\lambda,A}) c_{B,j}$

If the spectra for species or populations A and B cross at some wavelength λ_{iso}, there $\varepsilon_{\lambda,B} \equiv \varepsilon_{\lambda,A}$, and $A_{\lambda,iso}=\varepsilon_{\lambda,A} c_j^0$ is invariant. This is an *isosbestic point*, whose existence is *prima facia* evidence that in this data subset two and only two species or populations are interconverting.

4. MIC test

The MIC was determined by broth microdilution MIC test method, whereas the activity of an antimicrobial agent against different bacteria could be determined. Curcumin, CMC2.5 and

CMC2.24 dissolved in DMSO as stock solutions were used in the test, and doxycycline was used as a positive control. Serial dilutions were made to the final concentrations of 500, 250, 125, 62.5, 31.3, 15.7, 7.8, 3.9, 2.0, 1.0, 0.5, 0.25 μg/mL by broth media (containing a final concentration of 10ppm Mg^{2+} and 20ppm Ca^{2+}). Test bacterial *E. coli* (gram negative) and *S. aureus* (gram positive) were pre-incubated overnight on the sterilized plastic plate, and then inoculated to broth medium to grow at 37°C for 2 hours reaching the final concentrations of 1×10^8 cells/mL with the O.D. reading at 0.09 ±0.01. 100 μL of bacterial at a final concentration of 1×10^6 cells/mL was added to 100 μL solutions of antimicrobial agents into optical quality round bottom 96-well susceptibility microtrays. The trays were incubated at 37 ℃ for 18 hours for these bacteria, and minimum inhibitory concentration (MIC) was evaluated as the lowest concentration of the antimicrobial agents above the dilution at which overnight growth is observed.

5. *In vitro* β-galactosidase synthesis

In vitro β-galactosidase synthesis was carried out by 1 μL of β-galactosidase DNA plasmid (0.5 μg/μL), 5 μL of complete amino acid mix (2.5 μL of Amino Acid Mixture Minus Methionine, 1mM and 2.5 μL of Amino Acid Mixture Minus Leucine, 1mM), 20 μL of S30 Premix Without Amino Acid, 15 μL of S30 Circular Extract and 9 μL of inhibition solution to a final volume of 50 μL in PCR tube and incubated at 37°C for 1 hour. The inhibition solutions including doxycycline, curcumin and CMC2.24 in concentrations of 1 μM, 5 μM and 10 μM in DMSO (and DMSO only) were prepared separately. H_2O was used as a negative control. The reaction mixtures were vortexed gently, then centrifuged for 5 seconds before use. Cell lysates from the previous step was obtained. 15 μL of the cell lysates, 135 μL of 1X Reporter Lysis Buffer and 150 μL of 2X Assay Buffer were added together and vortexed gently. The reaction mixtures were

incubated in 37^{o}C water bath for 1 hour and a faint yellow color has developed. All the reactions were stopped by an addition of 500 µL of 1M Na_2CO_3. Absorbance was read at 420nm after a brief vortexing. The inhibition was calculated upon the percentage of absorbance decrease in contrast to the blank, whereas H_2O was used as a negative control.

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Chapter 6. An *in vitro* lipophilicity and an *in vivo* pharmacokinetic study of curcumin and CMC2.24

A. Introduction

Lead candidates in drug discovery usually have suitable physicochemical properties beyond their potency, e.g., bioavailability, appropriate *in vivo* pharmacokinetics, safety. Lipinski's rule of five considers a partition coefficient value (*log P*), a known molar refractivity, molecular weight, the number of atoms (including H-bond donors and H-bond acceptors) and polar surface, all of which are needed to assess the pharmacological profile of the active drug substance.[1] The partition coefficient (P) value determined as the distribution between two separate solvent systems, usually 1-octanol /aqueous solution (which are immiscible with each other) is regarded as the distribution of the drug substance to be expected between the physiological phases of lipophilicity or hydrophilicity. The logarithm of the ratio in equilibrium concentrations of a single species, *log P*, is used to assess the lipophilicity of a drug substance. Pharmacokinetics, including absorption, distribution, metabolism, and excretion (ADME), needs to be considered with regard to the drug disposition in the body. As mentioned in Chapter 2, curcumin is known to have poor bioavailability in the human system. Even with oral dosage up to 12g/day, only a small amount of curcumin (51.2ng/mL) can be detected in the serum.[2-6] Thus it is crucial to determine the biodistribution of the novel chemically-modified curcumins (CMCs) in comparison with curcumin, because they are more potent, more acidic, and more soluble at physiological pH. After considerable experimentation, ideal solution containing Solutol 15 and ethanol (v/v=1:1) was found to dissolve both curcumin and CMCs up to 40mg/mL, and this solution was used for the *in vivo* pharmacokinetics evaluation by intravenous (i.v.) route. No

precipitation occurred at the injection site. This is the first time that a CMC has been introduced intravenously.

B. *In vitro* lipophilicity

Prior to any *in vivo* pharmacokinetic study, the *in vitro* lipophilicity study was conducted by Simon Tong *et al* using the same evaluation method (HPLC) as was used for doxycycline.[7-9] Curcumin and CMC2.24 at a concentration of 1mg/mL were dissolved separately in methanol and then diluted to 10 μg/mL by PBS buffer saturated with 1-octanol (used to mimic the lipid) (v/v=1:1), the separated layers were evaluated by HPLC to determine the concentrations by comparing to the standard curves obtained earlier, in order to calculate *log P*. However, both the concentrations of curcumin and CMC2.24 in the aqueous layers were below the detection limit of HPLC, whereas those in 1-octanol layers were close to the injection concentration, so the partition coefficient cannot be deduced from this assay (data not shown). Therefore we adopted a UV-Vis scanning spectrophotometer to study the distribution concentrations of curcumin and CMC2.24, where doxycycline (hydrophilic) and CMT-3 (lipophilic) were used as the control compounds. Scans were carried out at wavelengths ranging from 300nm to 500nm. As shown from Figure 6.1, a relatively large amount of doxycycline was found in the aqueous layer in contrast to CMT-3, which is known to be lipophilic. Curcumin and CMC2.24 are both very lipophilic according to the data, given that at the level of detection the concentration in the aqueous solution was close to zero. This strongly suggests that the lead compound, CMC2.24 is still very lipophilic, as is curcumin.

Figure 6.1 *In vitro* lipophilicity of doxycycline, CMT-3, curcumin and CMC2.24

Note: Spectophotometric aborptions of compounds in methanol (blue line), PBS buffer (pink line) and 1-octanol (yellow line), wavelengths ranging from 300nm to 500nm.[9]

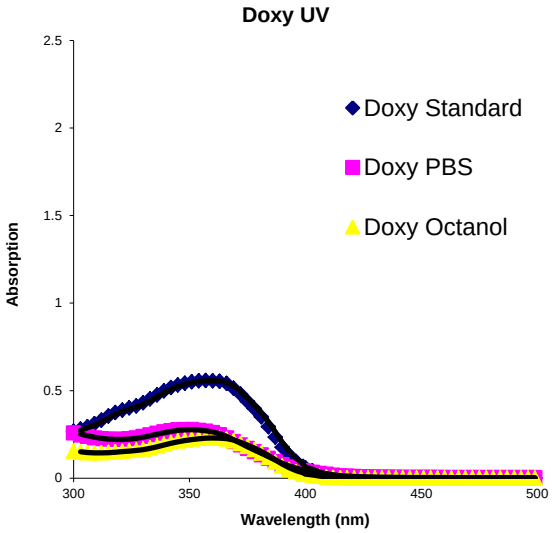


Figure 6.1a Lipophilicity study of doxycycline

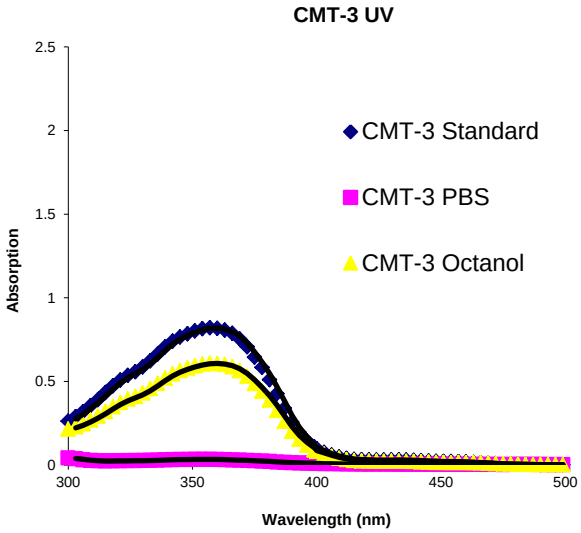


Figure 6.1b Lipophilicity study of CMT-3

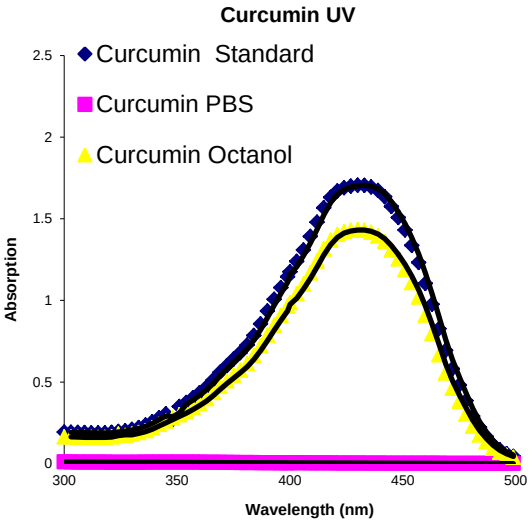


Figure 6.1c Lipophilicity study of curcumin

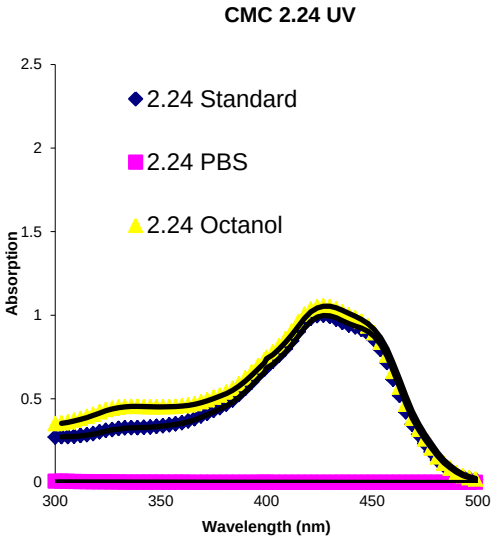


Figure 6.1d Lipophilicity study of CMC2.24

C. *In vivo* pharmacokinetics by oral route

In order to evaluate the *in vivo* pharmacokinetics of curcumin and the lead compound, CMC2.24, a preliminary study was carried out by oral route. Both curcumin and CMC2.24 were administered at 500mg/kg to rats, and the rats were sacrificed 6 hours after the dosage. Blood was drawn at 2 hours and 6 hours before the rats were sacrificed, intestines were harvested and feces samples were collected. Results show that the concentrations of both curcumin and CMC2.24 from serum samples were below the detection limits in the serum samples at 2 hours and 6 hours. Most of the compounds were found in the stomach, intestine and feces (Figure 6.2). Control samples were spiked with either curcumin or CMC2.24 (data not shown), and were used as reference to determine the concentration of the compound in serum. This result suggests that both curcumin and CMC2.24 have very poor absorption by the oral administration, which leads to the poor bioavailability accordingly.

Figure 6.2 HPLC spectra of rat serum samples after oral dosages of curcumin and CMC2.24

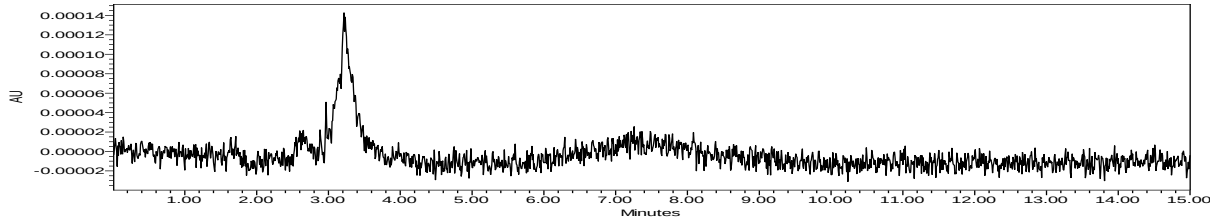


Figure 6.2a Blood sample from rats with no adminstration of curcumin or CMC2.24

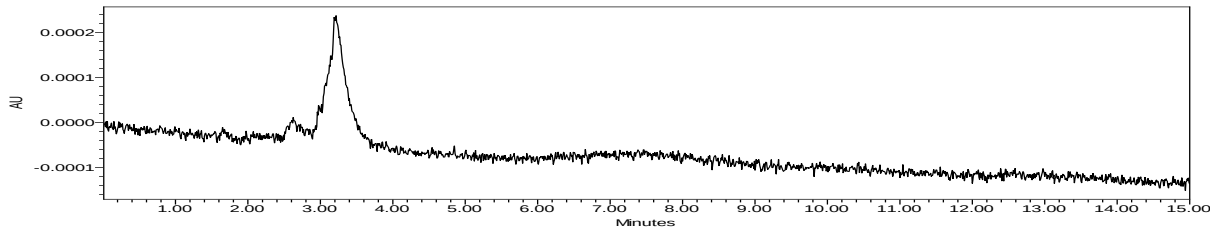


Figure 6.2b Blood sample from rats with oral adminstration of curcumin, 2h after dosage

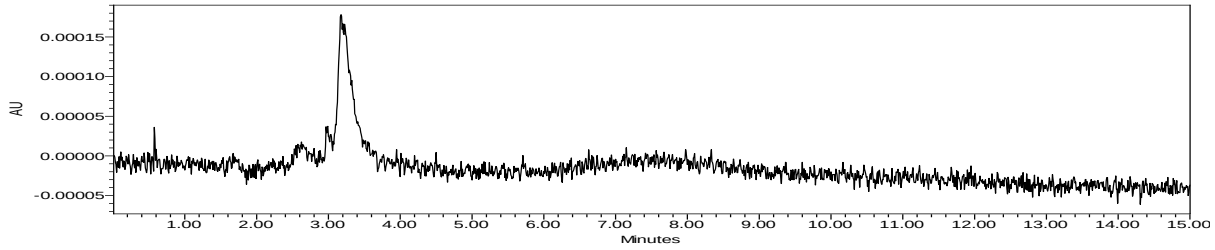


Figure 6.2c Blood sample from rats with oral adminstration of curcumin, 6h after dosage

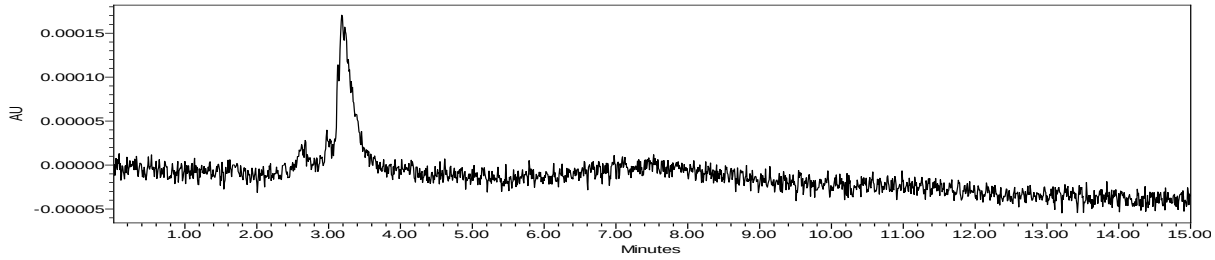


Figure 6.2d Blood sample from rats with oral adminstration of CMC2.24, 2h after dosage

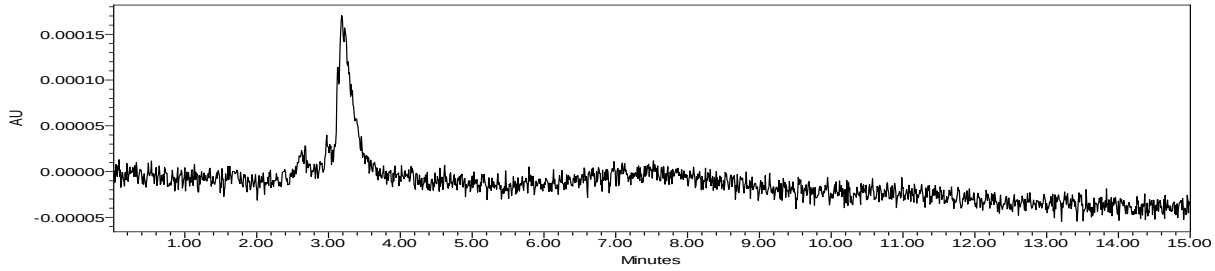


Figure 6.2e Blood sample from rats with oral adminstration of CMC2.24, 6h after dosage

D. *In vivo* pharmacokinetics by *i.v.* route

An *in vivo* pharmacokinetic study via the intravenous (*i.v.*) route was carried out for both curcumin and CMC2.24. Stock solutions were prepared in a combination of Solutol 15 and ethanol (v/v=1:1), the concentration of the drug substance being 40mg/mL.[10,11] Each rat was injected 20mg/kg (for a rat with the weight of 300g, 6mg of drug substance is injected). In the first pharmacokinetic study, rats were distributed to three groups, and each group contained three rats. Doxycycline was used as a control for the *in vivo* pharmacokinetics study, and blood was

drawn from retro-orbita (venous eye plexus) at 30min, 1h, 2h and 4h.[12] Serum samples was obtained for all the time points, and drug concentrations were calculated by comparison with standard curves. Curcumin, CMC2.24 and doxycycline showed relatively linear graphs for the standard concentrations ranging from 1 μM to 50 μM. When the rats were injected with curcumin, CMC2.24 and doxycycline, all of them remained alive and healthy, with no evidence of toxicity being observed. (Table 6.1a-6.1c, Figure 6.3a-6.3c)

Table 6.1 Standard curves for curcumin, CMC2.24 and doxycycline

Table 6.1a Standard curve for curcumin at concentrations at 1 μM, 5 μM, 10 μM and 50 μM

Curcumin (μM)	0	1	5	10	50
Area Under Curve	0	47441	115230	200551	972782
y=19523x, R^2=0.9982 (y: area in Area Under Curve; x: concentration of curcumin in μM)					

Table 6.1b Standard curve for CMC2.24 at concentrations at 1 μM, 5 μM, 10 μM and 50 μM

CMC2.24 (μM)	0	1	5	10	50
Area Under Curve	0	41003	116088	182532	810628
y=16366x, R^2=0.9950 (y: Area Under Curve; x: concentration of CMC2.24 in μM)					

Table 6.1c Standard curve for doxycycline at concentrations at 1 μM, 5 μM, 10 μM and 50 μM

Doxycycline (μM)	0	1	5	10	50
Area Under Curve	0	3241	16223	33819	169689
y=3391.9x, R^2=1.000 (y: Area Under Curve; x: concentration of doxycycline in μM)					

Figure 6.3 Standard curves for curcumin, CMC2.24 and doxycycline

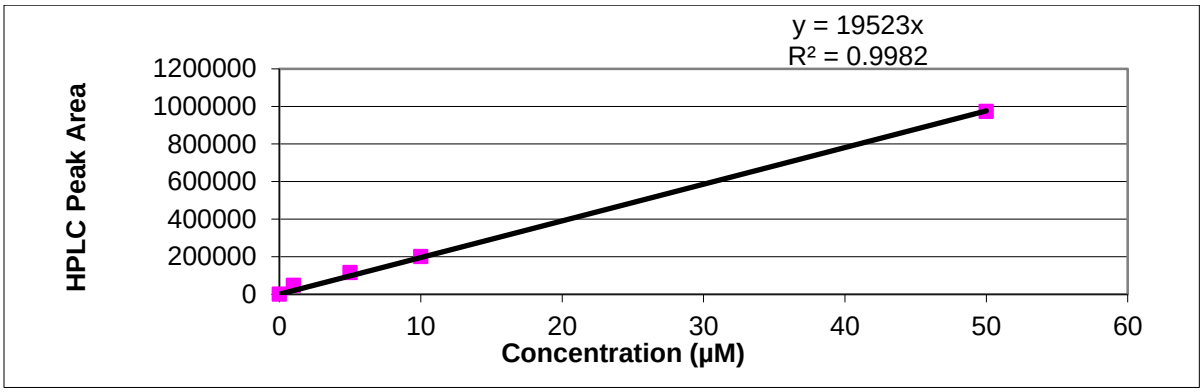


Figure 6.3a Curcumin standard curve

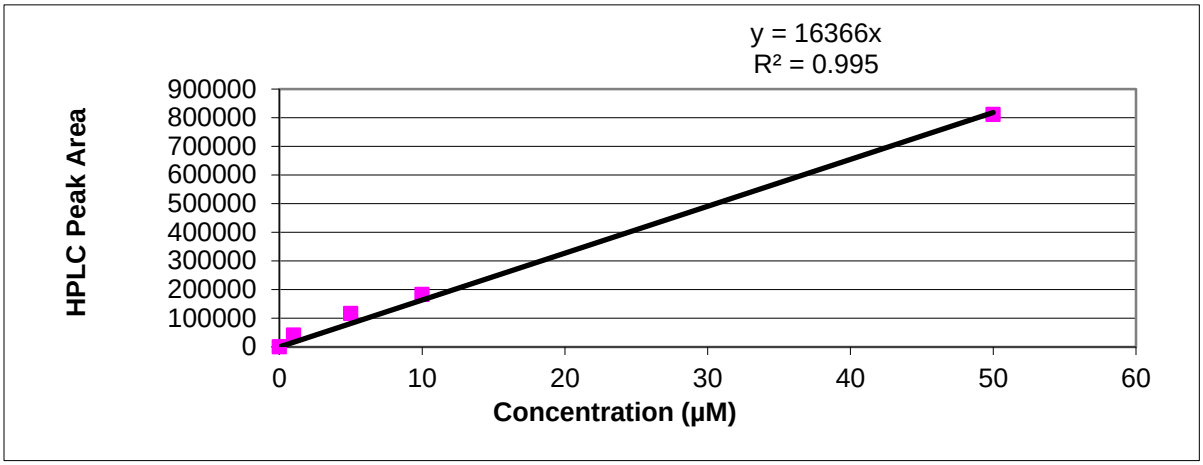


Figure 6.3b CMC2.24 standard curve

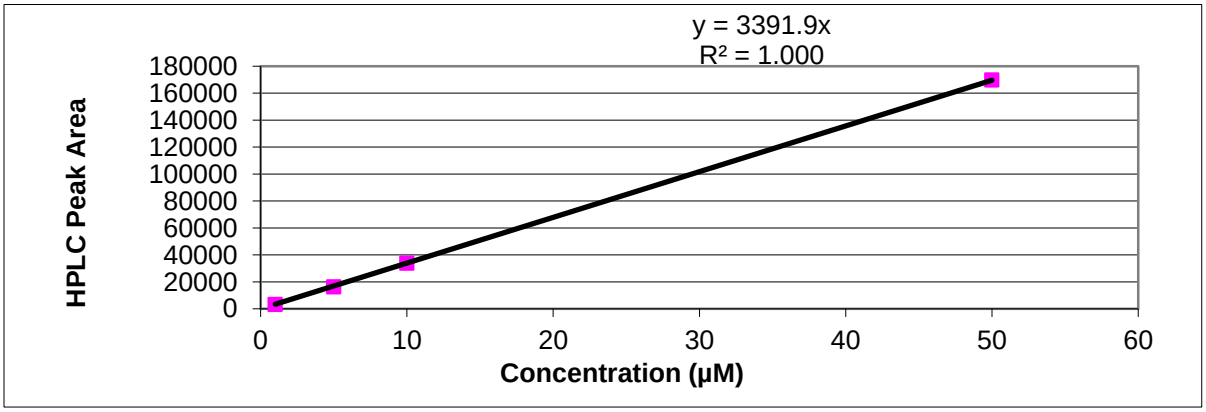


Figure 6.3c Doxycycline standard curve

According to the results (Table 6.4a-6.4c, Figure 6.2a-6.2c), doxycycline showed very high bioavailablility in all the time points (30min, 1h, 2h, 4h), especially in the first 30min when the concentration rose to 4.84 μg/mL in rat serum, and then decreased to 1.48 μg/mL at the 4h time point. Curcumin at 0.5h showed 0.02 μg/mL (0.07 μM) whereas CMC2.24 showed 0.71 μg/mL (1.66 μM), about 23-fold over curcumin (in molar amount). The concentrations of both compounds decreased very quickly in the serum from 1h to 4h, and went under the detection limit at 4h. However, CMC2.24 showed higher concentrations in serum samples at 0.2824 μM at 1h and 0.0561 μM at 2h in contrast to curcumin, where the values were 0.0766 μM at 1h and 0.0074 μM at 2h. The observed concentration of CMC2.24 is about 3.7-fold at 1h and 7.6-fold at the 2h time point compared to curcumin. The result confirms that curcumin has a very short half-life (about 13min) in blood serum, whereas CMC2.24 showed considerably greater bioavailability in the serum in contrast to curcumin.

Table 6.2 *In vivo* pharmacokinetics of curcumin, CMC2.24 and doxycycline

Table 6.2a *In vivo* pharmacokinetics of curcumin in rat serum at 0.5h, 1h, 2h and 4h

	Rat	0.5h	1h	2h	4h
Concentration of curcumin in serum(μg/mL)	1	0.040998	0.044824	0.008237	0
	2	0.014458	0.020659	0	0
	3	0.022846	0.019095	0	0
	AVE	0.026100	0.028193	0.002746	0
	S.D.	0.013566	0.014425	0.004756	----
	S.E.M.	0.007832	0.008328	0.002746	-----

Table 6.2b *In vivo* pharmacokinetics of CMC2.24 in rat serum at 0.5h, 1h, 2h and 4h

	Rat	0.5h	1h	2h	4h
Concentration of CMC2.24 in serum(μg/mL)	1	0.678227	0.019438	0.012915	0
	2	0.743715	0.147151	0.033735	0
	3	0.699961	0.195158	0.025491	0
	AVE	0.707301	0.120582	0.024047	0
	S.D.	0.033355	0.090823	0.010485	----
	S.E.M.	0.019258	0.052437	0.006054	----

Table 6.2c *In vivo* pharmacokinetics of doxycycline in rat serum at 0.5h, 1h, 2h and 4h

	Rat	0.5h	1h	2h	4h
Concentration of doxycycline in serum(μg/mL)	1	4.742970	4.707733	2.801545	1.739768
	2	5.396904	3.797942	3.516189	2.218084
	3	4.361591	1.363625	0.866771	0.477608
	AVE	4.833822	3.289767	2.394835	1.478487
	S.D.	0.523602	1.729002	1.370735	0.734173
	S.E.M.	0.302302	0.998239	0.791394	0.423875

Figure 6.4 *In vivo* pharmacokinetics of curcumin, CMC2.24 and doxycycline

Figure 6.4a *In vivo* pharmacokinetics of curcumin in rat serum at 0.5h, 1h, 2h and 4h

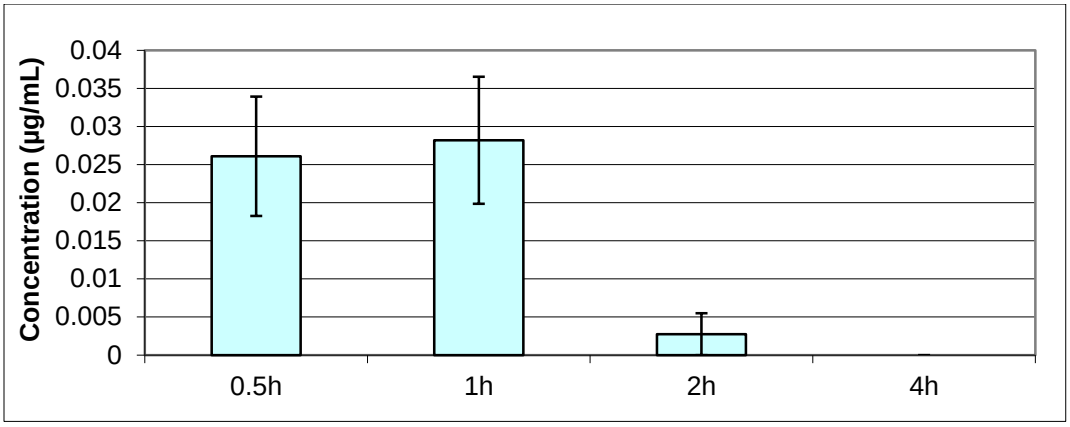


Figure 6.4b *In vivo* pharmacokinetics of CMC2.24 in rat serum at 0.5h, 1h, 2h and 4h

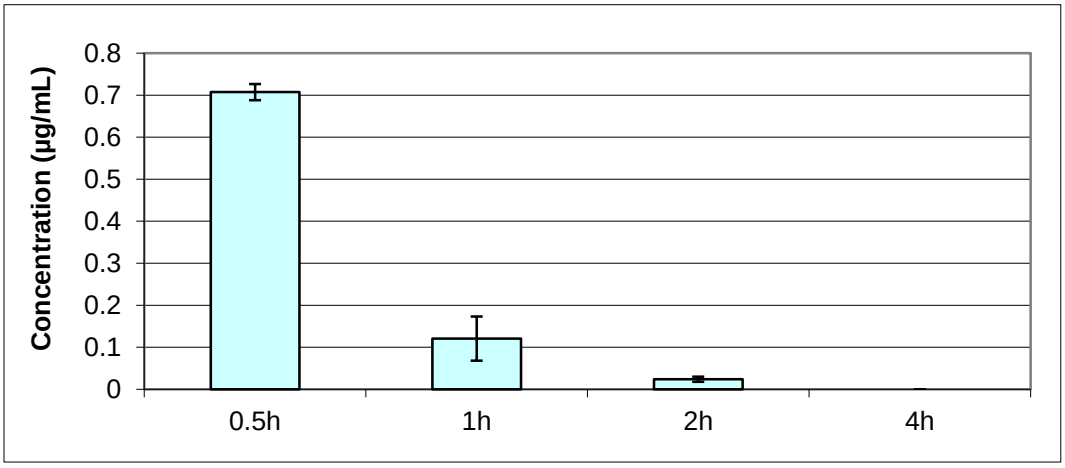
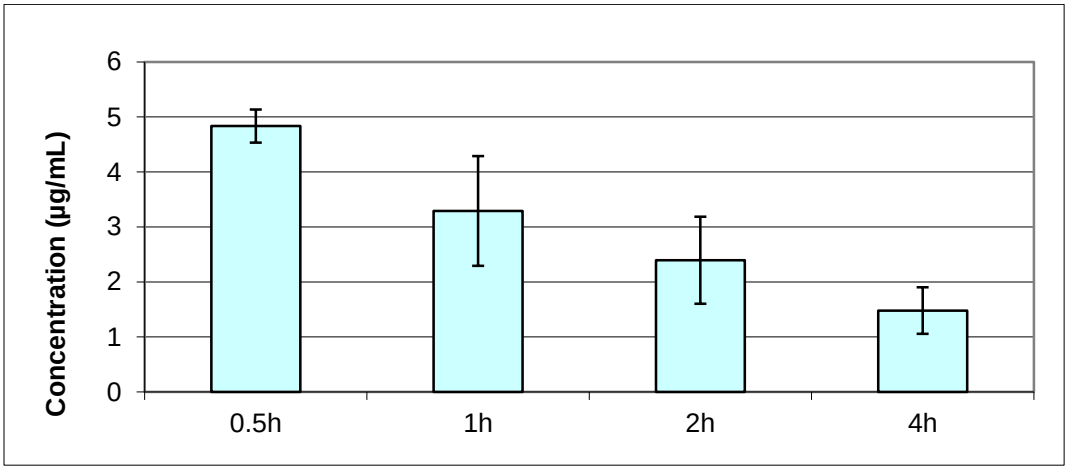


Figure 6.4c *In vivo* pharmacokinetics of doxycycline in rat serum at 0.5h, 1h, 2h and 4h



In order to study the dramatic changes of curcumin and CMC2.24 in serum during the first 0.5 hour, the second pharmacokinetics study with shorter intervals was carried out. Blood was taken at 5min, 10min and 20min. Blood pellets at each time point were collected and analyzed. All the rats were sacrificed at 1 hour time point, and organs including the liver, heart, spleen, kidney, lung and brain were collected and evaluated. From the data shown in Figure 6.5, Figure 6.6a-6.6b and Table 6.3a-6.3b, the 5min, 10min and 20min samples of both curcumin and CMC2.24 showed higher serum concentrations compared to the previous pharmacokinetics study. As for 5min sample, the concentration of CMC2.24 (11.00 μM) is about 2.2-fold in contrast to the one of curcumin (5.07 μM). Both 10min and 20min samples appeared to have decreased concentrations compared to 5min Samples.

Figure 6.5 HPLC analysis of rat serum samples by *i.v.* route of curcumin and CMC2.24

Figure 6.5a Blood sample from rats with no curcumin or CMC2.24

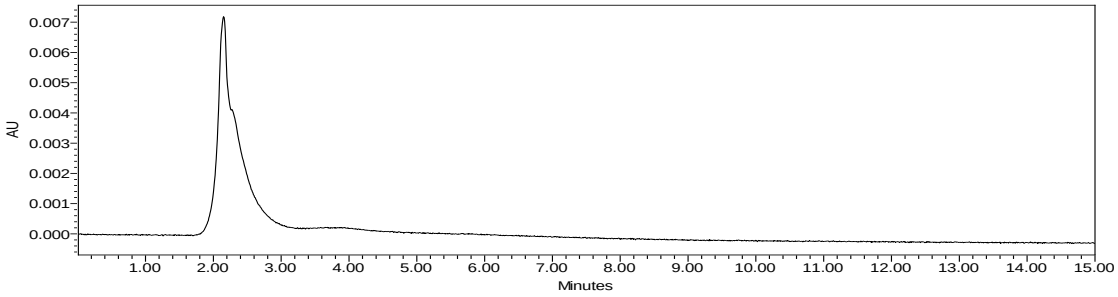


Figure 6.5b Blood sample from rats with *i.v.* adminstration of curcumin, 5min

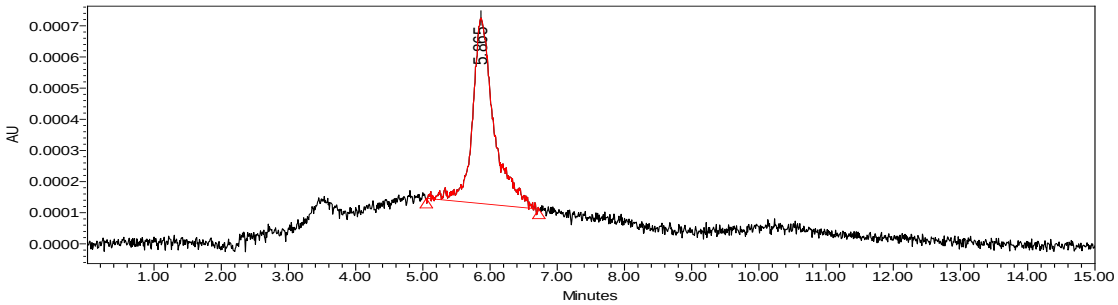


Figure 6.5c Blood sample from rats with *i.v.* adminstration of curcumin, 10min

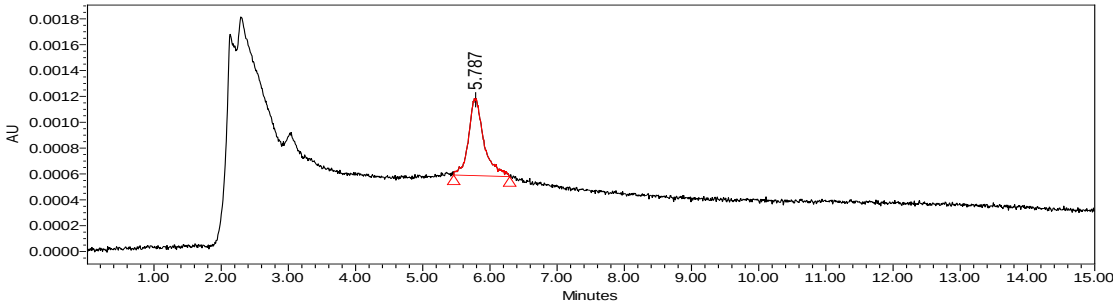


Figure 6.5d Blood sample from rats with i.v. adminstration of curcumin, 20min

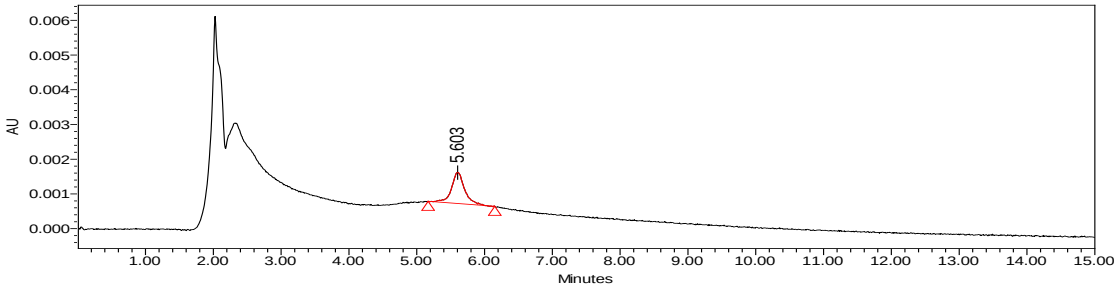


Figure 6.5e Blood sample from rats with i.v. adminstration of CMC2.24, 5min

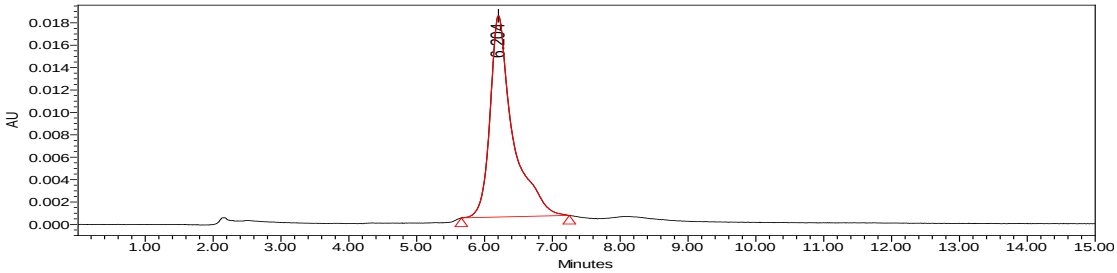


Figure 6.5f Blood sample from rats with i.v. adminstration of CMC2.24, 10min

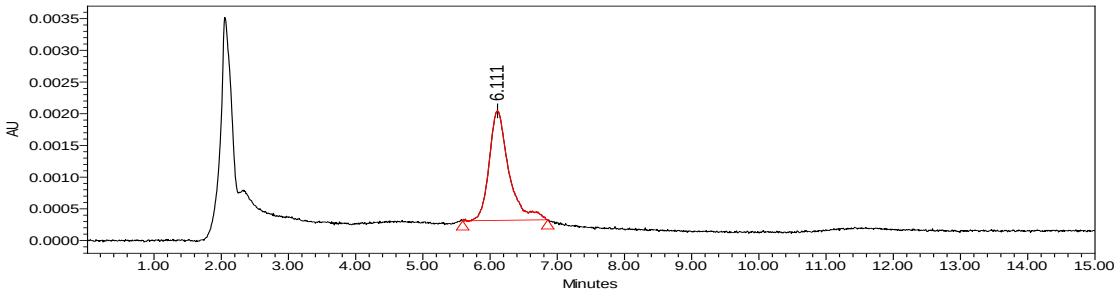


Figure 6.5g Blood sample from rats with i.v. adminstration of CMC2.24, 20min

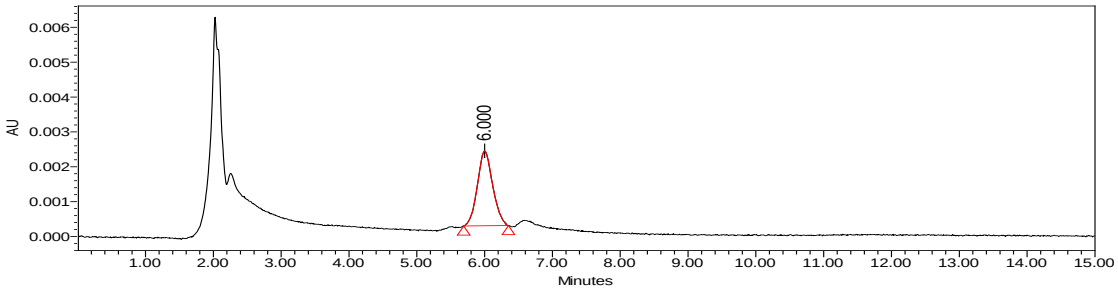


Table 6.3 *In vivo* pharmacokinetics of curcumin and CMC2.24 in 20min

Table 6.3a *In vivo* pharmacokinetics of curcumin in rat serum at 5min, 10min and 20min

*For Rat #2, there was no detection of compound, so the data are from the Rat #1 and Rat #3

	Rat	5min	10min	20min
Concentration of curcumin in serum(μg/mL)	1	0.264195	0.184707	0.051667
	2*	----	----	----
	3	1.865372	1.434979	0.172756
	AVE	1.064783	0.809843	0.112211
	S.D.	1.132202	0.884076	0.085623
	S.E.M.	0.653677	0.510422	0.049435

Table 6.3b *In vivo* pharmacokinetics of CMC2.24 in rat serum at 5min, 10min and 20min

	Rat	5min	10min	20min
Concentration of CMC2.24 in serum(μg/mL)	1	2.124512	2.058946	0.876829
	2	1.561866	0.844868	0.340222
	3	10.40994	1.575225	1.438222
	AVE	4.698774	1.493013	0.885091
	S.D.	4.954013	0.6112	0.549047
	S.E.M.	2.860201	0.352876	0.316992

Figure 6.6 *In vivo* pharmacokinetics of curcumin and CMC2.24 within 20min

Figure 6.6a *In vivo* pharmacokinetics of curcumin in rat serum at 5min, 10min and 20min

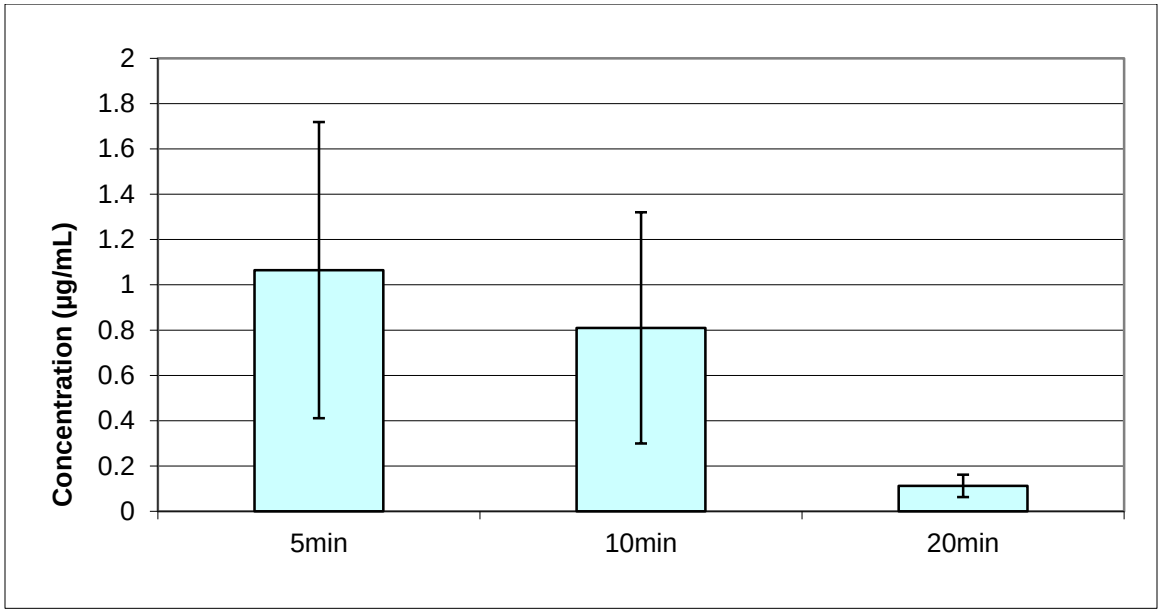
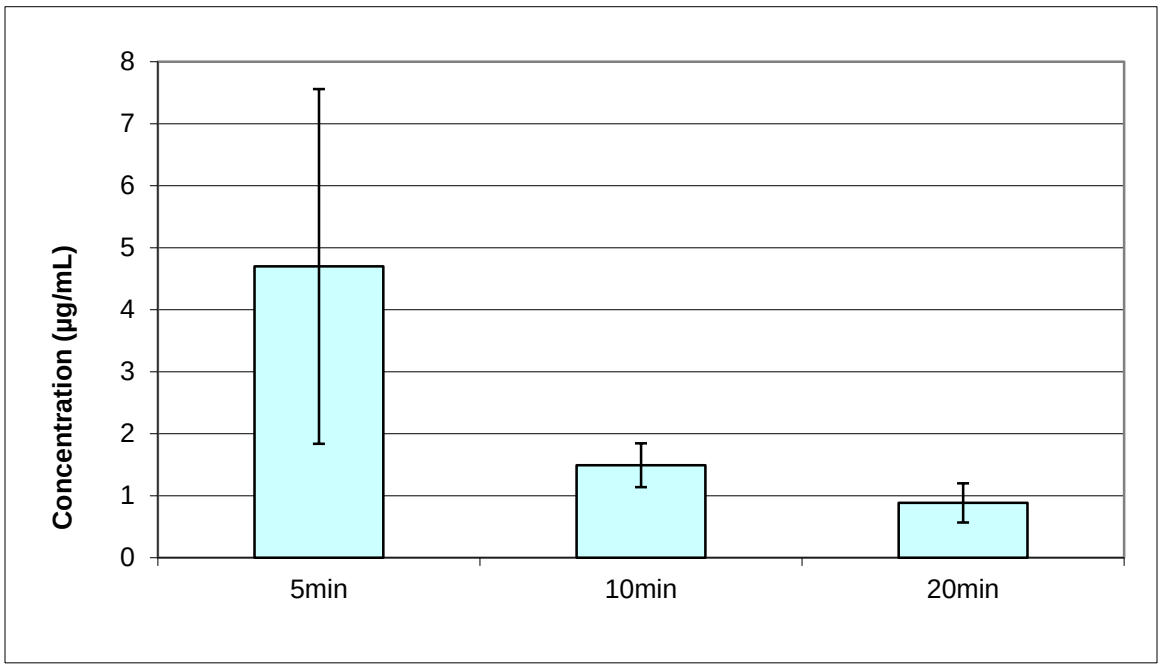


Figure 6.6b *In vivo* pharmacokinetics of CMC2.24 in rat serum at 5min, 10min and 20min



The concentrations of curcumin and CMC2.24 at 7 time points of 5min, 10min, 20min, 30min, 1h, 2h and 4h were shown in Figure 6.7, and then summarized in Table 6.4 and Figure 6.8. It appears that both these compounds have very short half-life within 30min. Pellets from rat blood (mainly blood cells), after removal of serum and washing three times by PBS buffer, were evaluated at these three time points, 5min, 10min and 20min. (Table 6.5, Figure 6.9) The data also indicate that only a relatively small amount of curcumin or CMC2.24 remains in the blood pellets. As for the 5min curcumin sample, about 1/5 of curcumin was found in the blood pellets (in μg/mL) compared to serum (in μg/mL) as well as CMC2.24. This finding contributes to a total blood distribution of curcumin and CMC2.24 in blood (not limited to serum).

Table 6.4 Summary of concentrations of curcumin and CMC2.24 in rat serum

	Curcumin(μg/mL)	CMC2.24(μg/mL)
5min	1.064783±0.653677	4.698774±2.860201
10min	0.809843±0.510422	1.493013±0.352876
20min	0.112211±0.049435	0.885091±0.316992
30min	0.026100±0.007832	0.707301±0.019258
1h	0.028193±0.008328	0.120582±0.052437
2h	0.002746±0.002746	0.024047±0.006054
4h	0	0

Figure 6.7 *In vivo* pharmacokinetics of curcumin and CMC2.24 in rat serum within 4h

Figure 6.7a *In vivo* pharmacokinetics of curcumin in rat serum within 4h

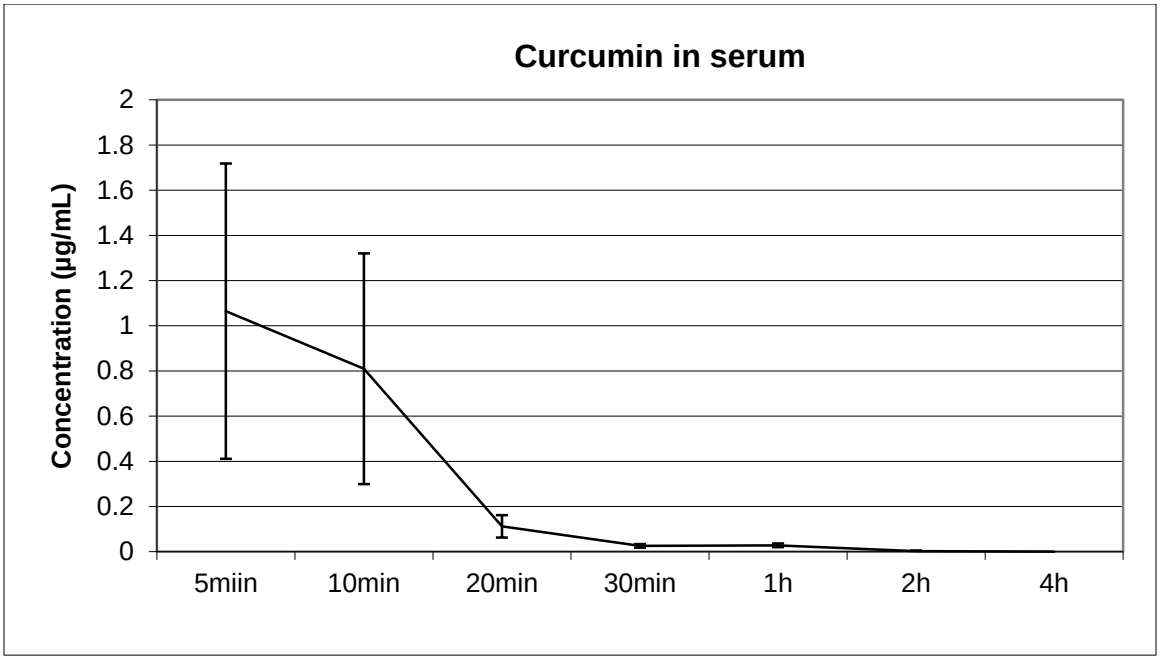


Figure 6.7b *In vivo* pharmacokinetics of CMC2.24 in rat serum within 4h

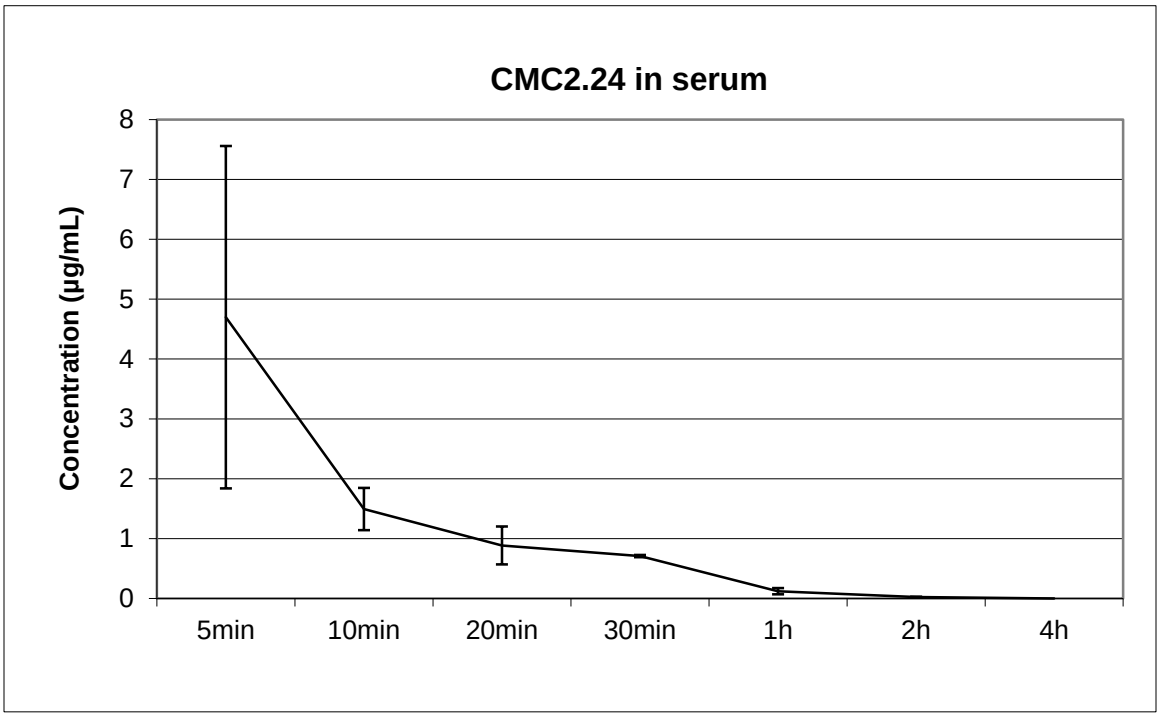


Figure 6.8 Comparison of curcumin and CMC2.24 in rat serum

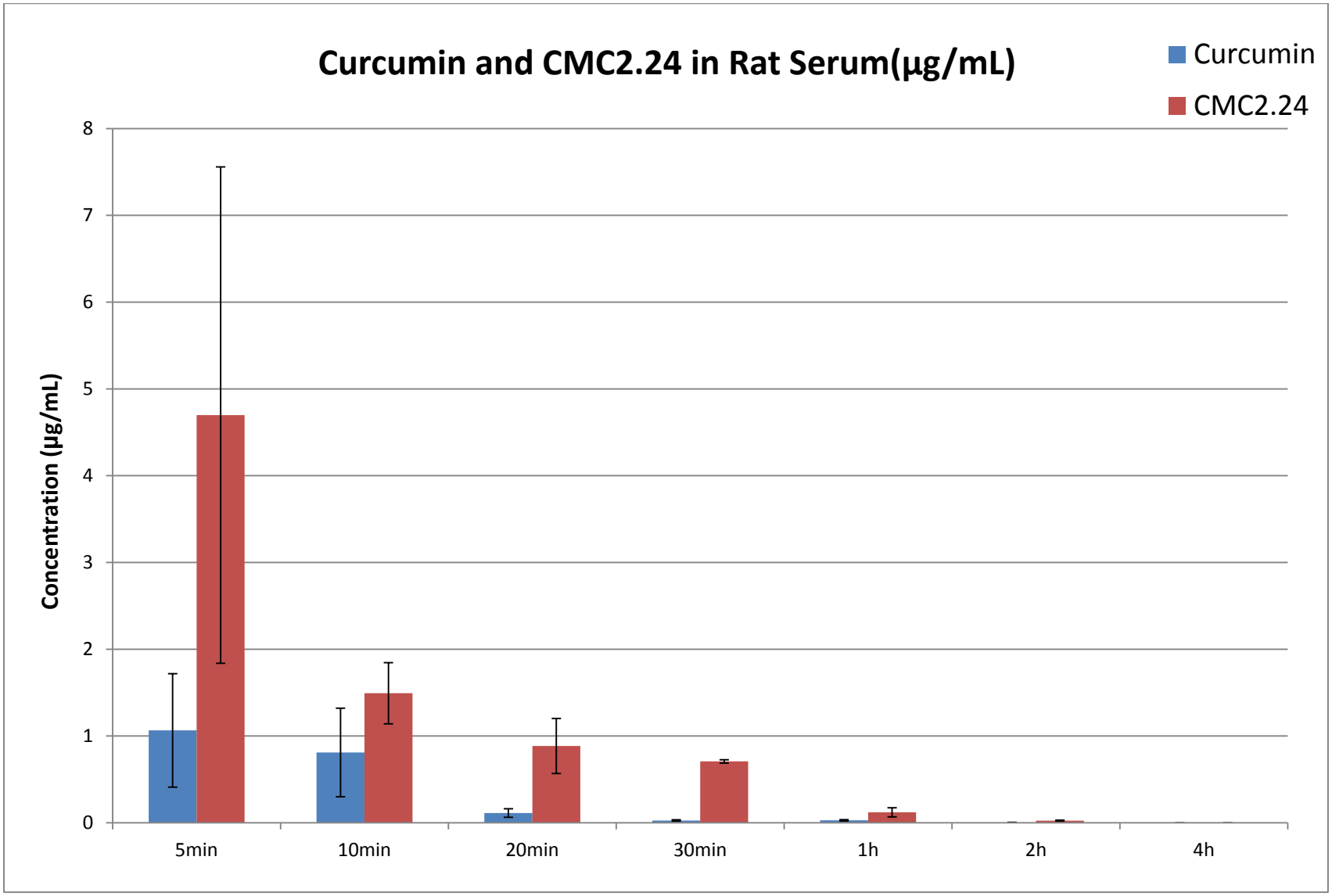


Table 6.5 *In vivo* pharmacokinetics of curcumin and CMC2.24 in rat blood pellets

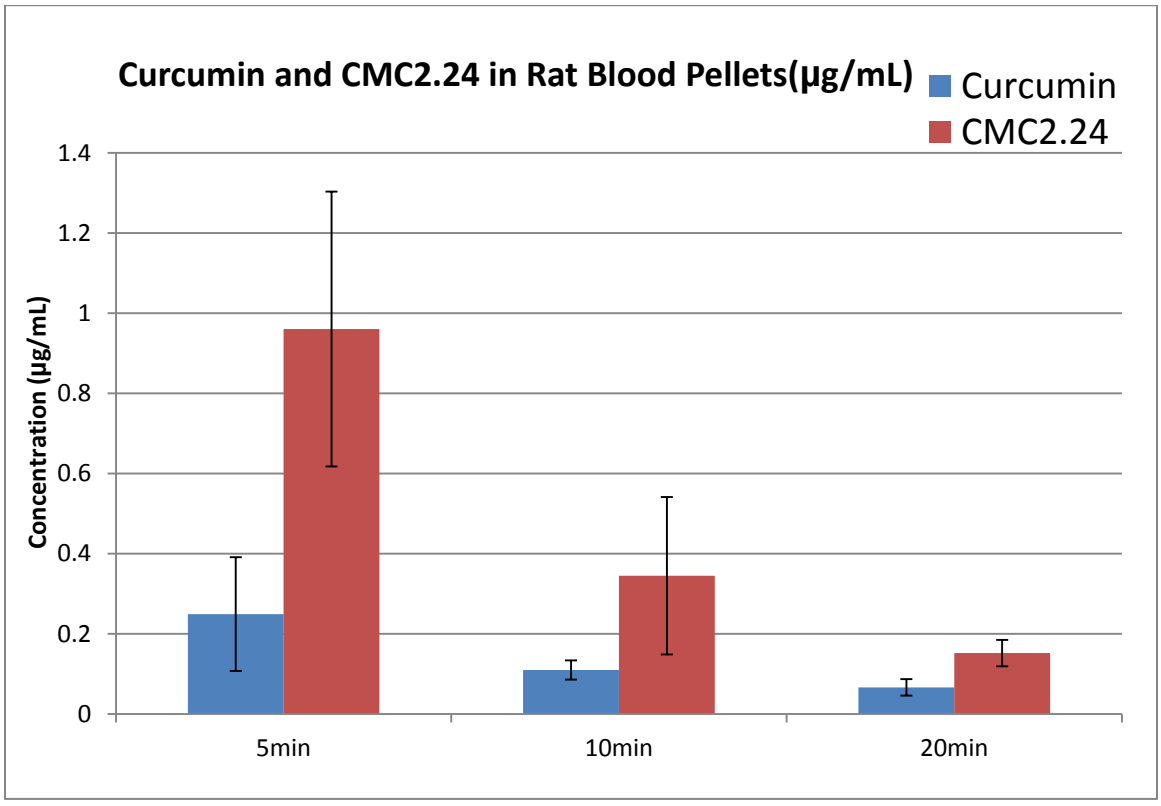
Table 6.5a *In vivo* pharmacokinetics of curcumin and CMC2.24 in rat blood pellets at 5min, 10min and 20min

		5min		10min		20min	
	Rat	Curcumin	CMC2.24	Curcumin	CMC2.24	Curcumin	CMC2.24
Concentration (μg/mL)	1	0.053853	1.077127	0.106198	0.074541	0.040018	0.146995
	2	0.168779	0.317158	0.070516	0.233616	0.052289	0.097605
	3	0.525262	1.487586	0.152776	0.727043	0.107348	0.211465
	AVE	0.249298	0.960624	0.10983	0.345067	0.066552	0.152022
	S.D.	0.245803	0.593848	0.04125	0.340229	0.03586	0.057096
	S.E.M.	0.141914	0.342858	0.023816	0.196431	0.020704	0.032964

Table 6.5b Comparison of curcumin and CMC2.24 in rat blood pellets at 5min, 10min and 20min

	Curcumin(μg/mL)	CMC2.24(μg/mL)
5min	0.249298 ± 0.141914	0.960624 ± 0.342858
10min	0.109830 ± 0.023816	0.345067 ± 0.196431
20min	0.066552 ± 0.020704	0.152022 ± 0.032964

Figure 6.9 Comparison of curcumin and CMC2.24 in rat blood pellets within 20min



Liver, heart, spleen, kidney, lung and brain were dissected from rats after 1 hour via i.v. injection of curcumin or CMC2.24. The samples were evaluated by HPLC, and the concentrations were calculated to μg/g for each organ. From the results obtained, it can be seen clearly that CMC2.24 has a significantly higher distribution in all the organs, compared to curcumin, generally about 1.5 folds. Also there are greater concentrations of both curcumn and CMC2.24 in spleen, compared to other organs including the heart and liver. It is also clear that the concentration of CMC2.24 in the lung is about 5 folds of that of curcumin, and also comparable to that of CMC2.24 in the spleen. Both curcumin and CMC2.24 showed low concentrations in brain tissue compared to other tissues at 1 hour time point. The results are shown in Table 6.6a-6.6b and Figure 6.10a-6.10b, and are summarized in Table 6.7 and Figure 6.11.

Table 6.6 *In vivo* pharmacokinetics of curcumin in rat organs

Table 6.6a *In vivo* pharmacokinetics of curcumin in rat organs including liver, heart, spleen, kidney, lung and brain at 1h after *i.v.* injection

Curcumin	Rat	Liver	Heart	Spleen	Kidney	Lung	Brain
Concentration (μg/g)	1	0.83041	2.841013	1.487927	0.403988	0.850426	0.095242
	2	0.012825	0.139508	2.468074	0.076898	0.307819	0.097746
	3	0.015486	0.152461	0.755557	0.014217	0.442845	0
	AVE	0.28624	1.044327	1.570519	0.165035	0.533696	0.064329
	S.D.	0.471267	1.555989	0.859241	0.2093	0.282482	0.055725
	S.E.M.	0.272086	0.898351	0.496083	0.120839	0.163091	0.032173

Table 6.6b *In vivo* pharmacokinetics of CMC2.24 in rat organs including liver, heart, spleen, kidney, lung and brain at 1h after *i.v.* injection

CMC2.24	Rat	Liver	Heart	Spleen	Kidney	Lung	Brain
Concentration (μg/g)	1	0.370603	1.253909	2.305669	0.50844	2.661765	0.099424
	2	0.729203	2.817656	2.588864	0.340268	1.503925	0.049081
	3	0.386824	1.620426	3.071122	1.347544	3.855265	0.106855
	AVE	0.495543	1.89733	2.655218	0.732084	2.673652	0.08512
	S.D.	0.202518	0.817822	0.387017	0.539596	1.175715	0.031431
	S.E.M.	0.116924	0.47217	0.223444	0.311536	0.678799	0.018147

Figure 6.10 *In vivo* pharmacokinetics of curcumin and CMC2.24 in rat organs

Figure 6.10a In vivo pharmacokinetics of curcumin in rat organs including liver, heart, spleen, kidney, lung and brain at 1h after *i.v.* injection

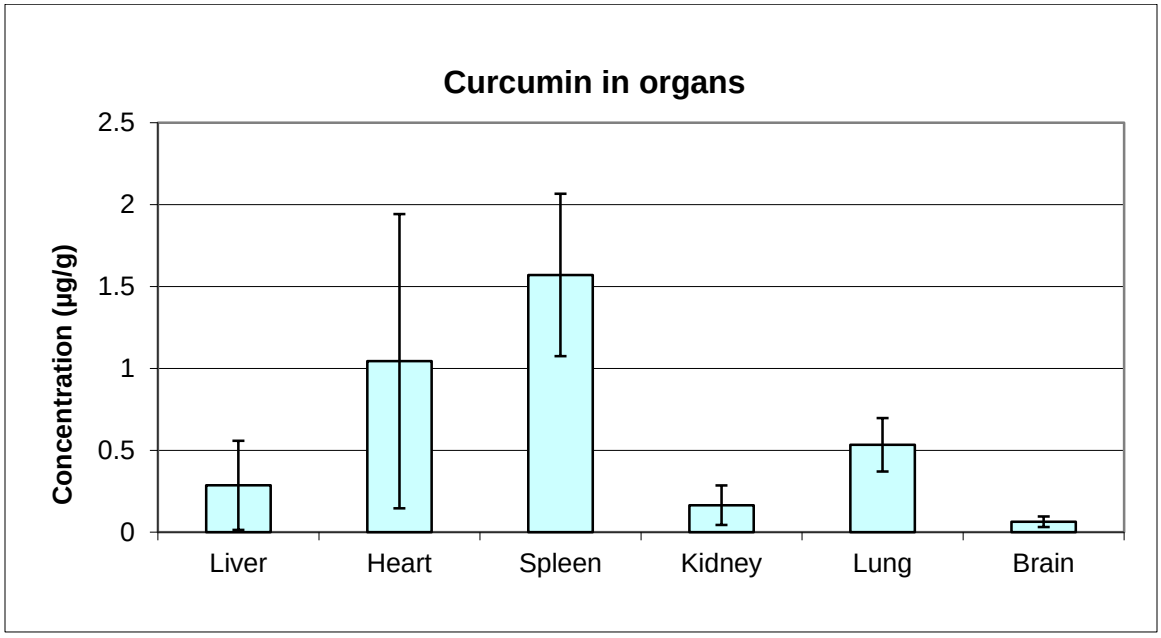


Figure 6.10b *In vivo* pharmacokinetics of curcumin in rat organs including liver, heart, spleen, kidney, lung and brain at 1h after *i.v.* injection

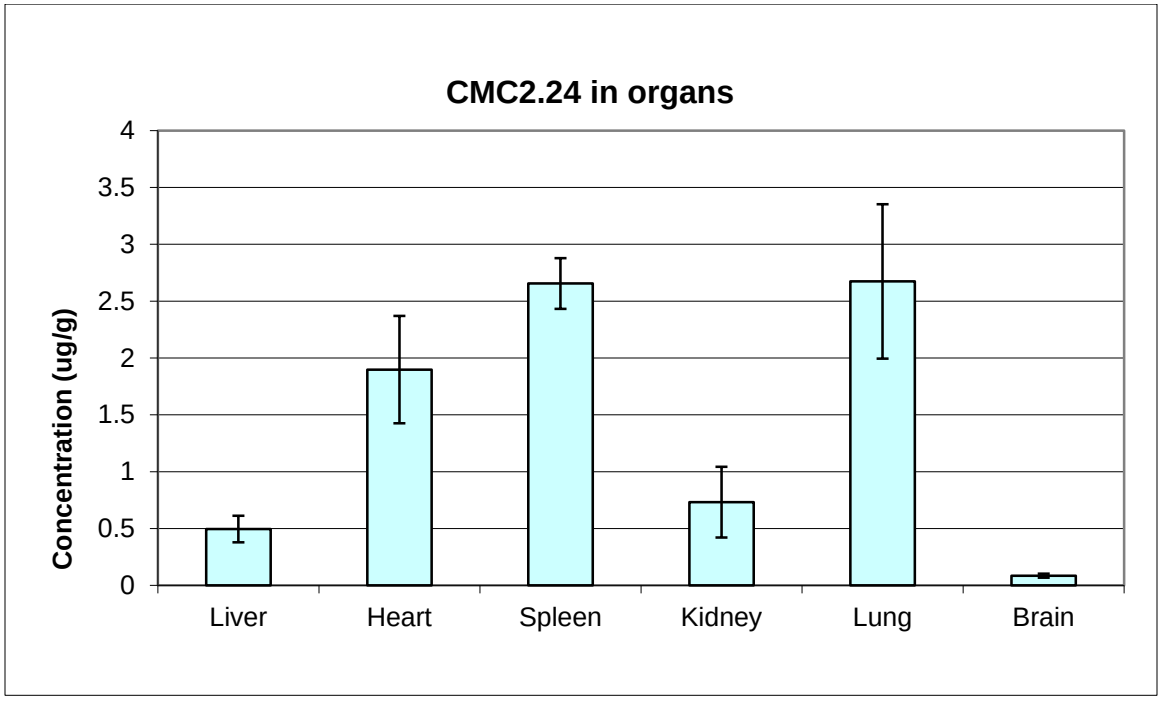
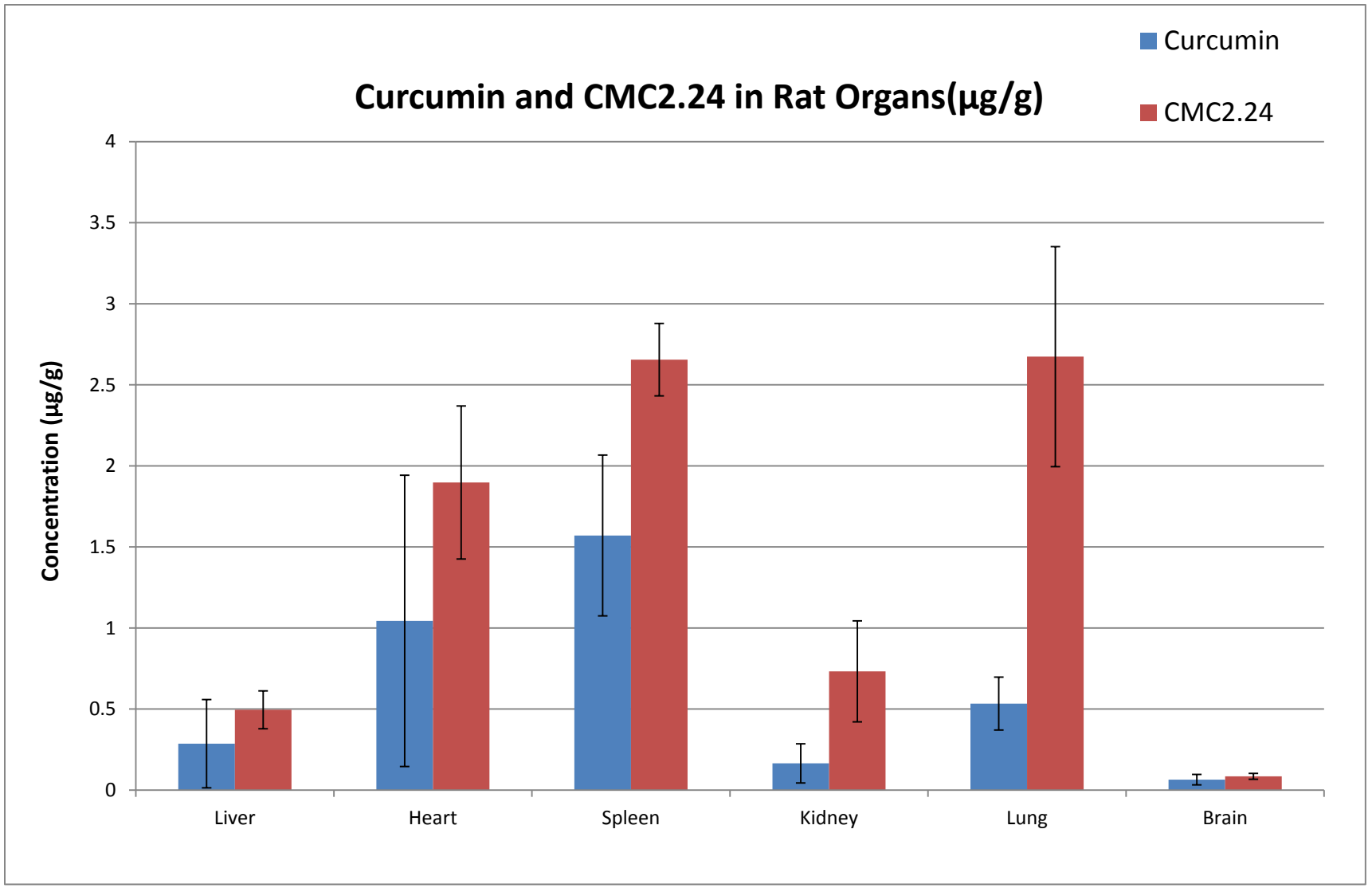


Table 6.7 Comparison of the distribution of curcumin and CMC2.24 in rat organs

	Curcumin(μg/g)	CMC2.24(μg/g)
Liver	0.286240±0.272086	0.495543±0.116924
Heart	1.044327±0.898351	1.897330±0.472170
Spleen	1.570519±0.496083	2.655218±0.223444
Kidney	0.165035±0.120839	0.732084±0.311536
Lung	0.533696±0.163091	2.673652±0.678799
Brain	0.064329±0.032173	0.085120±0.018147

Figure 6.11 Comparison of the distribution of curcumin and CMC2.24 in rat organs

Note: Liver, heart, spleen, kidney, lung and brain were dissected from rats after 1 hour via i.v. injection of curcumin or CMC2.24.



Materials and Methods

1. Standard curve

Curcumin and CMC2.24 were dissolved in Solultol HS 15 (BASF) and Ethyl alcohol (Fisher, 99.5%) (v/v =1:1) at a concentration of 100mM. Dilutions were made through Solultol 15 and ethanol mixture to 1 μM, 5 μM, 10 μM, 50 μM, where standard curves were extrapolated. 50 μL of each dilution was added to the extraction buffer containing 5mM oxalic acid in water, acetonitrile, and methanol with volume ratio of 2.5:1.5:1. Then each sample was centrifuged down at 10,000rpm for 10min, and 120 μL aliquot was taken for HPLC analysis. Both curcumin and CMC2.24 were eluted by HPLC for 20min with the mobile phase of 70% methanol and 30% distilled water eluting at 1.0mL per min, and the detection of peak was at 425nm. The retention time for curcumin and CMC2.24 are around 5.7min and 6.0min.Doxycycline was dissolved in 1X PBS buffer at concentration of 100mM, dilutions were made through PBS buffer to 50 μM, 10 μM, 5 μM, 1 μM, where the standard curve was extrapolated. Then each sample was centrifuged down at 10,000rpm for 10min, and 120 μL aliquot was taken for HPLC analysis. Doxycycline was eluted by HPLC for 20min with the mobile phase of 20% Methanol, 30%Acetonitrile and 50% 5mM Oxalic acid eluting at 1.0mL per min, and the detection of peak was at 362nm.

2. Serum analysis

Adult male Sprague-Dawley rats (297g average body weight, viral antibody free, Charles River Labs) were distributed into three groups: curcumin, CMC2.24 and doxycycline with three rats per group. Curcumin and CMC2.24 were dissolved in Solultol HS 15 and Ethyl alcohol (v/v=1:1) with the concentration of 40mg/mL, and doxycycline was dissolved in PBS buffer with the same concentration. 150 μL of Injection was made through tail vein to each rat (20mg/kg).

Blood was taken at time 5min, 10min, 20min, 30min, 1h, 2h and 4h from orbital sinus. Serum samples were collected and analyzed by HPLC C-18 reverse phase column. For curcumin and CMC2.24, the elution system is 70% Methyl alcohol in double-distilled water with the peak of curcumin appearing at 5.7min and CMC2.24 appearing at 6.1min, while for doxycycline, the elution system is 20% Methanol, 30% Acetonitrile and 50% 5mM Oxalic acid with the peak of doxycycline appearing at 3.2min.

3. Pellets analysis

200 μL of blood from rats was drawn at 5min, 10min and 20min after i.v. injection of curcumin and CMC2.24. Serum was removed and the pellets were washed three times by 200 μL of PBS buffer, then acetonitrile was added to each sample to reach a final volumn of 200 μL. 50 μL of supernatant, after vortexing and centrifuge at 10,000rpm for 10min, was added to extraction buffer containing 5mM oxalic acid in water, acetonitrile, and methanol with volumn raition of 2.5:1.5:1. 120 μL aliquot was taken for HPLC analysis. Both curcumin and CMC2.24 were eluted by HPLC for 20min with the mobile phase of 70% methanol and 30% distilled water eluting at 1.0mL per min, and the detection of peak was at 425nm.

4. Organ analysis

Liver, heart, spleen, kidney, lung and brain from rats by i.v. injection of curcumin and CMC2.24 were dissected after 1h of dosage. These organs were washed with PBS buffer for three times, and dried by blotting with filter paper. Wet tissue was homogenized in 5.0mL of acetonitrile for 4min by polytron. 0.3mL of the homogenate was added to 0.6mL of extraction buffer containing 5mM oxalic acid in water, acetonitrile, and methanol with volume ratio of 2.5:1.5:1. Then each sample was centrifuged down at 10,000rpm for 10min, and 120 μL aliquot was taken for HPLC analysis described above.

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Chapter 7. X-Ray Crystallography of the MMP-8 catalytic subunit with and without curcumin and CMC2.24 as inhibitors

A. Introduction

1. X-ray crystallography

X-ray crystallography as well as NMR spectroscopy is the advanced technology used to study the structural interactions between biologically-interesting molecules including proteins at an atomic level. Such information can help to elucidate the mechanisms of protein-protein or protein-inhibitor interactions.[1,2] The crystals that are needed for X-ray analysis would be obtained after protein expression, purification and crystallization under certain conditions with or without other bound molecules, including proteins, peptides, regulators or small drug molecules. These crystals are exposed to certain X-ray wavelengths in a beamline to obtain three-dimensional diffraction patterns from which the crystal packing symmetry aspects and repeating units can be determined, all of which contribute to understanding the physical properties of the crystals.[3,4] These data are processed by specific software such as Phenix Refinement to determine the crystal structures based on the electron density map. Figure 7.1 shows the general procedure of X-ray crystallography. Protein would be obtained after cloning (the process to create copies of DNA fragments), transformation (the genetic alteration of a cell resulting from the incorporation of exogenous DNA from its surroundings), expression (the process in which proteins are synthesized, modified and regulated in living organisms) and purification (the process intended to isolate a single type of protein from a complex mixture), and then crystallized in certain conditions, ready for X-ray diffraction and data process.

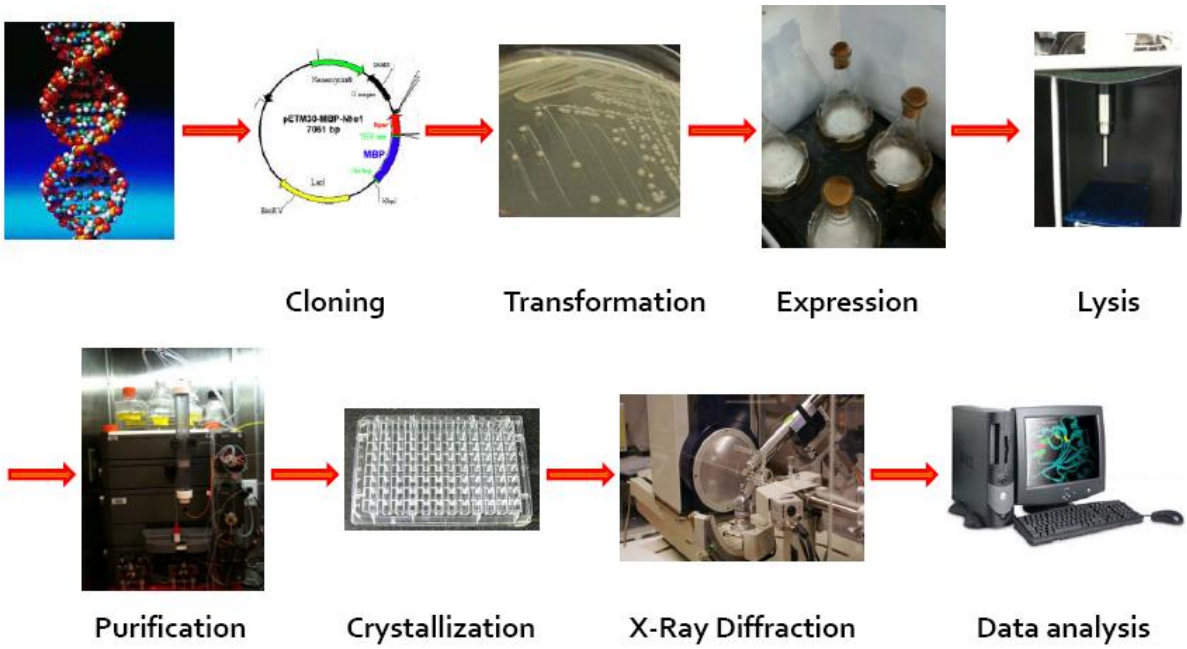

Figure 7.1 General procedures of X-ray crystallography

2. X-ray crystallography study of MMP-8

MMP-8, a neutrophil collagenase, possesses the ability to cleave the triple-helical domain of native fibrillar collagens I, II and III, generating the fragments of ¼ and ¾ length of a standard collagen monomer as mentioned in Chapter 2. Thus it is one of the collagenases of the matrix metalloproteinases family.[5,6] MMP-8, mainly produced by neutrophils, is crucial in a variety of pathological conditions related to inflammation and cancer because of its role in the regulation of immune response.[7,8] The glycosylated MMP-8 has a molecular weight of 75-80kD in the latent form. The catalytic subunit of MMP-8 lacking the hemopexin-like domain was first purified by Schnierer *et al*.[9], and since then the crystal structures of MMP-8 with a variety of inhibitors have been studied by X-ray crystallography.[10-24] The MMP-8 catalytic domain has a sphere-like structure consisting of a five-stranded β-sheet and two α-helices.[10-12] The two histidines and a catalytic glutamic acid residue of His197-Glu198-Xaa-Xaa-His201 (Xaa = acidic amino acid) comprise the zinc binding site, to the right of which sits the S1' pocket. Three amino acid

residues lie to the entrance to this pocket: Ile159, Leul60, and Ala161, and three other amino acid residues form the wall of the pocket: Tyr216, Pro217, and Asn218. Also in the interior of this pocket are the side chains of Tyr219-Tyr227, Leu214-APa213, and Leu193.[12] The crystal structure of the MMP-8 catalytic domain (Met80 to Gly242) bearing one hydroxmate inhibitor, namely Pro-Leu-Gly-NHOH, was first studied by Bode *et al.*[10] From their crystal structure (2.0Å resolution), penta-coordination with the catalytic zinc atom is formed by the two oxygens from the hydroxamic acid inhibitor as well as three histidines His197, His201 and His207 (Note: The example of penta-coordination with zinc is not known in inorganic chemistry, thus the assignment is questionable).[10-13]

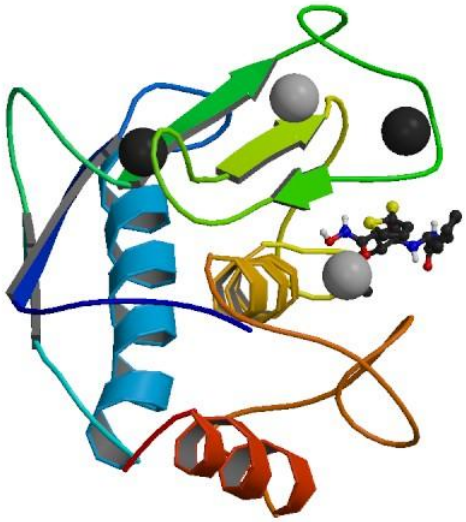

Figure 7.2 Crystal structure of MMP-8 catalytic domain with Batimastat

Note: This figure is adapted from Protein Data Bank (PDB) code: 1MMB. Five-stranded β-sheet and two α-helices are shown in the figure with two calcium atoms are shown black and two zinc atoms are shown in white. The inhibitior, Batimastat is interacting with the catalytic zinc atom.[13]

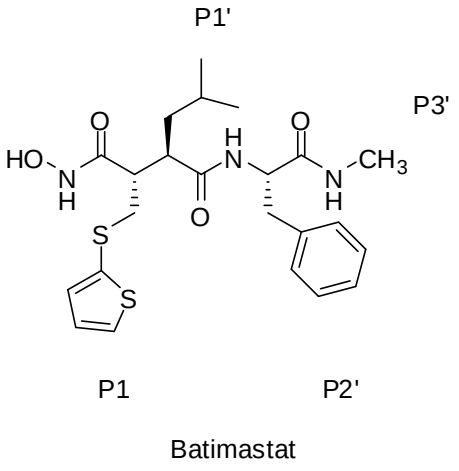

Figure 7.3 Structure of Batimastat (BB-94)

Batimastat (BB-94), {4-(N-hydroxyamino)-2(R)-isobutyl-3(S)-[(2 thienylthiomethyl) succinyll-L-phenylalanine-N-methylamide}, one of the most potent MMP inhibitors so far, was developed by British Biotech and evaluated in X-ray crystallography studies.[13] This hydroxymate inhibitor possesses the IC_{50} of 10nM against MMP-8.[14] Besides the penta-coordination between the catalytic zinc with its oxygens as well as the three histidine residues as mentioned above, the residues from Batimastat also displayed a strong interaction with MMP-8 in the pockets of catalytic domains. The P1' residue is the hydrophobic moiety of the compound (Figure 7.2), and it associates with the S1' pocket of MMP-8 catalytic domain. The P2' residue relates with the residues of Gly158, Ile159, Pro217 and Am218. The P3' residue was found to be responsible for solvent exposure (The solvent exposure of an amino acid in a protein defines as the extent to which the amino acid is accessible to the solvent, usually water, surrounding the protein).[13] Several other inhibitors have been crystallized together with MMP-8 catalytic domain, including phosphonate, carboxylate, peptide and non-zinc binding inhibitors.[15-24] Since curcumin and CMC2.24 are potent inhibitors of MMPs from the data discussed in Chapter 4, it is important to study the binding at the molecular level by X-ray crystallography. Although doxycycline as the MMP inhibitor was approved by the FDA at sub-antimicrobial doses for the treatment of

periodontal inflammation and chronic skin disease, the mechanism at the molecular level has still not been elucidated despite the proof by Golub *et al*.[24] that zinc binding is involved. It is important to attempt to define how the inhibitor binds in the catalytic pocket of the MMPs beyond the attachment to the zinc atom. Also the crystal structure of MMP-8 with curcumin and its analogues will definitely improve the further design and development of more specific inhibitors for different MMPs, based on the specific interactions between the inhibitors and their protein targets.

B. Recombinant protein expression with His-tag

The MMP-8 catalytic domain purification method reported in the literature utilizes a hydroxamate affinity column. However, after reviewing this method, the alternate method using recombinant protein, with a His-tag was chosen because of the experience of this procedure in our laboratory. Recombinant protein expression with polyhistidine residues have been developed during the past decades, whereby immobilized metal-affinity chromatography using Co^{2+}, Ni^{2+}, Cu^{2+} or Zn^{2+} ions was introduced for protein purification.[25,26] Because histidine can bind to the immobilized metal ion resins through its electron-donating imidazole group, it has the ability to retain the protein on the pre-loaded metal-affinity column. The pure protein then can be eluted out by free imidazole solution at a different pH after the elution of other protein fractions. The primary structure is given below for the catalytic domain of MMP-8.[9] Metal ions binding involved are as follows: H197, H201, H207 for catalytic zinc (Zn999); H147, D149, H162, H175 for structural zinc (Zn998); D154, G155, N157, 1159, D177, E180 for calcium site Ka997; D137, G169, G171, D173 for calcium site Ka996.

80
M L T P G N P K W E R T N L T Y R I R N Y T P Q L S E A E V E R A I K D A F E L W
121
S V A S P L I F T R I S Q G E A D I N I A F Y Q R D H G D N S P F D G P N G I L A H
162
A F Q P G Q G I G G D A H F D A E E T W T N T S A N Y N L F L V A A H E F G H S L
203
G L A H S S D P G A L M Y P N Y A F R E T S N Y S L P Q D D I D G I Q A I Y G

Figure 7.4 Primary structure of MMP-8 catalytic domain

PCR reaction was carried out by MMP8R255FWD (primer 1: 5'-GATGAAAAAGCCTCGCTGTG-3') and MMP81037REV (primer 2: 5'-CAGAGCCCAGTATTGGTTGC-3'). After purification by QIAGEN MINElute purification kit, the PCR product was then cloned into a pET-7 vector (Novagen) using the 5' NdeI and 3'Xhol restriction enzymes, so that a His-tag was encoded at the N-terminus. Protein with a His-tag was obtained after the insertion into a plasmid, digestion, ligation, transformation and expression in *E. coli*. Cells were harvested by centrifugation, and the cell pellets were resuspended and sonicated to remove the cell debris, followed by centrifugation. The clear supernatant was then loaded onto a His-bind column (1.5cm x 15cm) containing 4mL His-bind resin (Novagen) that had been charged with 10mL of buffer (Ni^{2+}). The eluted protein fractions were then collected and concentrated (Figure 7.5). TEV (tobacoo etch virus) protease was added to the desired protein fraction and left overnight in order to remove the His-tag. Following this the protein fraction was loaded on to a Q-Sepharose ion-exchange column because it carries a negative charge at this pH (pH=6.0), and the purity of the eluted fractions was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after Fast protein liquid chromatography (FPLC). However, the separation was not achieved, as can be seen from Figure 7.6, even after four additional purifications on a Q-Sepharose column. A second concern was the

low yield (about 30%) after the attempted purification. Thus, this method was not pursued further.

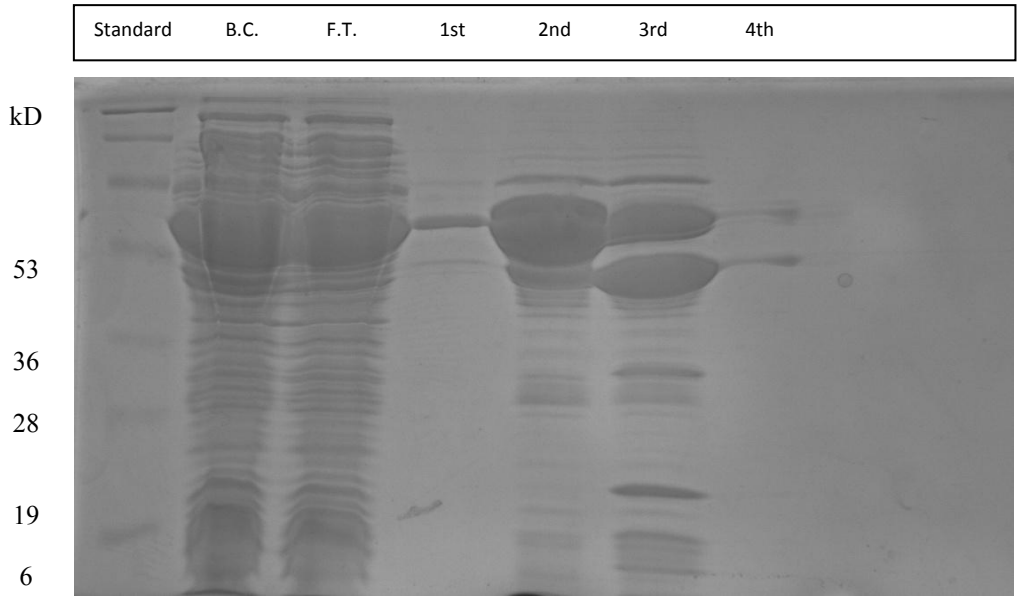

Figure 7.5 SDS gel of MMP-8 catalytic subunit after nickel column purification

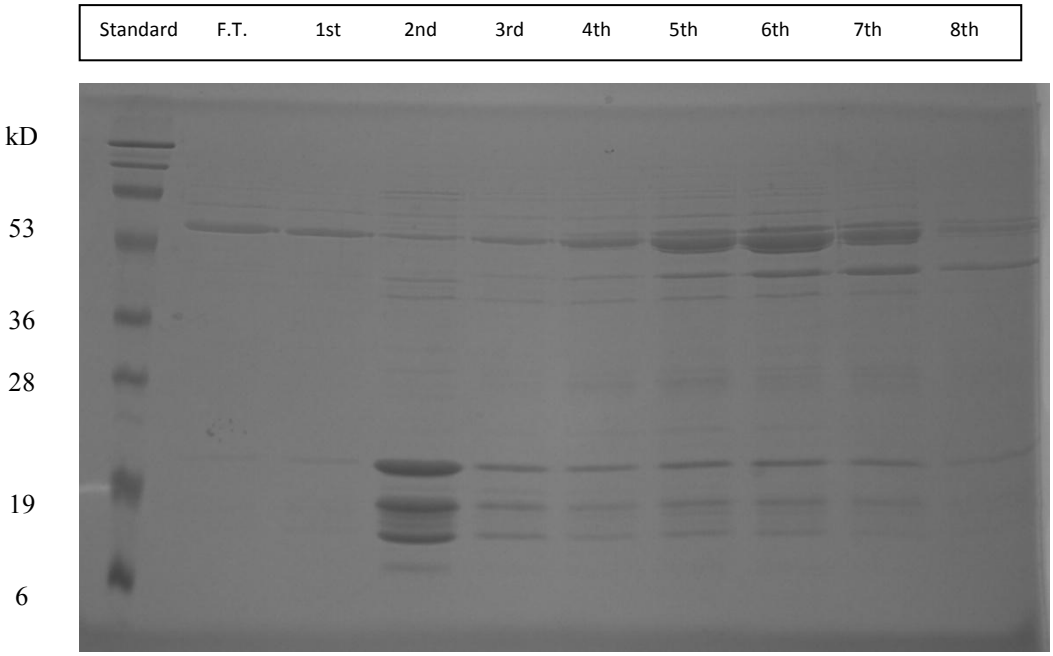

Figure 7.6 SDS gel of MMP-8 catalytic subunit after Q-Sepharose purification

C. Recombinant protein expression without His-tag

Since the protein expression with His-tag didn't give either a desirable yield or purity, a recombinant protein expression without His-tag attempted. Protein can be denatured by high concentrations of urea (above 6M), and thereafter dialyzed to refold the three-dimensional natural structure. The digestion of the plasmid insert was first transformed to the vector, namely pET-22b(+), and then transferred into TOP-10F' competent cells by electroporation. Expression of the MMP-8 catalytic subunit was carried out in *E.coli* strain BL21(DE3), and inclusion bodies were isolated. Pellets were resuspended, sonicated, and then dissolved in 6M urea to denature the protein. The stock protein solution was diluted to 1mg/mL and then dialyzed to refold to the three-dimensional structure in the absence of urea. Refolding was confirmed by Circular Dichroism, which indicated the reformation of the secondary structures, the α-helix and the β-sheet which corresponds to the native protein, as shown in Figure 7.7. The purity was determined by 15% SDS-PAGE (Figure 7.8) and MALDI (Figure 7.9). In Figure 7.8, the lower band is the *E. coli* heat shock protein with the MW of 16.0kD, and the upper band is the MMP-8 catalytic subunit with the MW of 18.5kD. The MMP-8 catalytic subunit was concentrated by centrifugation and loaded onto a superdex gel filtration column to remove the *E. coli* heat shock protein because of their differences in molecular weight. (Figure 7.10) The MMP-8 activity assay was also carried out for the MMP-8 catalytic subunit, which showed collagenase activity in its ability to degrade the fluorogenic substrate, Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 which was used in the *in vitro* inhibition assay as mentioned in Chapter 4. The MMP-8 catalytic subunit, the major peak in Frigure 7.8, was collected and concentrated for crystallization.

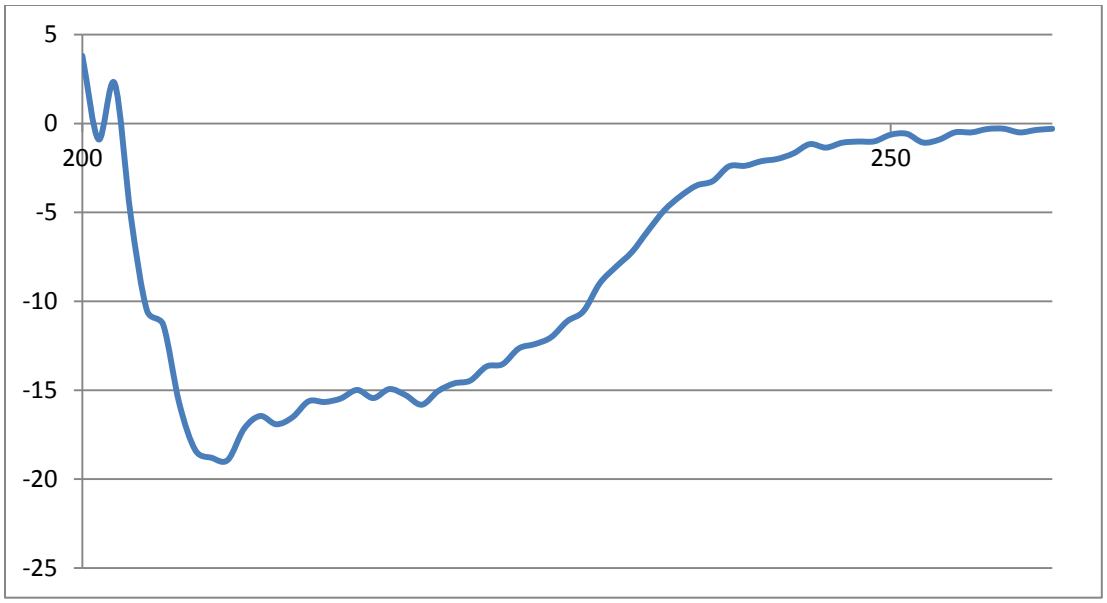

Figure 7.7 Circular Dichroism determination of MMP-8 refolding after dialysis

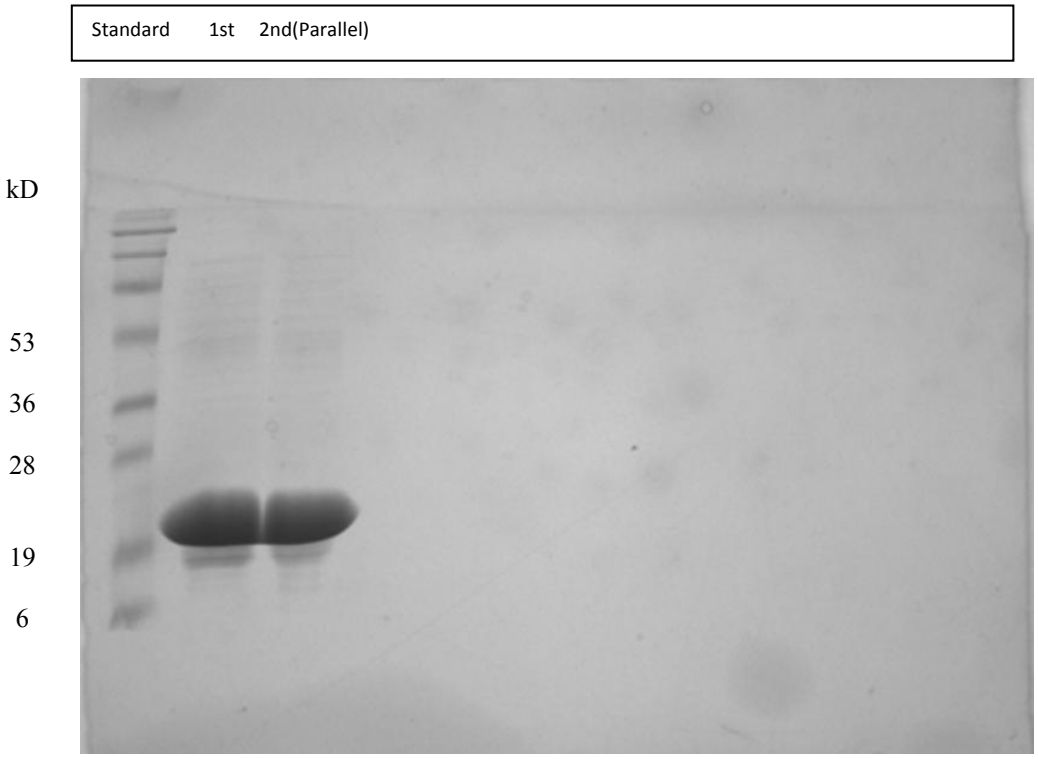

Figure 7.8 SDS gel of MMP-8 after dialysis

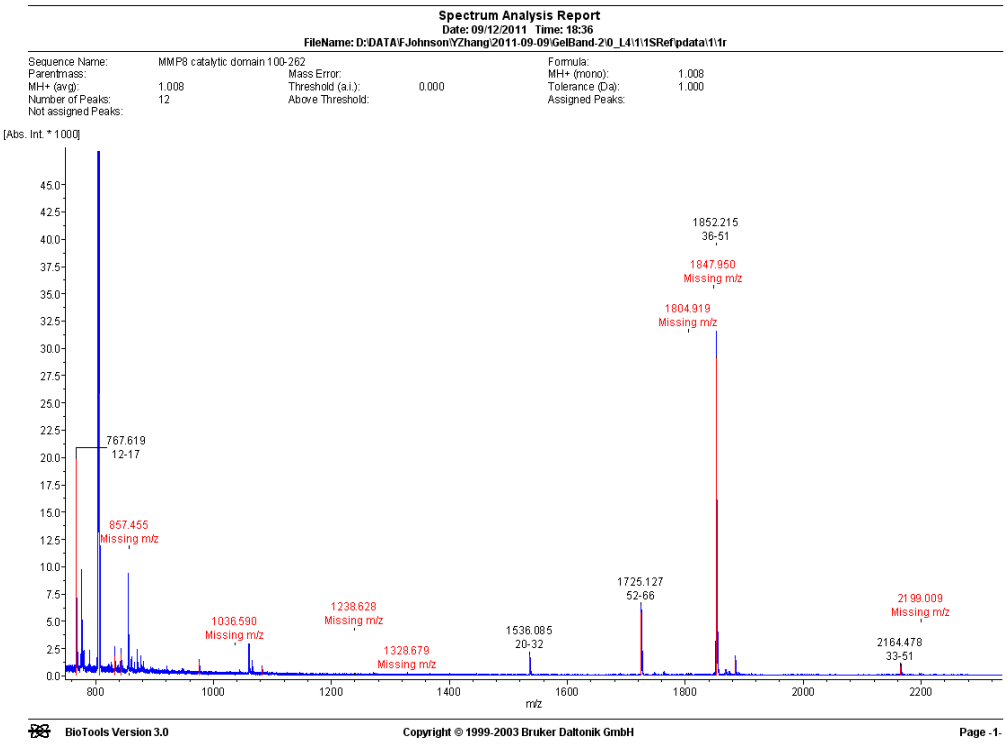

Figure 7.9 MALDI determination of MMP-8 after dialysis

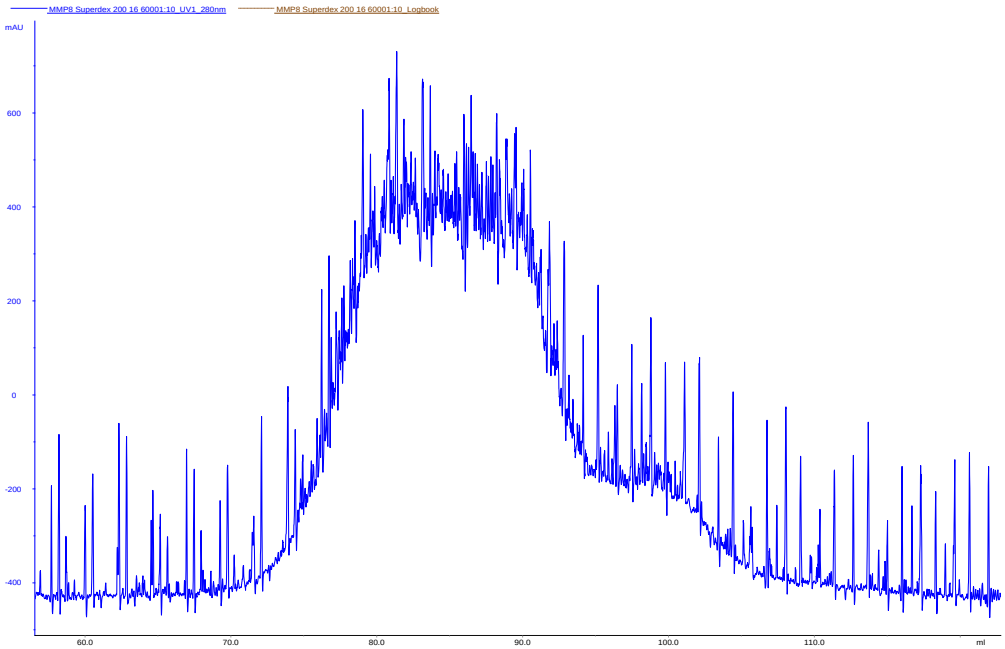

Figure 7.10 Gel filtration after dialysis

D. Crystallization of MMP-8 with or without the inhibitor present

The purified MMP-8 catalytic subunit was crystallized using the hanging drop method.[16,17] A 3-fold molar excess of curcumin or CMC2.24 (50mM) in DMSO was added to a solution of 12mg/mL MMP-8 catalytic subunit in the buffer containing 25mM MES, 100mM NaCl, 10mM $CaCl_2$, 0.1mM $ZnCl_2$ (pH=6.0) to give a total volume of 4 μL. The solution contained 10-25% (w/w) PEG6000, and was equilibrated against a 500 μL reservoir solution of 1M Na_2HPO_4/NaH_2PO_4 at pH 6.0. Parallel experiments were also carried out for the protein alone without any inhibitors to obtain the density map for structural analysis. Needle-shaped crystals appeared in 4 days. Crystal X-ray data were collected at 1.3-2.5Å on Beamline 25 from the National Synchrotron Light Source (NSLS) at Brookhaven Lab. From the results shown in Figure 7.11 and 7.12, the crystal structure without inhibitors showed a similar density in the catalytic zinc-binding domain as those harboring an inhibitor (either curcumin or CMC2.24). This strongly indicates that the protein itself needs further purification to remove what appears to be the endogenous inhibitor (peptide or protein fragment) in the catalytic domain of MMP-8 to allow the synthetic inhibitor, curcumin and CMC2.24, to bind the catalytic domain. A recent study (unpublished data) by Dr. Sanford R. Simon shows that Triton buffer (used as one component in the buffer for the MMP-8 catalytic subunit expression) could bind to the elastase and prevent the inhibitor from binding. Thus this fragment in the crystal structure of MMP-8 catalytic subunit might be the molecule from the buffers used for protein expression or purification.

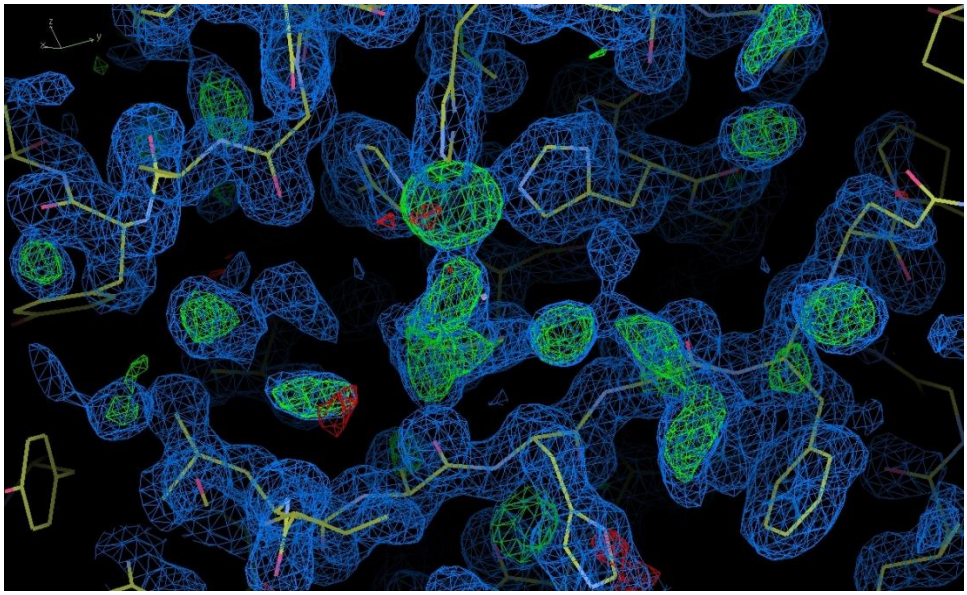

Figure 7.11 Density map from the X-ray data collection without any inhibitor

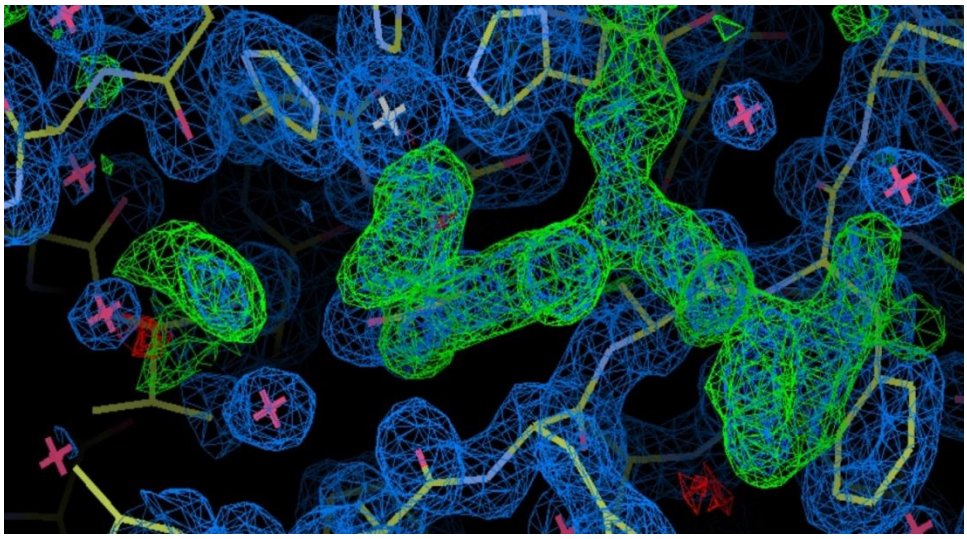

Figure 7.12 Density map from the X-ray data collection with an inhibitor CMC2.24

Note: The above figures seem the same, which strongly indicates that the protein itself needs further purification to remove what it appears to be as the endogenous inhibitor (or fragment) in the catalytic domain of MMP-8 to allow the synthetic inhibitor, curcumin and CMC2.24, to bind the catalytic domain.

Materials and Methods

1. Cloning and protein expression of MMP-8 in an MBP fusion system

The cDNA of MMP-8 was purchased from Thermo Scientific. For the sub-cloning of the 209bp fragment which corresponds to the 18.5kDa MMP-8 catalytic subunit, forward oligomers (5'-GATGAAAAAGCCTCGCTGTG-3') and reverse oligomers (5'-CAGAGCCCAGTATTGGTTGC-3') were used as primers. The PCR reaction contained 1X PFU buffer [200mM Tris-HCl (pH=8.8 at 25 ℃), 100mM $(NH_4)_2SO_4$, 100mM KCl, 1% (v/v) Triton X-100, 1mg/ml BSA](5 µL), 2.5mM dNTPs (5 µL), pfu (1 µL), cDNA of MMP-8(1 µL) forward and reverse oligomers (5 µL) at 100 µM and H_2O (28 µL) to a total of 50 µL .The 209bp fragment was amplified by polymerase chain reaction (PCR). After amplification the PCR product was purified by means of a QIAGEN MINElute purification kit and then digested with NdeI and Xhol restriction enzymes at the 5' and 3' end respectively, and thereafter purified by electrophoresis on agarose gel. The expression vector pET7-HMBP3 and the PCR product were digested in the following reaction: pET7-HMBP3 (7 µL), NdeI (1.5 µL), Xhol (1.5 µL), New England Biolabs (NEB) buffer 2 (5 µL) and H_2O (35 µL) at 37^oC, for 3 hours. The digestion was run on 1% agarose gel stained with ethidium bromide. The PCR and the pT7-HMBP3 were then cut out and purified by QIAGEN QIAQUICK Gel Extraction kit. The insert (PCR product) (5ng/ µL) and the vector (10.5ng/ µL) were used in ligation reactions of ratios 1:3, 1:7 and 1:10 in a T4 ligase Buffer (1 µL), containing T4 ligase (1 µL) and H_2O (6 µL). The ligation reaction mixture was then transfected into TOP10F' *E.coli* competent cells by electroporation. With the 1:7 ratio ligation reactions, a subcloned pT7-HMBP3 vector containing the MMP-8 catalytic subunit was obtained. The positive colonies containing the MMP-8 catalytic subunit were grown in order to amplify the DNA necessary for protein expression. The 209bp fragment was then

subcloned into pT7TEV-HMBP3 vector (Novagen) adjacent to the maltose binding protein (MBP) under the control of a T7 promoter, where MBP is responsible for the increase of the solubility of recombinant proteins expressed in *E. coli*. MMP-8 was expressed in (DE3)-RIL arctic express *E.coli* cells as a fusion protein with MBP containing a tobacco etch virus (TEV, used for the removal of affinity tags from purified proteins) protease restriction site. The pT7TEV-HMBP3 vector containing the MMP-8 gene was then transformed in DE3 arctic express cells which were plated on kanamycin-containing lysogeny broth (LB) plates. After 24 hours, one of the colonies that grew was selected for growth in a 300mL LB culture containing 100 µg/ml of kanamycin. This pre-culture was grown overnight, and 20mL were then used to inoculate a 1.5L 2XYT medium. A total of 12 flasks (1.5L each) were grown at 37^{o}C up to 0.8 O.D and then were induced for the protein expression with isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were grown at 14^{o}C for 18 hours. The cells were then harvested by centrifugation and frozen at -80^{o}C.

2. Nickel NTA chromatography for purification of the MBP-fusion protein

The first purification strategy selected was to bind the MBP-MMP-8 fusion protein containing six histidine residues onto a Nickel-NTA column. This step removes most of the impurities from *E.coli* and allows the purification to continue using the isoelectric point of MMP-8. The cell pellets were resuspended in 30mL of lysis buffer (5% Glycerol, 20mM imidazole, 1M KCl, 20mM Tris-HCl, pH 7.0). In order to degrade further the cell walls, they were sonicated for 4min at 0^{o}C. Cell debris was removed by centrifugation at 20,000rpm for 30min at 4 ℃ and the clear supernatant was loaded onto a Nickel-NTA (nitrilotriacetic acid) column 4mL (1.5cm x 15cm). Before loading the lysate, the Nickel column was equilibrated by 5% glycerol, 20mM imidazole and 0.5M KCl buffer. After adsorption on the column the protein was then eluted by using buffer

containing 5% glycerol, 0.5mM imidazole, 0.5M KCl, and 20 mM Tris-HCl, at pH 7.0. After the protein was eluted TEV (2.5mg) protease was added to the protein solution in order to separate MBP from the MMP-8 catalytic subunit.

3. Q-Sepharose for purification of the MMP-8 catalytic subunit

After the MBP-MMP-8 fusion protein had been cleaved with TEV, the protein solution was loaded to a Q-Sepharose ion exchange column based on the isoelectric point (IP) of MMP-8 (-8.9) at pH 7.0, where the charged group of Q-Sepharose is a quarternary amine which carries a non-titratable positive charge, in order to separate the MMP-8 catalytic subunit which carries a negative charge at pH 7.0. The protein fraction was loaded onto a Q-Sepharose (1.5cm x 55cm) fast protein liquid chromatography (FPLC) column and the protein was eluted in a linear gradient by means of buffer A (5% Glycerol, 1mM DTT and 20mM Tris/HCl, pH 7.0) then buffer B (5% Glycerol, 1mM DTT and 1M KCl and 20mM Tris/HCl, pH 7.0). The purity of MMP-8 catalytic subunit was determined by 15% SDS-PAGE which gave a molecular weight as 18.5kDa. The yield of this protein expression method is only about 10%, and the purity is only 30% accordingly.

4. Cloning and protein expression of MMP-8 in pET-22b(+) vector

Because the protein expression with His-tag didn't give the desired yield or purity, the recombinant protein expression without MBP was carried out. By using the same primers as before, the MMP-8 catalytic subunit was cloned into pET-22b(+) vector under the control of T7 promoter. The protein expression was accomplished in BL21(DE3) for 4 hours at 37^{o}C after IPTG.

5. Protein purification of MMP-8 catalytic subunit by purification of inclusion bodies

In order to purify the inclusion bodies pellets which contain the MMP-8 catalytic subunit and are found in the cell debris, the *E.coli* cells was resuspended in a 1.5L flask and sonicated for 4min in the following buffer: 50mM Tris/HCl (pH=8.0), 10mM DTT and 100mM NaCl. The lysate was then centrifuged and spun-down. The material that was collected, was washed by 30mL of 50mM Tris/HCl (pH=8.0), 1%Triton X-100, 100mM NaCl and 1mM DTT and then washed three times with 50mM of Tris/HCl (pH=8.0), 100mM NaCl and finally 1mM DTT. In order to denature the protein after the washing procedures, the pellets were dissolved in 50mL of 6M urea, 50mM Tris/HCl (pH=8.0), 200mM NaCl, 5mM $CaCl_2$, 0.1mM $ZnCl_2$ and 1mM DTT and held at 37^{o}C for 4 hours. To renature the protein, the solution was diluted to 1.5mg/mL, and then 10mL of the protein solution was dialysed for 2 days against 4L of 50mM Tris/HCl (pH=7.5), 200mM NaCl, 5mM $CaCl_2$, 0.1mM $ZnCl_2$ and 0.005% Brij-35 buffer at 4^{o}C. This buffer was changed twice. Refolding of the MMP-8 catalytic subunit was confirmed by Circular Dichroism (CD).

6. Gel Filtration Chromatography

After the dialysis, the MMP-8 catalytic subunit was concentrated by centrifugation and loaded onto a Superdex 200 gel filtration column 16/60, pre-equilibrated with buffer of 25mM MES (pH=6.0), 100mM NaCl, 10mM $CaCl_2$, and 0.2mM $ZnCl_2$. The eluted fractions from the gel filtration were collected and the purity was determined by 15% SDS-PAGE and MALDI. The protein solution was concentrated to 12mg/mL for use. The yield is about 80% in total, and the purity is about 95%.

7. Crystallization

The purified MMP-8 catalytic subunit was crystallized by using the hanging drop method.[16,17] A 3-fold excess of curcumin or CMC2.24 (50mM) in DMSO was added to a solution of 12mg/mL of the protein (MMP-8 catalytic subunit) in buffer (25mM MES, 100 mM NaCl, 10mM $CaCl_2$, 0.1mM $ZnCl_2$, pH 6.0) to give a total volume of 4 μL. The solution contained 10-25% (w/w) PEG6000 (Polyethylene Glycol with a molecular weight of 6000), and equilibrated against a 500 μL reservoir solution of 1M Na_2HPO_4/NaH_2PO_4 at pH 6.0 to initiate the crystallization in the protein solution. Parallel experiments were also carried out with the MMP-8 catalytic subunit without inhibitors. Needle-shaped crystals appeared in 4 days. The crystals diffracted to 1.3-2.5Å and data was collected at Beam line X25 and X29 in Brookhaven National Laboratory. Data processing was carried out using Phenix (this is a software suite for the automated determination of macromolecular structures using X-ray crystallography and other methods) and by model building by the Crystallographic Object-Oriented Toolkit (Coot) program.

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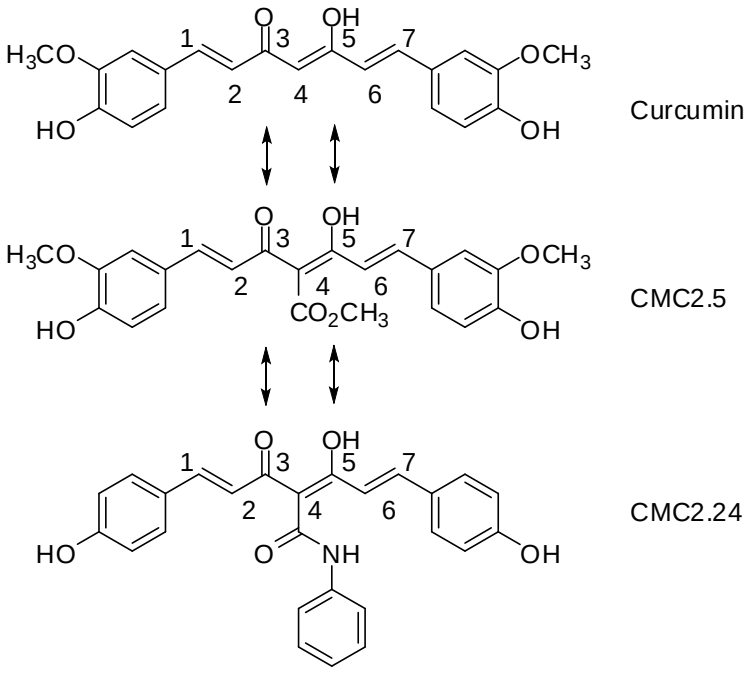
Chapter 8. Collateral studies and conclusion

A. Introduction

Because several chemically-modified curcumins (CMCs) have shown excellent inhibitory activity against nine different matrix metalloproteinases, namely MMP-1, -2, -3, -7, -8, -9, -12, -13 and -14, as well as better zinc and serum albumin binding activity in contrast to curcumin in the previous chapters, both CMC2.5 and CMC2.24 were selected as lead compounds. In particular CMC2.24, the current lead compound, was evaluated for its efficacy in the reduction of chemokines and cytokines in cell culture (human peripheral blood monocytes) and tissue culture (bovine cartilage).[1-7] Also, curcumin and a group of CMCs were tested for their therapeutic potentials as inhibitors of inflammatory mediators (cytokines and prostaglandins) in a variety of *in vivo* disease models including osteoarthritis, periodontitis, acute respiratory disease syndrome (ARDS) and type-I diabetes. Of extreme importance, CMC2.24 is highly efficacious and non-toxic regardless as to whether it is administered systemically, by either the oral or the intraperitoneal (i.p.) route, or topically, in successful wound healing studies.[7]



Scheme 8.1 Structures of curcumin, CMC2.5 and CMC2.24

B. Cell Culture Studies

CMC2.5 and CMC2.24 were found to inhibit the production of various mediators of inflammation and connective tissue destruction, induced in human monocytes either by endotoxin from *E. coli*, or by a complex of C-reactive protein (CRP) oxidized low-density lipoprotein (LDL) cholesterol from the studies carried out by Dr. Ying Gu (Department of Dentistry, Stony Brook University). These mediators include not only pro-inflammatory cytokines and mediators such as TNF-α (Figure 8.2a) and IL-1β (Figure 8.2b) but also PGE2, MCP-1 and several others (data not shown).[1-4] The expression of pathologically-excessive levels of MMP-2, -8 and -13 (data not shown) by monocytes in culture, was also reduced to normal levels by treatment with CMC2.24 at a low concentration. Regarding the effect on viable cells, as illustrated in Figure 8.1a and 8.1b, human peripheral blood monocytes were cultured in serum-free media with and without lipopolysaccharide (LPS). Curcumin at 2 or 5 μM did not appear to

decrease the MMP-9 levels in the conditioned media Figure 8.1a. In contrast, when CMC2.24 was added to the culture at the same concentration either 2 or 5 µM, the extracellular MMP-9 levels were reduced in a dose-dependent manner Figure 8.1b with a lesser effect at 2 µM, but dramatically reduced at the 5 µM concentration. CMC2.24 (2 and 5 µM) can inhibit the activities of inflammatory mediators, TNF-α (Figure 8.2a) and IL-1β (Figure 8.2b).

Figure 8.1 Illustration of the attenuating effect of curcumin and CMC2.24 on MMP-9 levels in conditioned media from human monocytes stimulated by LPS in cell culture

Note: Human peripheral mononuclear cells (PBMC) (5x 10^5 cells/well) were cultured in serum-free media (37^{o}C, 5% CO_2) overnight with LPS (*P. gingivalis*, 50 ng/mL) containing vehicle alone or curcumin or CMC2.24 at different concentrations (Figure 8.2a and 8.2b). Conditioned medium from both experiments were analyzed for MMP-9 levels by gelatin zymography.

Figure 8.1a The effect of curcumin on MMP-9 levels

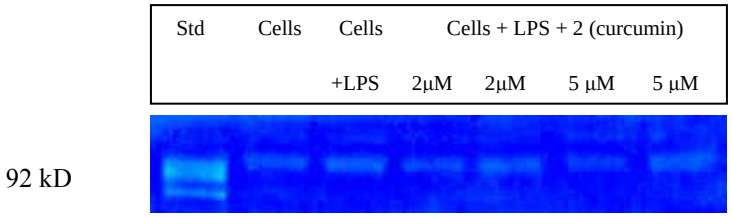


Figure 8.1b The effect of CMC2.24 on MMP-9 levels

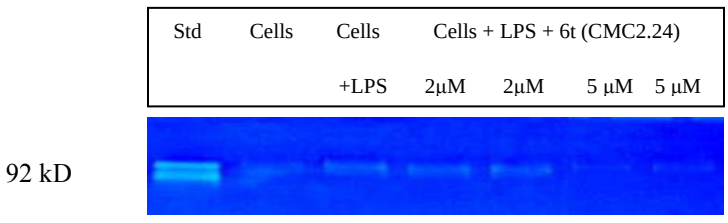


Figure 8.2 CMC2.24 (2 and 5 µM) inhibits the activities of inflammatory mediators (cytokine levels) TNF-α and IL-1β produced by human monocytes stimulated with *P.gingivalis* LPS (50ng/ml). Each bar represents the mean of 3 cultures ± S.E.M.

Figure 8.2a CMC2.24 (2 and 5 μM) inhibits the activities of TNF-α

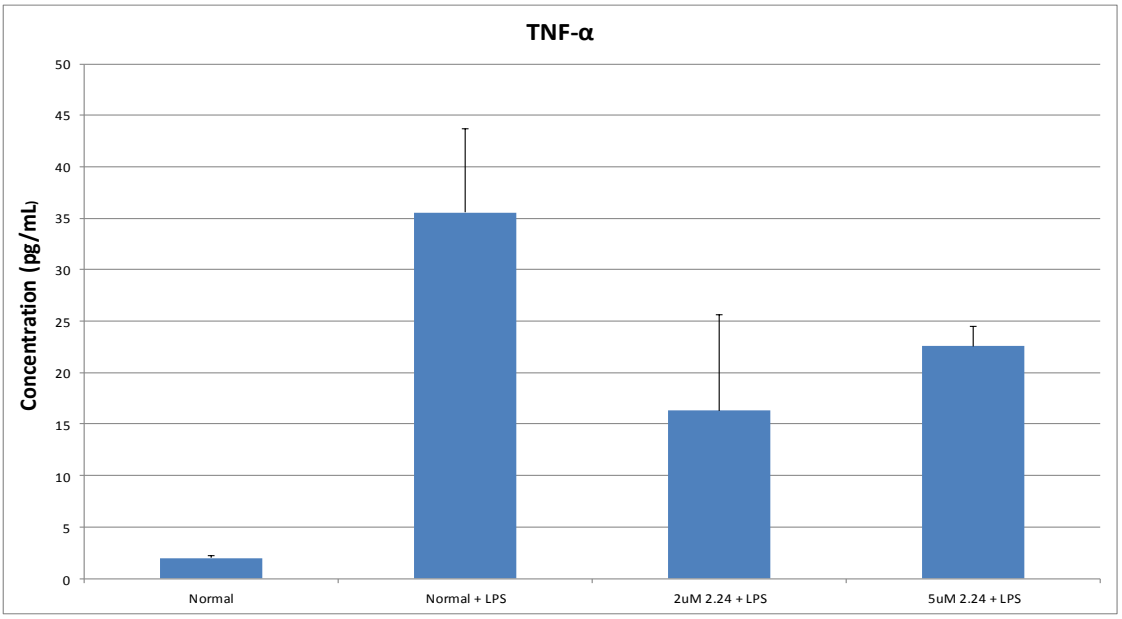
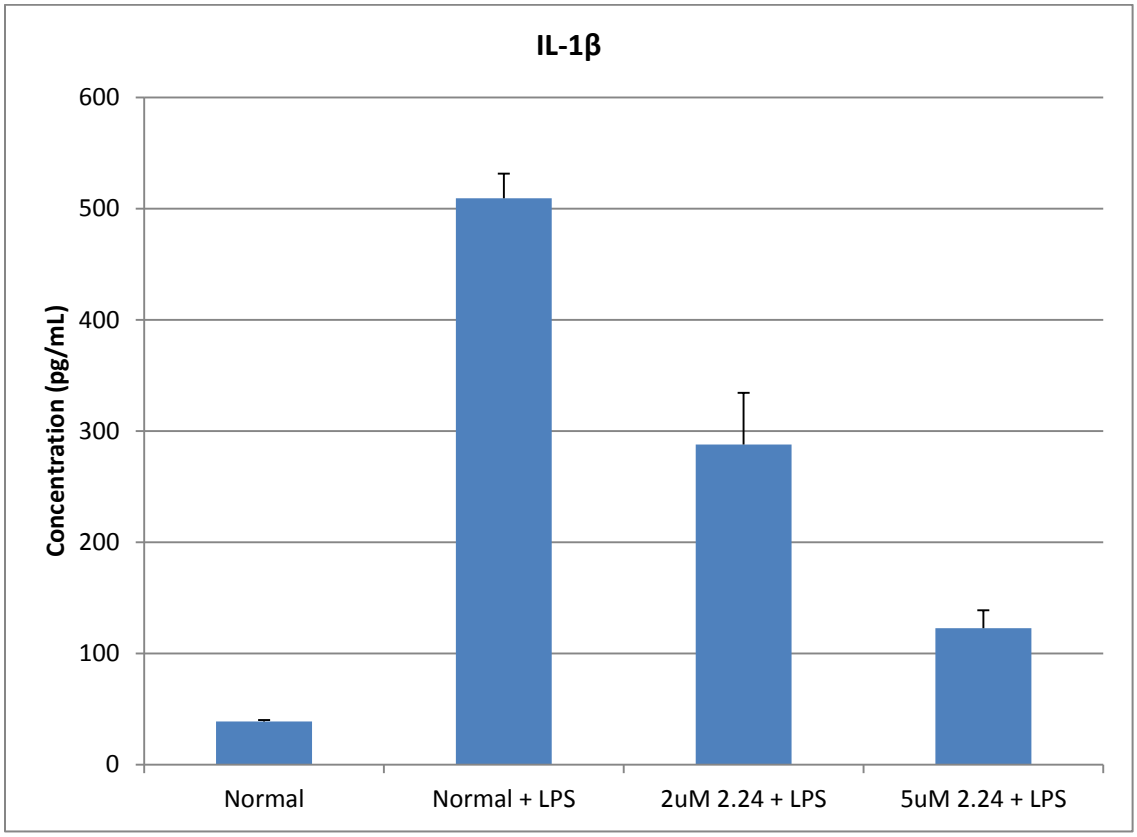


Figure 8.2b CMC2.24 (2 and 5 μM) inhibits the activities of IL-1β



CMC2.23 and CMC2.24 were also studied by Dr. Balakrishna L. Lokeshwar (School of Medicine, University of Miami) in cancer cells in culture, and the result revealed that CMC2.24 was more effective in terms of inhibiting the cancer cell growth, than CMC2.23 in the study of prostate and pancreatic cancer cell lines (unpublished). (Figure 8.3)

Figure 8.3 Effect of CMC2.23 and CMC2.24 (μM) against prostate (PC-3) and pancreatic (MiaPaCa) cancer cells in culture

Figure 8.3a Effect of CMC2.23 and CMC2.24 (μM) against prostate (PC-3) cancer cells in culture

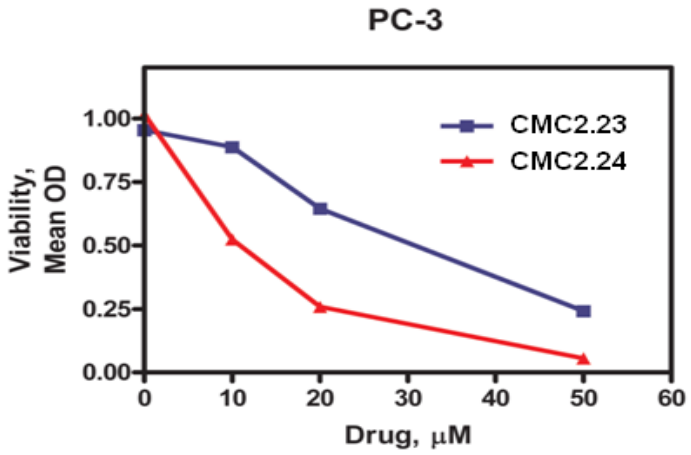
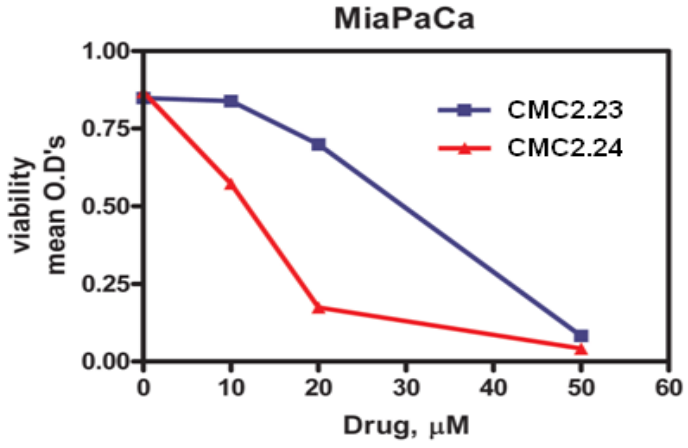


Figure 8.3b Effect of CMC2.23 and CMC2.24 (μM) against pancreatic (MiaPaCa) cancer cells in culture



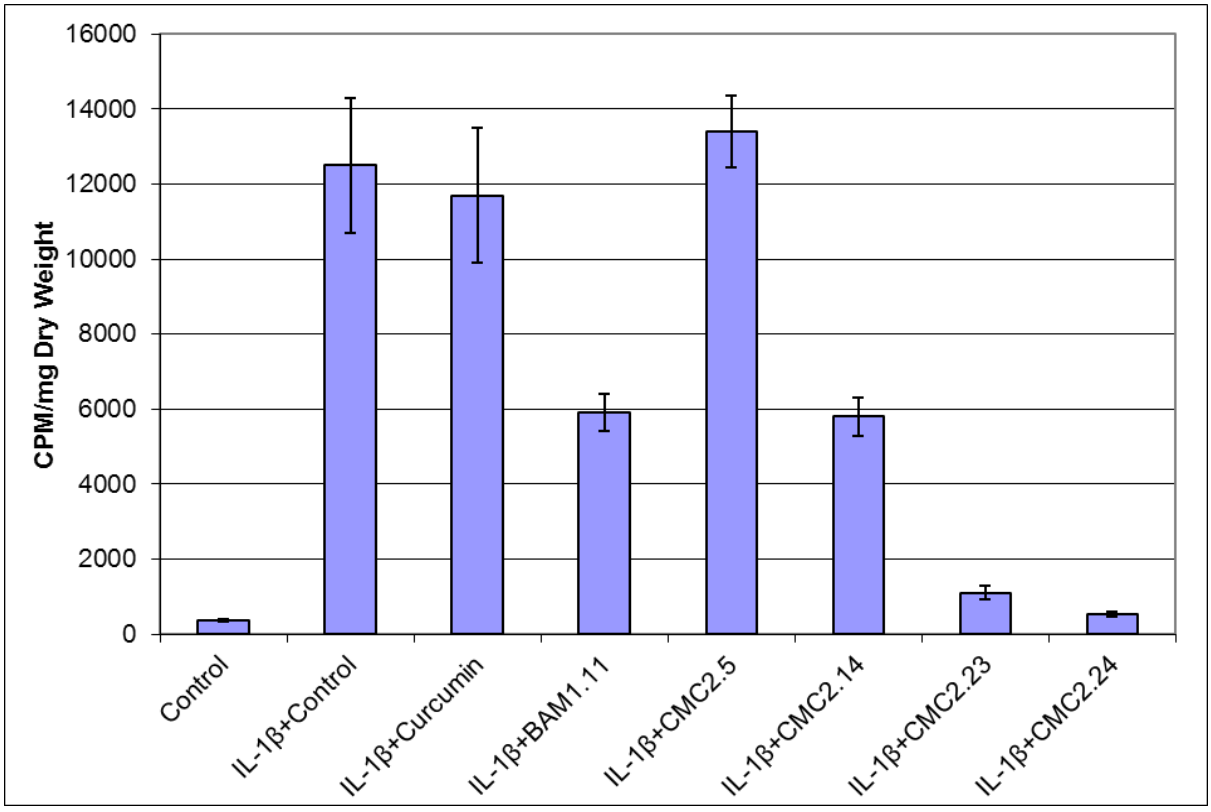
C. Tissue Culture Studies

Osteoarthritis (OA) is considered to be the result of aging, and one of the leading causes of disability among the elder people.[8, 9] OA is characterized by continuous degradation of articular cartilage. Once the chondrocytes (the only cells exist in healthy cartilage, that produce and maintain the cartilaginous matrix, comprising mainly of collagens and proteoglycans) are stimulated by pro-inflammatory cytokines including but not limited to interleukin-1β (IL-1β), the release of cartilage-degrading enzymes such as MMPs will be triggered. Chondrocytes are responsible for the synthesis of proteoglycans, which are initially synthesized in endoplasmic reticulum, then go through the addition and sulfation of the glycosaminoglycan chains (within Golgi apparatus). As for the synthesis of proteoglycans, sulfation is the crucial and final step, so radioactive labeling with $^{35}SO_4^{2-}$ has been utilized to monitor the synthesis of proteoglycan.[10] Dr. Daniel Grande (Department of Orthopaedic Surgery, Long Island Jewish Medical Center) reported recently that IL-1β-induced excessive proteoglycan (S^{35}-labeled) degradation of bovine cartilage in culture (a model for osteoarthritis) was reduced to basal/control levels by CMC2.23 and CMC2.24 but not by curcumin or CMC2.5.[7] In this experiment, three millimeter full-thickness bovine articular cartilage explants were trephined from femoral condyles of the knee and placed in tissue culture. Explants were labeled with ^{35}S sulfate control cartilage explants (media only, negative control) demonstrated a baseline level of ^{35}S sulfate release throughout the 72-hour period. IL-1β and Oncostatin M (OsM) -challenged explants (positive control) demonstrated high release of ^{35}S compared to that of negative controls at all time-points. Curcumin derivatives, specifically CMC2.14, CMC2.23, and CMC2.24 exhibited significant chondroprotective effect, as evidenced by lower release of ^{35}S. The results demonstrate improved chondroprotective effects of novel curcumin derivatives against IL-1β and OsM-induced

chondrolysis. This new evidence allows for speculation that these compounds may play a role in the future treatment of OA.

Figure 8.4 Analysis of $^{35}SO_4^{2-}$ release following treatment with curcumin and CMCs (10 µM) after 72 Hours

Note: Three millimeter full-thickness bovine articular cartilage explants were trephined from femoral condyles of the knee and placed in tissue culture. Explants were labeled with ^{35}S sulfate and separated into eight study groups: negative/positive controls, curcumin, novel derivatives labeled. Explants were challenged with IL-1β and OsM or with IL-1β, OsM and one of the derivatives. ^{35}S sulfate release was evaluated at 24, 48, and 72 hours post-treatment by scintillation counting. Explants were then lyophilized to obtain dry weights (mg) and data expressed as counts per minute (CPM)/mg dry weight. Mean and standard deviation of CPM/mg for each group were used for statistical analysis using unpaired t-tests ($\alpha = 0.05$).



D. *In Vivo* Studies.

1. Type-I diabetes

One of the leading causes of mortality for diabetic people is cardiovascular complication, characterized by the changes in vascular structure, including ECM deposition, intimal proliferation (the characteristic feature of the response of human saphenous vein to arterial implantation) and media-to-lumen ratio (the basis that the force-producing smooth muscle cells lie within the media) increase.[11-13] As discussed in the previous chapters, MMPs are essential in the regulation of ECM turnover. Nephropathy, the damage to kidney, is mainly due to the dysregulation of MMPs and TIMPs, characterized by collagen deposition in basement membrane.[11] When rats are rendered diabetic by LPS, we found that the diabetics exhibited a 4-fold increase in MMP-9 levels as estimated, from their peritoneal macrophages in cell culture, compared to the non-diabetic control rats (Figure 8.5a). However, when the diabetic rats were administered CMC2.24 (30mg/kg) orally, the pathologically-elevated levels of MMP-9 were reduced to normal levels (Figure 8.5b) despite the severely hyperglycemic state of these animals which remained unchanged (Figure 8.5c). We had previously demonstrated that either inducing diabetes with streptozotocin (STZ), or causing periodontitis locally by endotoxin injection into the gingiva, leads to an increase in the production of pro-inflammatory cytokines and MMPs. In these studies, both CMC2.5 and CMC2.24 were found to inhibit the production of pathologically-excessive levels of these inflammatory mediators and MMPs in tissue from rats that had been orally-administered these curcumin analogues (data not shown). Of particular significance, administration of CMC2.5 or CMC2.24 by the oral route in doses as high as 500mg/kg body-weight per diem, showed no evidence of toxicity in these already seriously-ill

animals. This suggests that the continuing tissue-degradation associated with diabetes, might be arrested by the use of CMC2.24 (unpublished).

Figure 8.5 Effect of CMC2.24 on levels of MMP-9 secreted by peritoneal macrophages from diabetic rats

Note: Thioglycollate-induced peritoneal macrophages derived from N, D and D rats treated with CMC2.24 daily for three weeks were isolated as described in the methods section. Cells at 1 x 10^6 cells/mL were cultured in serum-free media (37^oC, 5%CO_2/95%O_2) overnight, and conditioned media were analyzed for MMP-9 levels by gelatin zymography. **Std:** MMP standards; **N:** Non-diabetic control; **D:** Diabetic control; **D+2.24**: diabetics orally administered CMC2.24 (30mg/kg) daily for three weeks. Pro-MMP-9 has a MW of 92kD, while activated MMP-2 is 72kD (Representative zymogram is shown in Figure 8.5a). The band densities from the gelatin zymogram were measured by scanning on a laser densitometer, and image analysis was performed using Image J (Figure 8.5b). Each value represents the mean of 3 analyses ± S.E.M. Blood glucose levels (mg/100mL) for the three groups of rats are shown in Figure 8.5c. Each value represents the mean ± S.E.M. for 3 analyses per group.

Figure 8.5a Effect of CMC2.24 on levels of MMP-9 secreted by peritoneal macrophages from diabetic rats

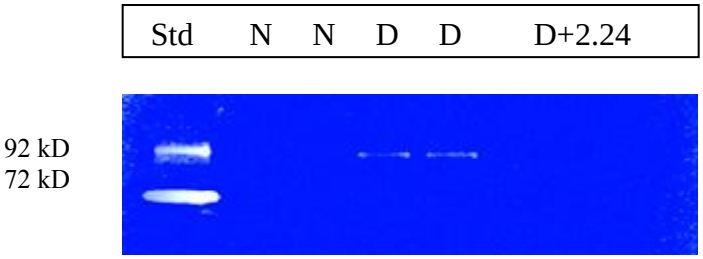


Figure 8.5b Densitometric scanning of CMC2.24 on levels of MMP-9 secreted by peritoneal macrophages from diabetic rats

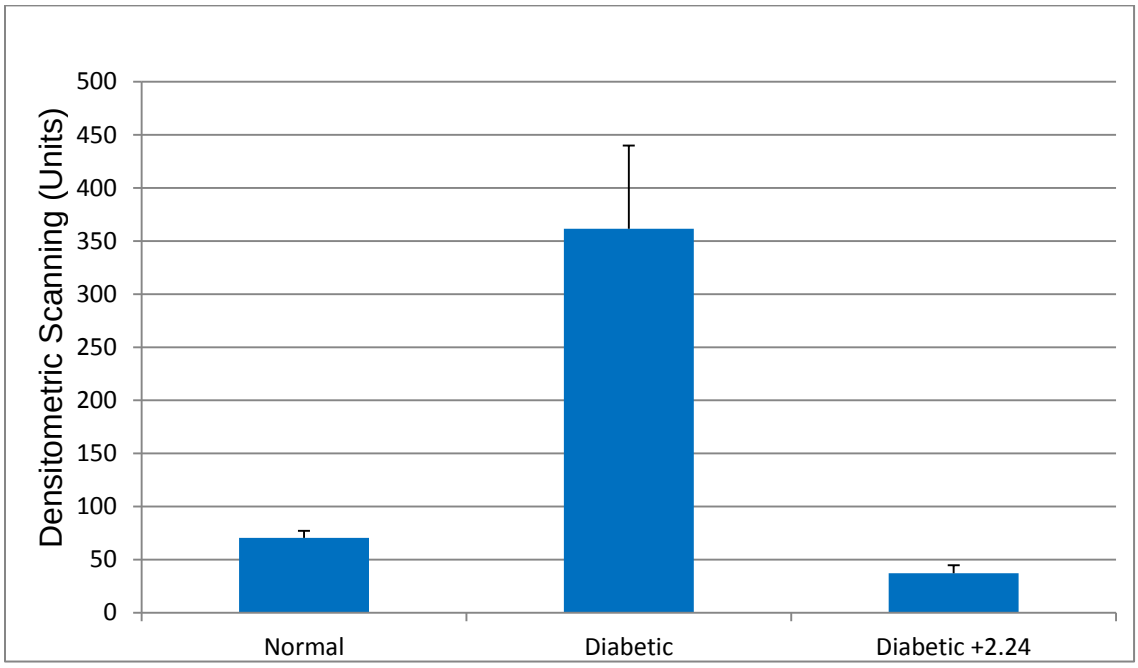
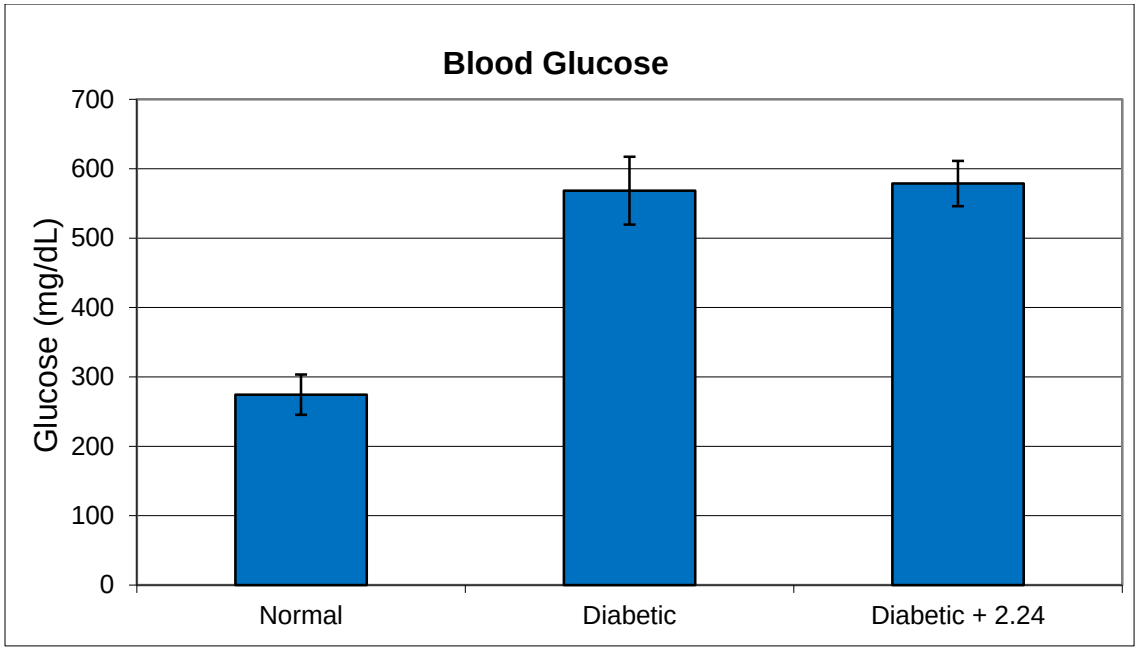


Figure 8.5c Blood glucose levels of normal and diabetic rats



2. Wound-healing

Figure 8.6 Chronic wounds

Wound-healing is a complex and dynamic process, which involves various types of cells, chemokines, cytokines, growth factors and the extracellular matrix (Figure 8.6). For the normal wound healing, there are general three distinct phases: inflammatory, proliferation and remodeling (Figure 8.7).[14-18]

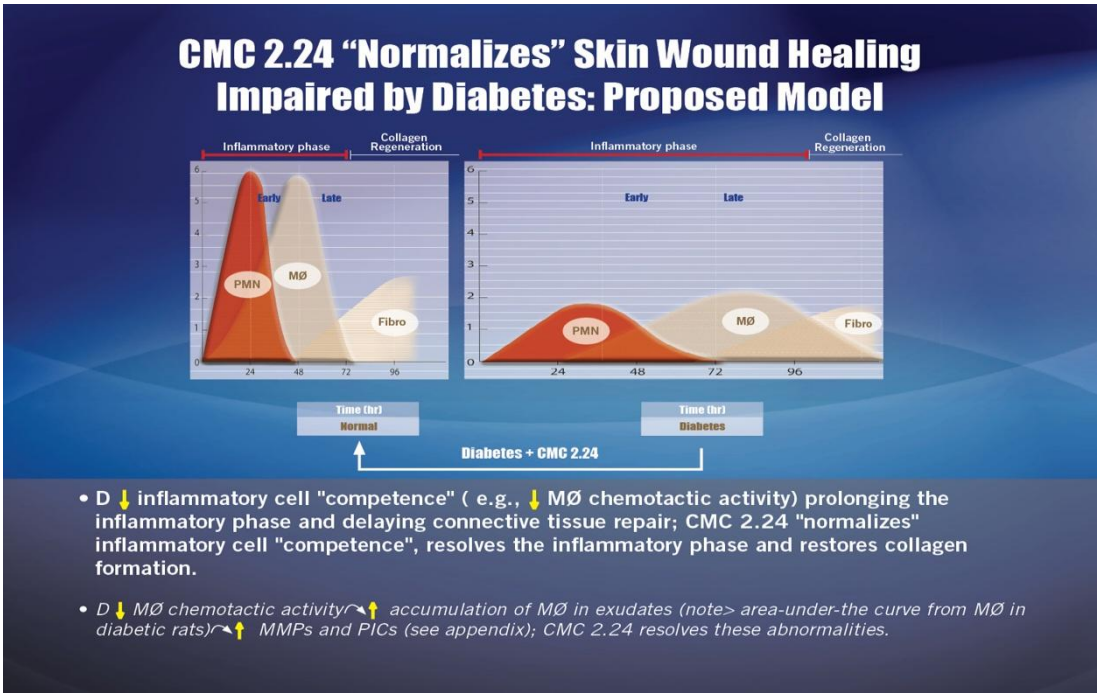


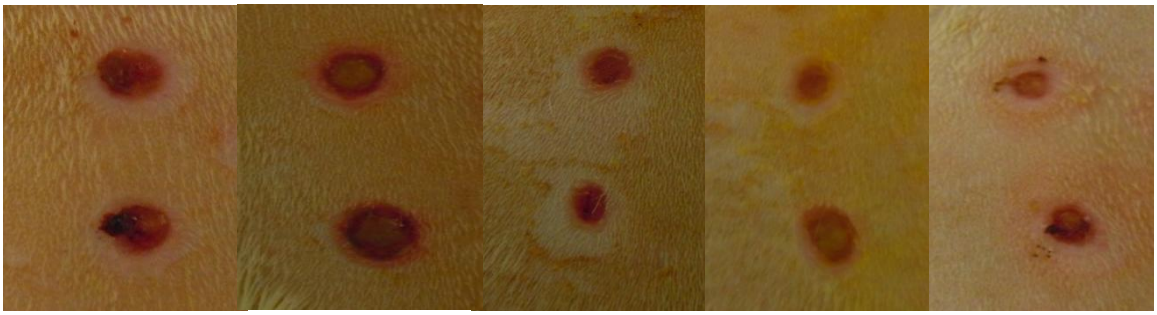
Figure 8.7 Proposed mechanism of wound healing

The healing cascade starts immediately with hemostasis and fibrin deposition, which release the clotting factors to prevent bleeding and generate cellular signals for the healing process. Then in the inflammatory phase, neutrophils, macrophages and lymphocytes sterilize and clear tissue debris, and then secret additional chemokines and cytokines to promote healing. This is followed by the proliferation phase, whereas fibroblasts proliferate and synthesize collagen, producing a matrix for the injured tissue. The final stage is the remodeling phase, whereas collagen deposition and cross-linking occur to create new epithelium and seal the wound permanently. Because MMPs are responsible for the degradation of ECM, and they are secreted by various types of cells in wound healing process, they play an essential role in the regulation of wound repair.[14-16] In our current study, CMC2.24 was either topically (1% or 3% CMC2.24 in petrolatum jelly) or systematically (30mg/kg) administered to Type-I diabetic rats for 7 days to evaluate the efficacy on impaired wound-healing. Diabetic rats were divided to five groups: normal; diabetic; diabetic treated with 1% CMC2.24 in petrolatum jelly for topical administration; diabetic treated with 3% CMC2.24 in petrolatum jelly for topical administration; diabetic treated with 30mg/kg of CMC2.24 for oral route. The result revealed that 1% topical treatment of CMC2.24 produced the greatest improvements (about 34% reduction in healing) in healing of both the surface epithelium and the underlying connective tissue, according to the data on the biochemical and histological measurements (Figure 8.8).

Figure 8.8 Clinical appearances of wound healing studies

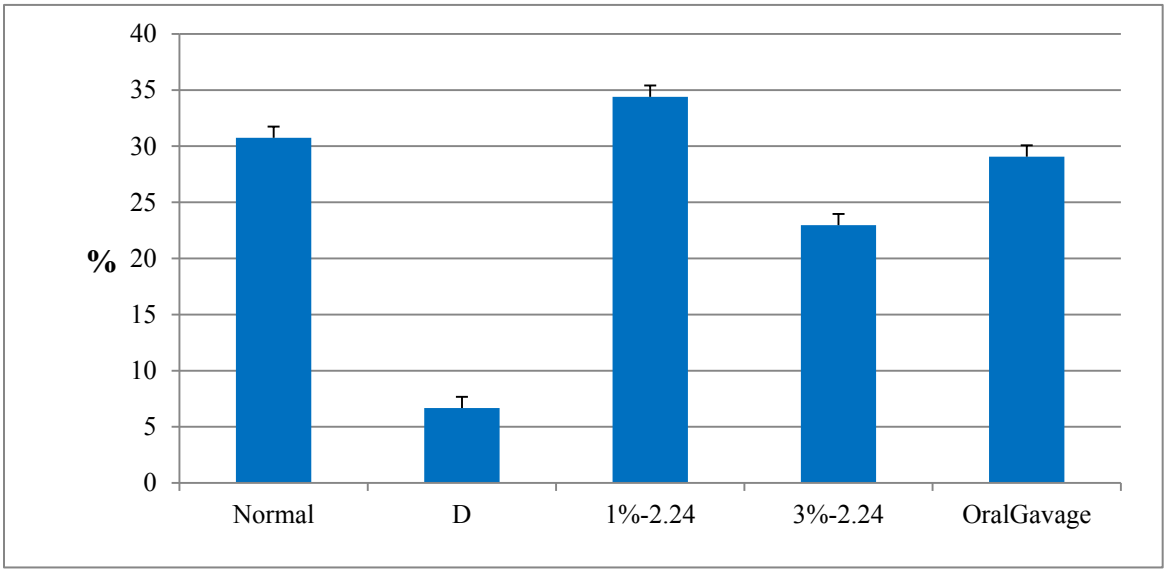
Note: Figure 8.6a Clinical appearance of standardized skin wounds in normal and diabetic rats after 7 days of healing: effect of topical and systemic administration of CMC2.24; Figure 8.6b Healing (% reduction) of skin wounds in Normal, Diabetic, and Diabetic Rats Treated with Vehicle Alone or with topical or Systemic Administration of CMC2.24.

Figure 8.8a Standardized skin wounds in normal and diabetic rats



Normal	D	D+1% CMC2.24	D+3% CMC2.24	D+CMC2.24 Oral 30mg/kg

Figure 8.8b Healing (% reduction) of skin wounds in normal, diabetic, and diabetic rats treated with vehicle alone or with topical or systemic administration of CMC2.24



3. Acute respiratory distress syndrome (ARDS)

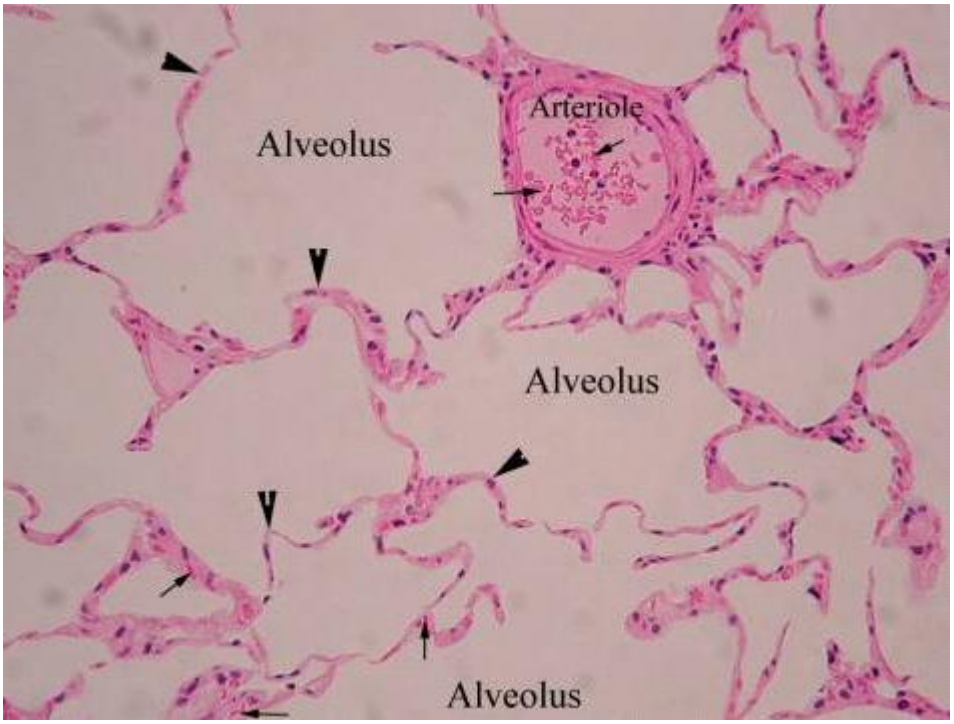


Figure 8.9 Normal lung histology

Note: There are large alveolar volumes, the septal spaces are very thin, and there is no cellular congestion.

Acute respiratory distress syndrome (ARDS) is a severe lung disease that prevents oxygen from passing into the blood thus rendering breathing difficulty. The morality varies from 40-60%, and is caused by the accumulation of protein-loaded fluid in the alveolar spaces, which leads to a dramatic decrease in diffusing capacity and results in hypoxemia.[19-20] Studies have shown that sepsis, including but not limited to pneumonia, is one of the leading causes of ARDS, however other conditions such as multiple transfusions and trauma can also initiate the ARDS syndrome.[21-22] ARDS is generally characterized by alveolar inflammation, including thickened septal caused by protein release, congestion and decreased alveolar volume, while in the normal lung, septal spaces are very thin, and show no cellular congestion. A preliminary study

(unpublished) by Dr. Lucille London (Department of Oral Biology and Pathology, Stony Brook University) has been carried out by using a mouse model of ARDS. The condition is induced by reovirus, and the *in vivo* model was used to study the efficacy of curcumin, CMC2.5 and CMC2.24 in preventing the progression of the disease. The results revealed that 2% CMC2.24 in carboxymethyl cellulose administered 40mg/kg body-weight per diem over 19 days by the intraperitoneal route was able to completely suppress the lung inflammation and prevent the lung tissue destruction (Day 14). However, it did not suppress the initial inflammatory response in the lungs (Day 9) as can be seen from Figure 8.10, the alveolar space is large and clear. Fibrosis is not observed in Day 14 with the treatment of CMC2.24. When tested, CMC2.5 was proved to be less effective in this mouse model compared to CMC2.24. Curcumin was also able to suppress both the lung fibrosis and inflammatory response (Day 9 and Day 14).

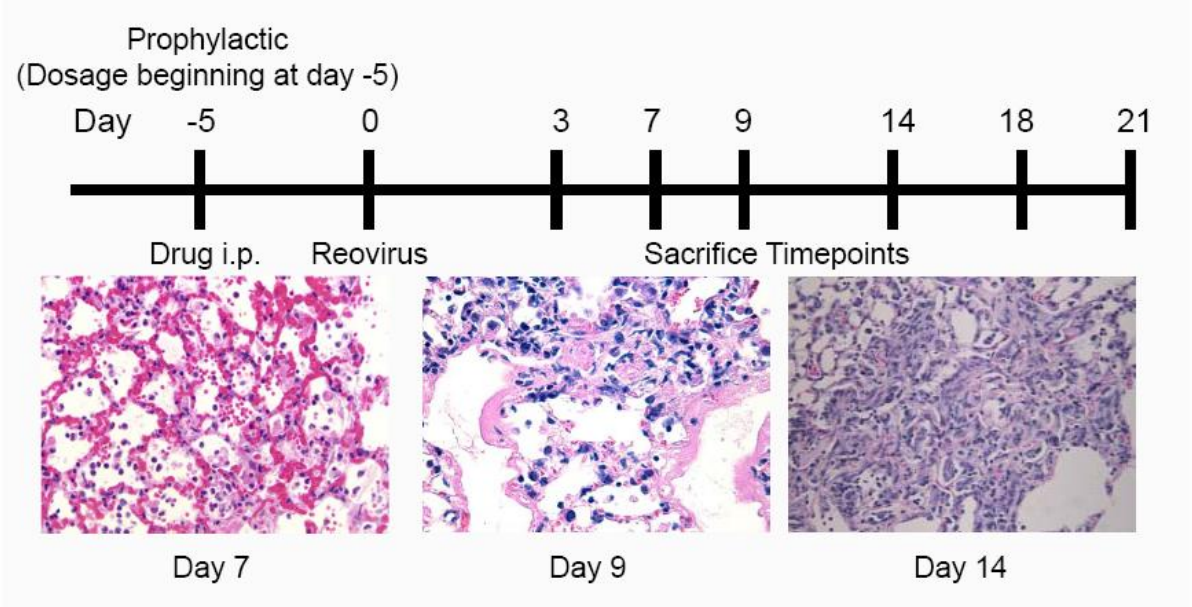


Figure 8.10 Histology of lung tissue in the mouse model of ARDS

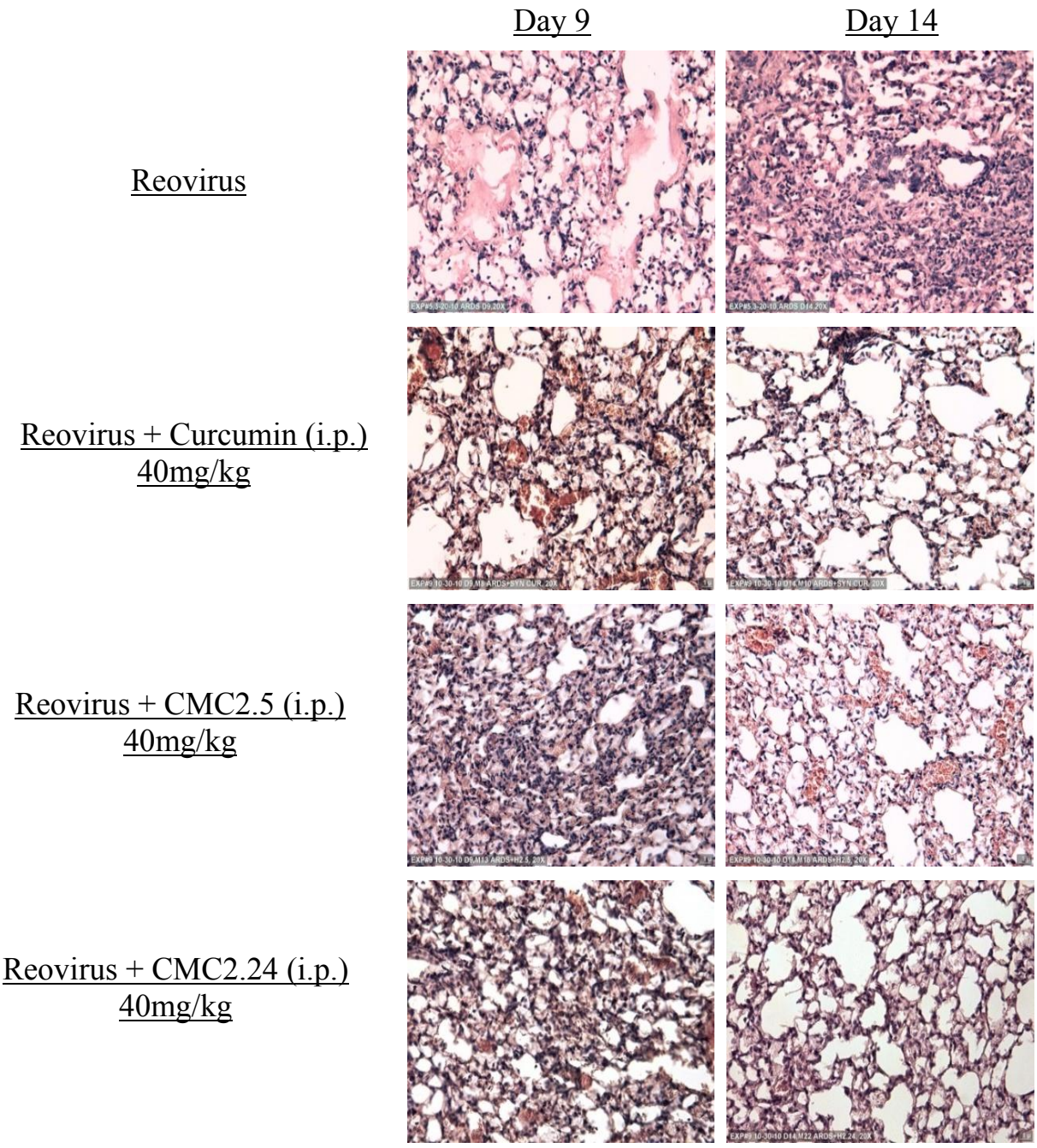


Figure 8.11 ARDS treatment with curcumin, CMC2.5 and CMC2.24

Note: All three compounds were administered to the mice 5 days ahead of the induction of reovirus. Lung tissues were obtained at Day 9 and Day 14. The group induced by reovirus without treatment of any compound was used as a control.

E. Conclusion

In general, curcumin and chemically-modified curcumins required for the structure-activity relationship (SAR) studies were synthesized by a modified Pabon reaction. The synthetic route is shown in Chapter 3, and a series of the fully characterized compounds are documented. MMP inhibition assays were then carried out for pre-selected compounds with MMPs -1, -2, -3, -7, -8, -9, -12, -13 and -14. CMC2.24, the current lead compound, exhibited inhibitory IC_{50} values *in vitro*, ranging from 2-8μM against two collagenases (MMPs-8, -13), two gelatinases (MMPs-2, -9), and MMPs- 3, -7 and -12. In addition, the membrane-type MMP, MMP-14, was also effectively inhibited more by CMC2.24 (IC_{50}=15.3 μM) than by the other known MMP inhibitors such as 1,10-phenanthroline (IC_{50}=43.8 μM) and curcumin (IC_{50}=29.5 μM). These MMPs are significantly up-regulated during a variety of tissue-destructive diseases such as (but certainly not limited to) arthritis, cardiovascular disease, periodontal disease, and cancer. In contrast, MMP-1 is often considered constitutive due, in part, to its involvement in the degradation of the triple-helical collagen molecule during physiological connective-tissue turnover. This collagenase requires much higher concentrations of CMC2.24 to inhibit its activity (IC_{50}=69.8 μM). As illustrated in Figure 8.1, human peripheral blood monocytes were cultured in serum-free media and stimulated by lipopolysaccharide (LPS). Curcumin at 2 or 5 μM did not appear to decrease the MMP-9 levels in the conditioned media (Figure 8.1a). In contrast, when CMC2.24 was added to the culture at a final concentration of 2 or 5 μM, the extracellular MMP-9 levels were reduced in a dose-dependent manner (Figure 8.1b) and dramatically so at the 5 μM concentration. When rats were rendered severely diabetic, we found that they exhibited a 4-fold increase in MMP-9 levels (Figure 8.2) produced by peritoneal macrophages in cell culture, compared to the non-diabetic control rats; These rats when administered CMC2.24 (30mg/kg

body weight) show normal levels of MMP-9 despite the severely hyperglycemic state of these animals. A significant number of CMCs are, in general, much more active than curcumin and do not share the latter's insolubility characteristics. Significantly, these CMCs appear to have the ability to reduce the pathological levels of only the inducible and not the constitutive MMPs to essentially normal homeostatic levels, which may be one of the reasons that these substances show little or no demonstrable toxicity at oral doses as high as 500mg/kg body-weight per diem. We have found that these compounds have no antibiotic or photosensitizing properties (similar to curcumin), and we are currently studying the potential of these compounds to control diseases of tissue loss/breakdown, especially those associated with aging, inflammation and cancer. Future studies will focus on the larger animals (e.g., pigs, dogs) to determine the toxicity and pharmacokinetic characteristics of chemically-modified curcumins in preparation for an Investigational New Drug (IND) application to the FDA.

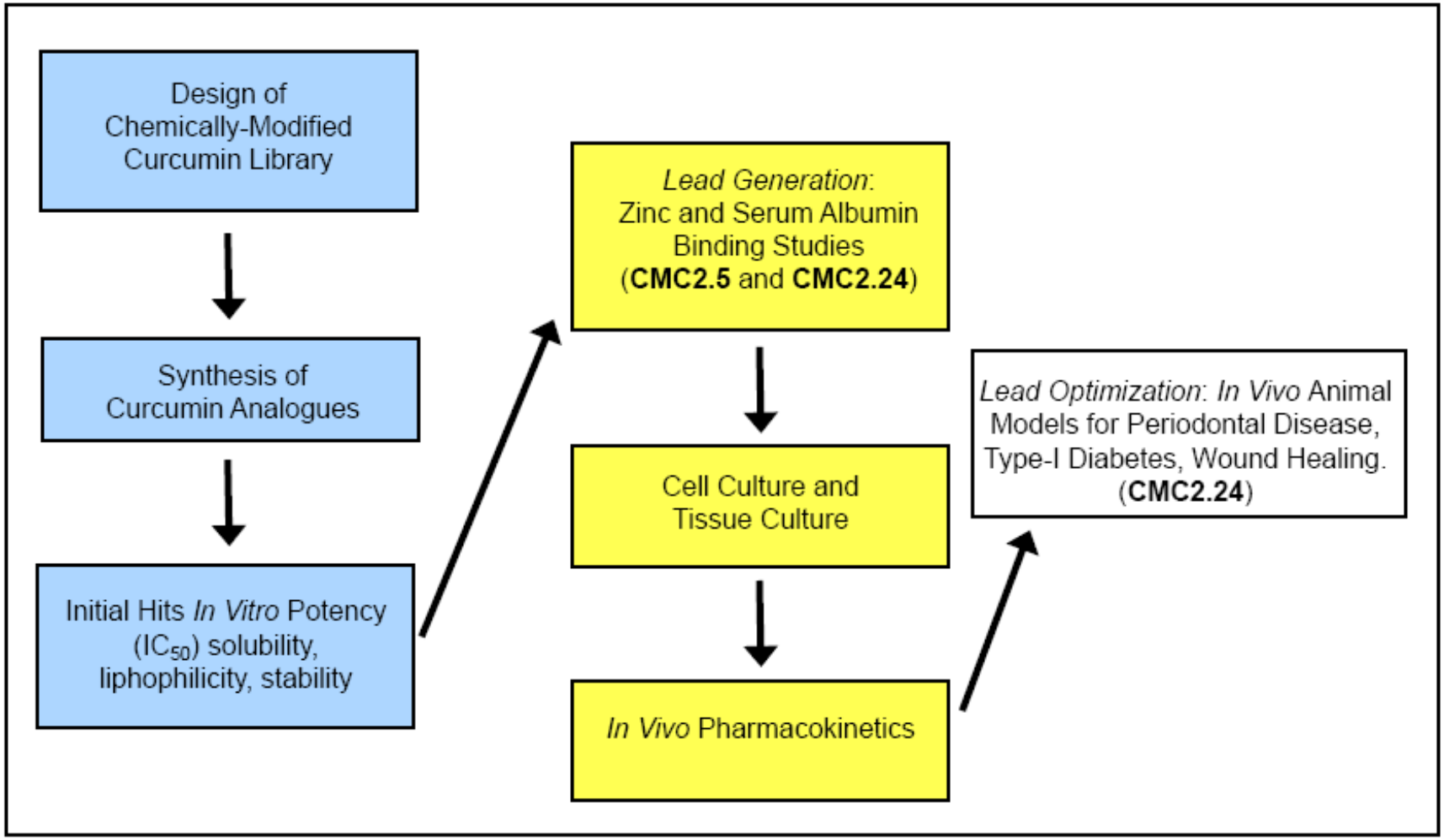


Figure 8.12 Pre-clinical developments of novel curcumin analogues as inhibitors of MMPs

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Appendix

A. Report on the boron analysis of CMC2.24

Report Number: 38546 GALBRAITH LABORATORIES, INC. Report Date: 2012-01-05

Laboratory Report

Report prepared for:
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Sample: C2.24
Lab ID: 2011-P-9675 **Received:** 2011-12-13

Analysis	Method	Result	Basis	Amount	Date (Time)
B : Boron					
	GLI Procedure ME-70	< 28 ppm	Dried	90.59 mg	2012-01-03
ZZX: Dry before analysis					
	GLI Procedure 3.2.3.6	Completed	As Received	Direct	2011-12-19

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