# DEVELOPMENT OF HYPO- AND HYPERURICEMIA IN ZEBRAFISH (*Danio rerio*) AS A POTENTIAL ANIMAL MODEL FOR GOUT STUDIES

BY

KEVSER IRFAN UNAL

A thesis submitted in fulfillment of the requirement
for the degree of Master in Pharmaceutical Sciences
(Pharmaceutical Chemistry)

Kulliyyah of Pharmacy
International Islamic University Malaysia

JULY 2018

# ABSTRACT

Gout disease is becoming more common in most countries around the world. Gout is caused by elevated levels of serum uric acid that are deposited in joints and cause inflammation. Treatment for gout is continuously developed and new drugs are being formulated, tested, and commercialised. The use of zebrafish is gaining popularity in exploring disease mechanisms, which are complementary to other animal models such as rodents. The transparent and ex-utero development of embryo, ease of maintenance and drug administration, and cheaper and faster bioassays make zebrafish suitable for several assays. Given the specific advantages of zebrafish, the zebrafish model could have promising outcomes in giving insights to gout. The objectives of this study is to develop a zebrafish hypo- and hyperuricemia model and to identify the biomarkers by using LC-MS based metabolomics. In this study, zebrafish embryos (ZFE) were used to induce hypo- and hyperuricemia by administering allopurinol (AP) and potassium oxonate (PO), respectively, through static immersion and observed under specified parameters, namely phase of development (number of days post fertilization), days of immersion, and concentration of AP or PO. The set of parameters that has yielded the best results was selected for the LC-MS metabolomics studies. The results show that the optimum conditions were achieved at 3 dpf immersed for 2 days for the hypouricemia model, and at 4 dpf immersed for 3 days for the hyperuricemia model. The PCA score plot of the metabolomics data showed that the analysed groups were separated and distinguished. The identified metabolites which distinguish the normal zebrafish and hypo- and hyperuricemia zebrafish are Docosahexaenoic acid, Eicosapentaenoic acid, Dihydroceramide, and PE(20:4(5Z,8Z,11Z,14Z)/15:0) for AP exposed zebrafish, and Methyl (9Z)-10'-oxo-6,10'-diapo-6-carotenoate, 3-Oxooctadecanoic acid, (9S,10S)-9,10-dihydroxyoctadecanoate, and N-(2-Hydroxyethyl)-morpholine for PO exposed zebrafish respectively.

# خلاصة البحث

أصبح داء النقرس أكثر شيوعا في معظم البلدان في جميع أنحاء العالم. ينجم داء النقرس عن المستويات المرتفعة لحمض اليوريك في الدم والتي تترسب في المفاصل مسببة حالات من الالتهاب. علاجات داء النقرس يتم تطويرها بشكل مستمر ويجري حاليا صياغة واختبار وتسويق أدوية جديدة لهذا المرض. استخدام أسماك الزيبرا اكتسب شعبية كبيرة في عمليات استكشاف كيفية تكون الأمراض، وتعتبر هذه الأسماك المخبرية مكملة للحيوانات المخبرية الأخرى مثل القوارض. المظهر الشفاف ونمو الأجنة خارج الرحم وسهولة التعامل معها ويسر إعطاء الأدوية، وسرعة ورخص الفحوصات الحيوية جعل أسماك الزيبرا مناسبة لعدة فحوصات مخبرية. وبالنظر إلى المزايا المحددة لأسماك الزيبرا فإنه بامكان نموذج سمك لزيبرا أن يكون له نتائج واعدة في إعطاء أفكار عن داء النقرس. هدفت هذه الدراسة لتطوير نموذج لمرض الفرط وأيضا النقص في حمض يوريك الدم، وتحديد المؤشرات الحيوية بواسطة الميتابولوميات المعتمدة على الكروماتوغرافيا السائلة – مطياف الكتلة (LC–MS). تم استخدام أجنة أسماك الزيبرا لتطوير حالتي فرط ونقص في حمض يوريك الدم عن طريق إعطاء عقار الألوبورينول (AP) وحالة نقص حمض يوريك الدم عن طريق إعطاء أوكسونات البوتاسيوم (PO) عن طريق الغمر الساكن، ومراقبتها على حسب معايير محددة، وهي مراحل النمو (عدد الأيام بعد الإخصاب، dpf)، عدد أيام الغمر، وتركيز AP أو PO. تم اختيار الأجنة ذي المعايير التي أسفرت عن أفضل النتائج لإجراء دراسات تحليل ميتابولوميات الـ LC–MS عليها. أظهرت النتائج أن الظروف المثلى تحققت في 3 dpf والمغمورة لمدة يومين لنموذج نقص حمض اليوريك في الدم، و 4 dpf المغمورة لمدة 3 أيام لنموذج فرط حمض يوريك الدم. أظهر تخطيط نقاط الـ PCA لبيانات الميتابولوميات أن المجموعات المحللة تم فصلها وتمييزها. نواتج الأيض التي تم تحديدها والتي ميزت بين أسماك الزيبرا العادية والمصابة بفرط حمض يوريك الدم هي حمض الدوكوساهيكسينويك، حمض الإيكوسابنتاينويك، ديهيدروسيراميد، و PE(20:4(5Z,8Z,11Z,14Z)/15:0) للأسماك المعرضة للألوبيورينول، أما بينها و بين الأسماك المصابة ب نقص حمض يوريك الدم فكانت ميثيل (9Z)-10'-أوكسو-6،10'-ثنائية الأبو-6-كاروتينوإيت، وحمض 3-أوكسو أوكتاديكانويك، و (10S،S9)-9،10-ثنائي هيدروكسي أوكتاديكانويك، و إن-(2-هيدروكسي إيثيل)-مورفولين للأسماك المعرضة للأوكسونات البوتاسيوم .

# APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

……..
Bisha Fathamah Uzir
Supervisor

……..
Alfi Khatib
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

……..
Abdul Razak Kasmuri
Internal Examiner

……..
Mohd. Zaini Asmawi
External Examiner

This thesis was submitted to the Department of Pharmaceutical Chemistry and is accepted as a fulfillment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

……..
Mohamed Sufian Mohd. Nawi
Head, Department of
Pharmaceutical Chemistry

This thesis was submitted to the Kulliyyah of Pharmacy and is accepted as a fulfillment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

……..
Juliana Md. Jaffri
Dean, Kulliyyah of Pharmacy

# DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Kevser Irfan Unal

Signature ..... Date .....

**INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA**

**DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH**

**DEVELOPMENT OF HYPO- AND HYPERURICEMIA IN ZEBRAFISH (*Danio rerio*) AS A POTENTIAL ANIMAL MODEL FOR GOUT STUDY**

I declare that the copyright holder of this thesis are jointly owned by the student and IIUM.





By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Kevser Irfan Unal

……..…….. ……..

Signature Date

# ACKNOWLEDGEMENTS

Alhamdulillah, all glory and praise is due to Allah, the Almighty, whose Grace and Mercy have been with me throughout the duration of my programme. Through His blessings and guidance, I found the strength and persistence in completing this thesis.

I am very thankful to my supervisors, Dr Bisha Fathamah Uzir and Dr Alfi Khatib, who kindly agreed to supervise this work. I will always be grateful for their support and encouragement despite the technical hitches and hurdles faced in conducting this research. My sincere thanks to my friends and lab mates for their kindness and moral support throughout my study.

My deepest gratitude to my beloved parents and family - had it not been for their prayers and understanding, this work may have not been completed. Finally, special thanks and appreciation to my dearest husband, Majid, for his love, care, patience, and unwavering belief in my ability to accomplish this study. Thank you for being on this journey with me.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AP | Allopurinol |
| AMP | Adenosine monophosphate |
| CVD | Cardiovascular disease |
| FET | Fish embryo test |
| FT-MS | Fourier transform mass spectrometry |
| GC-MS | Gas chromatography–mass spectrometry |
| GMP | Guanosine monophosphate |
| GWAS | Genome-wide association study |
| HIU | 5-hydroxyisourate |
| H-NMR | Hydrogen nuclear magnetic resonance |
| HPGPRT | Hypoxanthine-guanine phosphoribosyltransferase |
| HPLC | high performance liquid chromatography |
| IACUC | Institutional Animal Care and Use Committee |
| ICRACU | Integrated Centre for Research Animal Care & Use |
| IM | Intramuscular |
| IMP | Inosine monophosphate |
| IP | Intraperitoneal |
| IV | Intravascular |
| LC-MS | liquid chromatography–mass spectrometry |
| MS | Mass spectrometry |
| NAD | Nicotinamide adenine dinucleotide |
| NMR | nuclear magnetic resonance |
| OECD | Organisation for Economic Co-operation and Development |

| | |
|---|---|
| OHCU | 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline |
| OMPDC | Orotidine-5′- monophosphate decarboxylase |
| OPRT | Orotate phosphoribosyltransferase |
| PNP | Purine nucleoside phosphorylase |
| PO | Potassium oxonate |
| PRPP | Phosphoribosyl pyrophosphate |
| RAS | Renin-angiotensin system |
| SU | Serum urate |
| SUA | Serum uric acid |
| UA | Uric acid |
| ULT | Urate-lowering therapy |
| UOX | Urate oxidase |
| XDH | Xanthine dehydrogenase |
| XMP | Xanthosine monophosphate |
| XO | Xanthine oxidase |
| YSL | Yolk syncytial layer |

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND OF THE STUDY

In recent years, the prevalence of gout inflammatory disease has an increasing trend in the population of developed countries (Kuo et al., 2015). Poor dietary habits such as consumption of fast foods, alteration in lifestyle due to lack of exercises, increased rate of obesity and metabolic syndrome are among the causes of the gradual increase in the worldwide incidence of gout. In the general population, the prevalence of gout is 1-4 %, 3-6% in men and 1-2% in women in western countries, and possible increase of up to 10% in other countries (Ragab, Elshahaly, & Bardin, 2017). The prevalence of gout show variation based on region and ethnicity in the Asia-Pacific population, which offer studies on genetics insight of the disease (Paul & James, 2017).

Gout can lead to severe damage in joints and associated muscles if it persists for a long time, and it can also lead to permanent disabilities. The burden of the disease is substantial and worsening; while available drugs are limited, most of them have serious side effects (Sutaria, Katbamna, & Underwood, 2006). Therapeutic failure in the management of gout is frequent due to poor adherence to urate-lowering drugs which can cause risk of gout flares (Ragab et al., 2017). Thus, there is a need for more effective and much safer alternatives to these drugs. There is a need to conduct experimental trials on animal models before vending them for human use, and even before running clinical trials. Rodents have been the common choices to model human diseases, however, there is a need for a vertebrate model that is physiologically simple yet similar to humans before proceeding with more complex animals such as mice and rats, hence, the use of zebrafish is rapidly increasing and gaining popularity.

Zebrafish is a promising animal to develop models of human diseases, including metabolic conditions, due to its rapid breeding and development, and morphological and physiological similarity to mammals, which accelerates the process of drug screening and discovery. In the current study, the development and evaluation of a zebrafish hyperuricemia model was proposed. A novel zebrafish animal model was used to assess and demonstrate its potential for modeling human behavioral and metabolic disorder, and the identification of possible biomarkers and pathways involved in hyperuricemia.

## 1.2 STATEMENT OF THE PROBLEM

Gout is a disease caused by high levels of uric acid, a condition known as hyperuricemia, which may cause the deposition of uric acid crystals in tissues. Elevated uric acid levels precipitates into uric acid crystals and deposits in joint structures and in tissues in the form of tophi that result in acute inflammatory attacks (Keenan & Pillinger, 2009). Acute arthritis, soft tissue inflammation, chronic tophus formation, gouty nephropathy, and nephrolithiasis are some of the clinical manifestation of gout (Ferri, 2013).

New treatments for hyperuricemia are being developed as the prevalence of gout increases. In Malaysia, anti-inflammatory agents are generally used for the alleviation of acute and chronic gout, with prescription of corticosteroid at a low level (Yeap, Goh, & Gun, 2010). Conventional medications for the treatment of hyperuricemia, such as allopurinol and benzbromarone, have some clinical effectiveness, and are mostly used to inhibit uric acid production and promote uric acid excretion. However, these drugs have adverse side effects, such as liver and kidney damage, bone marrow suppression, gastrointestinal reactions, and relapse after

withdrawing the medication. Thus due to the side effects, long-term use of these medications is not suggested (Pan et al., 2013). Thus, drug discovery research is still ongoing in order to produce drugs with minimum side effects.

Some animal studies have demonstrated the condition of gout and the effects of hyperuricemia on the kidneys as well as highlighting certain mechanisms related to the disease (Martin et al., 2011; Mazzali et al., 2001; Pineda et al., 2015; Torres et al., 2009). Most animals models of induced hyperuricemia demonstrate a common feature of hypertension, with afferent glomerular arteriolopathy, which could be corrected by xanthine oxidase inhibitor and urate lowering drugs that may reduce the hyperuricemia (Mazzali et al., 2002, 2010; Perlstein et al., 2006; Sánchez-Lozada et al., 2002). Nevertheless, most studies showing that hyperuricemic animals can cause development of hypertension and renal disease does not address serum uric acid levels and lack definite interpretation on how it may relate to human condition. Furthermore, there are not enough studies that are able to confirm the findings of improved renal function and reduced uric acid levels in patients with gout (Johnson et al., 2003)

Since rodents have uricase which metabolizes uric acid, unlike humans, there is a need for a hyperuricemic animal model that may help to further investigate hyperuricemia and its relationship with other related renal problems. Chen & Xu (2004) stated that there are three major methods for establishing models of hyperuricemia; 1) by feeding or administering purine or uric acid and adding certain excretory inhibitors, if necessary, to increase serum uric acid level, 2) by inhibiting uricase activity to decrease uric acid decomposition, and 3) by splicing the urate oxidase gene and rearrange it, or in other words, knocking out the urate oxidase gene to induce hyperuricemia in mice or rats. Inducing hyperuricemia by using the urate oxidase inhibitor, potassium oxonate, which will raise the serum uric acid levels

(SUA) level by inhibiting the oxidation of uric acid, is among the most widely applied method and is also used to evaluate whether drugs have anti-hyperuricemic effects in animal studies (Chen, Wei, & Xu, 2006).

There are a few setbacks with the use of rodents as models for hyperuricemia and gout. Firstly, they are significantly hypouricemic, i.e. they have very low levels of uric acid because of the enzyme uricase that is missing in humans and the higher primates (Liu et al., 2016). These rodents can only become hyperuricemic after they are treated with a uricase inhibitor (Feig, Kang, & Johnson, 2008). Thus, when a uricase inhibitor such as oxonic acid is directly administered or mixed with the diet over a short time or a period of few weeks, SUA levels which are normally relatively low in rats and mice can be raised (Edwards, Weaver, & Schumacher, 2006). Low doses of oxonic acid can cause mild hyperuricemia in rats without intrarenal deposition of urate crystals (Filiopoulos, Hadjiyannakos, & Vlassopoulos, 2012). To obtain a prolonged sustained hyperuricemic condition, a higher concentration of oxonic acid is needed to make the SUA levels become even higher, which could cause another setback, which is crystals formation and precipitation that could arise when investigators want to look only at the effect of uric acid on renal function, without the crystals.

Although there are several potential studies on animal hyperuricemia models (Wu et al., 1994; Tang & Yang, 2000; Chen et al., 2001; Yu et al., 2002; Mazzali et al., 2002; Chen, et al., 2003; Chen & Xu, 2004; Zhu et al., 2004; Sánchez-Lozada et al., 2005; Xu & Shi, 2006; Xu et al., 2007; Hu et al., 2010) data from animal studies are yet to be made conclusive. Outcomes from animal models are usually species specific, and may provide some evidence, but do not necessarily translate into the core and basic to the disease process in humans. The role of uric acid as an independent

risk factor for kidney disease development and progression is pointed out by large epidemiological studies but it is not definitively conclusive. Clinical interventional trials to explain whether lowering of uric acid results in inhibition or, at least, slowing of renal disease is needed (Filiopoulos et al., 2012). Extrapolation of findings of experiments in animals to human conditions is not always straightforward. (Dousdampanis et al., 2014). Thus, it is important to be careful in interpreting and extrapolating animal models to human conditions (Mazzali et al., 2002; Kang & Nakagawa, 2005).

In the oxonic acid induced-hyperuricemia rat model, inflammatory differentiation of cultured macrophage-lineage cells and modulates renal transport of uric acid can be promoted by oxonate alone, and thus possibly can affect intracellular handling and effects of uric acid in renal proximal tubular cells, which calls for caution with interpreting this model. Thus, some mechanisms of renal disease in the oxonic acid–induced hyperuricemia in vivo model continue to be resolved; preferably, an alternative model of hyperuricemia would be useful on this challenge (Neogi et al., 2012).

## 1.3 PURPOSE OF THE STUDY

The establishment of animal models is important to study the vital functions and conditions in humans. According to Simmons (2008), animal models are employed in the study of human disease because of their similarities to humans in terms of genetics, anatomy, and physiology, and their unlimited supply and ease of manipulation. Animal models of mice and rat are widely used and have been established to study human disease and drug screening. Similar studies are now being done on zebrafish to examine alternative functions in vivo at high throughput.

Zebrafish have unique features and they share significantly similar amount of genetic identity as well as organ system with humans (Seth, Stemple, & Barroso, 2013). Its inexpensive husbandry maintenance and production of large numbers of offspring as well as rapid growth development give zebrafish the advantage in reducing time and cost of carrying out in vivo studies, thus, making it an attractive research tool to study human diseases (Tsang et al., 2017). Zebrafish models have been developed for several metabolic disorders, such as diabetes, liver disease, and obesity. However, no zebrafish model on hyperuricemia has been developed yet. There is still a need for a novel understanding of the pathogenesis of hyperuricemia, and novel agents for the management of hyperuricemia and gout. Hence, the development of a valid zebrafish model for hyperuricemia will give a better understanding of its pathological condition and metabolite profiling and, thus, aid in the development of novel agents, in addition to the efficacy in toxicity testing for drug discovery to treat hyperuricemia and gout with greater efficacy and safety.

## 1.4 RESEARCH OBJECTIVES

The present study has been undertaken to develop a zebrafish hypo- and hyperuricemia model. More specifically, the objectives of this study were as follows:

1. To induce hypouricemia in the developing zebrafish by using allopurinol
2. To induce hyperuricemia in the developing zebrafish by using potassium oxonate
3. To identify any possible biomarkers involved in hypo- and hyperuricemia induced zebrafish using LC-MS

## 1.5 RESEARCH QUESTIONS

The research questions of this study were as follows:

1. Can hypouricemia be induced in the developing zebrafish using allopurinol?
2. Can hyperuricemia be induced in the developing zebrafish using potassium oxonate?
3. What are the possible biomarkers involved in hypo- and hyperuricemia induced zebrafish using LC-MS?

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 HYPERURICEMIA AND GOUT

Gout is a metabolic disease associated with increased concentrations of uric acid in the blood (Choi, Mount, & Reginato, 2005). Gout is also defined as a disease of purine metabolism (Jiménez & Puig, 2012) During purine metabolism, the enzyme xanthine oxidase converts the purine bases xanthine and hypoxanthine to uric acid. Inborn errors metabolism can lead to overproduction or under excretion of uric acid can subsequently to hyperuricemia. In gout, serum uric acid concentration increases more than its solubility threshold, exceeding 6.8 - 7.0 mg/dL (~408-420 μM) in vitro, hence defined "hyperuricemia" (Terkeltaub, 2011).

Chronic hyperuricemia can cause uric acid precipitation in tissues and joints. Usually before gout is clinically expressed, a prolonged asymptomatic hyperuricemia often precedes. Initially this period can be recognized as the first stage, followed by self-resolving acute attack of gout, which involves inflammation and pain around joints. The persistent deposition of uric acid crystal in joints then can lead to advanced gout and tophi (Grassi et al., 2013). Acute gout usually resolves in 5 to 6 days but repeated attack is of high probability. Chronic gout can lead to complications and deformities like permanent damage to joints, dysfunction joints and tophi. (Burns & Wortmann, 2012).

### 2.1.1 Purine Metabolism in the Pathogenesis of Hyperuricemia

Purines are molecules with various roles in cell physiology and are important for nucleic acid synthesis, energy-requiring and cofactor reactions, and intercellular and intracellular signaling. Purine metabolism synthesize the ribonucleotides adenosine monophosphate (AMP), inosine monophosphate (IMP), xanthosine monophosphate (XMP), and guanosine monophosphate (GMP), which in return produces deoxyribonucleotides which are essential for the synthesis of nucleic acids (DNA and RNA) (Jiménez & Puig, 2012). Uric acid is the final compound of purine metabolsim in humans. Enzymes in the purine metabolism retain a balanced ration between their synthesis and degradation in the cell under physiological conditions (Maiuolo et al., 2016).

Purine in the cells are produced through three different processes (Figure 2.1): 1) purine synthesis de novo from smaller organic molecules i.e. interconversion of purine nucleotide (de novo purine synthesis), 2) salvage of preformed purine bases (salvage purine synthesis), and 3) purine uptake from the extracellular medium i.e. degradation of purine nucleotide (pyrimidine synthesis) (Terkeltaub, 2011). The product of breakdown of synthesized purine nucleotides, which is the uric acid, its level in serum is determined by its rate of formation and excretion (Anzai & Endou, 2007). Maiuolo et al. (2016) also described in his review the enzymatic degradation of purines in humans, their structure and biochemistry until the formation of uric acid. Excess uric acid in humans generated from purine metabolism has shown to have rising roles in human disease, with increase of SUA being inversely associated with severity of disease (Maiuolo et al., 2016).