UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Synthesis and evaluation of 5-substituted (thio)barbiturates as proteasome inhibitors for cancer therapy

Experiência Profissionalizante na vertente de Farmácia Comunitária, Hospitalar e Investigação

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Relatório para obtenção do Grau de Mestre em
Ciências Farmacêuticas
(Ciclo de estudos Integrado)

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Dedications

To my parents,

to my sisters and brothers,

to my family and friends,

to the Global Platform for Syrian Students,

to Syria,

to the martyrs of Syria,

to Allah.

Acknowledgments

This dissertation is the culmination of five years of hard work and challenges, which would not have been achieved without the support and assistance I have received.

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I would also like to thank the entire staff of Holon Covilhã Pharmacy for all the knowledge and skills they have transmitted to me, for being always available when I had any doubts, for their friendship and for the hospitality. A special thanks to my supervisor Dr. Patrícia Pais for Her willingness to give her time so generously to teach me.

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Most importantly, I would like to express my sincere gratitude to the Global Platform for Syrian Students, its founder His Excellency Dr. Jorge Sampaio, his diplomatic adviser, Dr. Helena Barroco and all the staff for the generous opportunity they have given me to finish my studies in Portugal. My educational pursuits would not be possible without their endless

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Resumo alargado

O presente relatório de estágio foi elaborado com o objectivo de obter o grau de Mestre em Ciências Farmacêuticas. Encontra-se subdividida em três capítulos que abordam cada um dos períodos de aprendizagem inseridos na unidade curricular "Estágio" do Mestrado Integrado em Ciências Farmacêuticas.

No primeiro capítulo é descrito todo o trabalho realizado no âmbito da área de investigação desenvolvida no Centro de Investigação em Ciências da Saúde da Universidade da Beira Interior, na área da química farmacêutica, sendo este intitulado "Synthesis and evaluation of 5-substituted (thio)barbiturates as proteasomal inhibitors for cancer therapy".

O sistema ubiquitina-proteassoma foi definido como um potencial alvo terapêutico no tratamento de uma série de condições clínicas, tais como inflamação, doenças neurodegenerativas e cancro, particularmente, malignidades hematológicas. Uma variedade de produtos sintéticos e naturais têm sido associados a atividade inibitória do proteassoma, dos quais três compostos, bortezomib, carfilzomib, e ixazomib foram aprovados para uso clínico no tratamento do mieloma múltiplo. Os derivados de arilaldeídos de 2-tioxoimidazolidin-4-ona foram descritos como novos inibidores não covalentes de proteassoma num estudo recente. Face a isso, e considerando a similaridade estrutural dos últimos com os ácidos (tio)barbituricos, hipotetizou-se que derivados 5-substituídos de ácidos (tio)barbitúricos também poderiam ter atividade inibitória do proteassoma. Neste contexto, é também conhecido que vários derivados de barbituratos demonstraram efeitos antiproliferativos promissores em diferentes linhas celulares de cancro.

No presente trabalho, foram desenvolvidos varios derivados 5-benzilideno(tio)barbitúricos utilizando como precursores ácidos (tio)barbitúricos e benzaldeídos e água como solvente. Os compostos obtidos foram devidamente caracterizados por determinação dos seus pontos de fusão, e espectroscopia de ressonância magnética nuclear de protão e carbono-13. Verificou-se, durante o estudo, a degreadação dos derivados 5-benzilideno tiobarbitúricos e a formação de bis-(tio)barbiturates em soluções aquosas, levando à exclusão destes compostos da avaliação biológica. Assim, tentou-se sintetizar seletivamente os bis-(tio)barbiturates puros, embora sem sucesso. Alternativamente, realizou-se a redução da dupla ligação exocíclica de 5-benzilidenotiobarbituratos como uma tentativa de aumentar a estabilidade dos compostos sintetizados. Os compostos obtidos foram incluídos nos estudos biológicos.

Nos estudos da avaliação biológica foram incluídos os derivados 5-benzilidenobarbitúricos e os 5-benzyl(thio)barbituratos, determinado-se a sua atividade como inibidores do proteassoma e da xantina oxidase, tendo sido também analisando os seus efeitos citotóxicos em linhas celulares saudáveis (NHDF) e cancerígenas (Caco-2 e PC-3).

A atividade inibitória do proteasoma dos compostos foi determinada mediante *o bioluminescent Proteasome-Glo™ Assay*. Num screening inicial, incubaram-se os compostos com a enzima em duas concentrações (10 e 100 μM), tendo sido utilizado o bortezomib como controlo positivo. Para o 5-[1-[2-(4-nitrofenil)hidrazinil]etilideno]-barbiturato **9b**, composto que demonstrou a melhor atividade inibitória, procedeu-se a ensaio posterior para determinar a curva concentração-resposta. Os valores de IC_{50} foram calculados por um ajuste sigmoidal dos resultados obtidos, considerando-se um intervalo de confiança de 95%. Adicionalmente, os compostos foram avaliados quanto à capacidade para inibir a xantina oxidase, não se verificando atividade inibitória da mesma.

A similaridade estrutural de **9b** e bortezomib foi verificada visualmente, sendo realizados estudos *in silico* de superposição bidimensional e tridimensional.

Os estudos de viabilidade celular foram realizados em fibroblastos normais da derme humana em células de cancro da próstata, e em células de cancro de cólon. Num screening inicial, as células de NHDF foram expostas aos compostos, em concentração de 10 e 100 μM durante 72 horas, tendo sido utilizado o 5-fluorouracilo como controlo positivo. A determinação da proliferação celular foi realizada mediante o ensaio do brometo de [3-(4,5-dimetiltiazol-2-yl)-2,5-difenil tetrazólio]. Para o composto **9b**, que apresentou a maior atividade inibitória no ensaio do proteassoma e para bortezomib, efectuaram-se estudos de concentração-resposta. Para tal, os compostos foram incubados com as células, em seis concentrações distintas de cada composto no mesmo período de 72 horas, e os valores de IC_{50} foram calculados por um ajuste sigmoidal, considerando-se um intervalo de confiança de 95%. Adicionalmente, as células PC-3 e Caco-2 foram expostas a **9b** e a bortezomib em diferentes concentrações, igualmente durante um período de 72 h. O composto **9b** demonstrou citotoxicidade nas linhas celulares saudáveis e cancerígenas mas, contudo, esta foi inferior à citotoxicidade apresentada pelo bortezomibe.

Como trabalho futuro, propõe-se estudos de viabilidade celular linha celular Jurkat, linha de células T leucémicas. Adicionalmente, propõe-se realizar estudos do *docking molecular* para prever o modo de ligação e afinidade de ligação do **9b** com o proteassoma. Por outro lado, será igualmente importante sintesar mais derivados destes compostos, variando os substituintes no seu esqueleto molecular de modo a otimizar os resultados biológicos obtidos.

O segundo capítulo é dedicado ao estágio em Farmácia Comunitária orientado pela Dr.ª Patrícia Pais e realizado na Farmácia Holon Covilhã. O terceiro capítulo refere-se ao estágio em Farmácia Hospitalar, que foi realizado no *Barts Heart Centre*, no Hospital *St Bartholomew*, em Londres, Reino Unido orientado pelo Dr.Paul Wright, *Lead Cardiac Pharmacist* em *Barts Health NHS Trust*. Em ambos os relatórios são abordados as atividades desenvolvidas, os conhecimentos adquiridos ao longo dos meus estágios em farmácia comunitária e em farmácia hospitalar.

Palavras-chave

Inibidores do proteassoma, Cancro, (tio)barbituratos 5-substituídos, Citotoxicidade, Farmácia Comunitária, Farmácia Hospitalar.

Abstract

The present training report was developed to obtain the integrated master's degree in pharmaceutical sciences. It is subdivided in three chapters that address the three main activities enclosed in the curricular unit "Internship" of the Integrated Master in Pharmaceutical Sciences.

The first chapter detailed the laboratorial research component, developed at the Health Sciences Research Center from University of Beira Interior in the area of pharmaceutical chemistry. The ubiquitin proteasome system has been defined as potential target in the treatment of a range of clinical conditions, such as inflammation, neurodegenerative diseases and cancer, particularly, haematological malignancies. A variety of chemical synthesized and natural products have exhibited proteasome inhibitory activity from which three were approved for use in multiple myeloma treatment. 2-Thioxoimidazolidin-4-one arylaldehyde derivatives were described as novel noncovalent proteasome inhibitors in a recent study. Considering the structural similarity of thiobarbituric acid and 2-thioxoimidazolidin-4-one systems, 5-substituted (thio)barbiturate derivatives where designed and efficiently synthesized in this work as a potential novel class of proteasome inhibitors with anticancer interest. In this context, several barbiturate derivatives demonstrated promising antiproliferative effects in different cancer cell lines. Stability study of the 5-benzilidene(thio)barbiturates derivatives in solution was performed and led to the exclusion of 5-benzilidene thiobarbiturates due to their instability. Then, a xanthine oxidase and proteasome inhibition assay were performed. None of the compounds showed relevant inhibition of xanthine oxidase enzyme. However, 5-[1-[2-(4-nitrophenyl)hydrazinyl]ethylidene]barbiturate, **9b**, showed interesting inhibitory activity in the proteasome inhibition assay. Cytotoxicity of the assayed compounds was evaluated by the MTT assay in healthy (NHDF) and antiproliferative effect of **9b** in cancer cell lines (Caco-2 and PC-3) was assessed and compared with the effect of bortezomib, an approved proteasome inhibitor. Although **9b** showed cytotoxicity against healthy and cancer cell lines, it was less potent than bortezomib.

The second chapter describes the activities accomplished during the internship in Community Pharmacy that took place in the Pharmacy Holon Covilhã under the supervision of Dr. Patrícia Pais. It is divided to present generally the operations performed at the pharmacy, the legislation that regulates the sector, and the tasks and activities perform.

The third chapter describes the hospital pharmacy internship at The Barts Heart Centre in St Bartholomew's Hospital in London, UK that was guided by my main supervisor and contact person, Paul Wright, the Lead Cardiac Pharmacist at Barts. It is organized based on the

activity I observed or performed myself. It is divided into four main subchapters, The Barts Heart Centre, Oncology, Clinical trials, and Dispensary.

Keywords

Proteasome inhibitors, Cancer, 5-Substituted (thio)barbiturates, Cytotoxicity, Community Pharmacy, Hospital Pharmacy.

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List of Acronyms, Abbreviations and Symbols

ACE	Angiotensine Converting Enzyme
ACS	Acute Coronary Syndrome
AF	Atrial Fibrillation
ANF	*Associação Nacional de Farmácias*
AR	Aortic Regurgitation
ARIA	Electronic Prescribing Platform
AS	Arterial stiffness
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BPF	*Boas Práticas Farmacêuticas*
BP	Blood Pressure
BD	Twice daily
C-L	Caspase-Like
Caco-2	Human Epithelial Colorectal Adenocarcinoma Cells
CCF	Invoice Conference Center
CIMPI	*Centro de Informação de Medicamentos de Preparação Individualizada*
Cr	Creatinine
CT-L	Chymotrypsin-Like
CTA	Clinical Trial Agreement
CVD	Cardiovasculer Diseases
DAPT	Dual Antiplatelet Therapy
DCM	Dichloromethane
DES	Drug Eluting Stent
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
DMSO-d_6	Deuterated DMSO
DVT	Deep Vein Thrombosis
E1	Ubiquitin-activating enzymes
E2	Ubiquitin-transferring enzymes
E3, E4	Ubiquitin ligases
ECG	Electrocardiogram
EF	Ejection Fraction
FBS	Fetal Bovine Serum
FCH	*Farmácia Holon Covilhã*
FDA	The Food and Drug Association
FEFO	First Expired, First Out
FIFO	First In, First Out
GP	General Practitioner
GTN	Glyceryl Trinitrate Spray
HF	Heart Failure
HR	Heart Rate
HTN	Hypertension
Hz	Hertz
IC_{50}	Half of the maximal inhibitory concentration

IMP	investigational medicinal products
INE	National Statistical Institute
INR	International Normalized Ratio
IPTB	Ankle-Brachial Pressure Index
IV	Intravenous
J	coupling constants
LAD	left anterior descending coronary artery
LV	Left Ventricle
mAVR O-nX	On-X mechanical Aortic Valve Replacement
MI	Myocardial Infarction
mp	Melting Points
MTT	3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NHDF	Normal Human Dermal Fibroblasts
NHS	National Health System
NOAC	Novel Oral Anticoagulants
NSTEMI	Non-ST-segment elevation myocardial infarction
OD	Once a Day
ON	Once at Night
OTC	Over-The-Counter
PC-3	Human Prostate Cancer Cell
PCI	Percutaneous Coronary Intervention
PE	Pulmonary Embolism
ppm	Parts Per Million
PODs	Patients' Own Medications
PRN	As Needed
PVP	*Preço de Venda ao Público*
PVF	*Preço de Venda à Farmácia*
QID	Four Times a Day
RCA	Right Coronary Artery
RP	Regulatory Particle
SNS	*Sistema Nacional de Saúde*
SPO_2	Peripheral Capillary Oxygen Saturation
STEMI	ST-Elevation Myocardial Infarction
T-L	Trypsin-Like
TDM	Therapeutic Drug Monitoring
THF	Tetrahydrofuran
TID	Three Times a Day
TLC	Thin Layer Chromatography
TTA	To Take Away
Ub	Ubiquitin
UPP	Ubiquitin Proteasome Pathway
VTE	Venous Thromboembolism
XO	Xanthine Oxidase
5-FU	5-Fluorouracil
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
δ	chemical shift

Chapter 1 - Synthesis and evaluation of 5-substituted (thio)barbiturates as proteasome inhibitors for cancer therapy

1.1 Introduction

Proteostasis, cellular protein homeostasis, is a key requirement for cell viability.[1] Proteostasis is achieved by interacting pathways that maintain the balance between normal protein synthesis and abnormal protein degradation.[2] Various conditions such as oxidative stress, aging, ultra-violet light exposure, fluctuating nutrient supply and diseases may increase proteins' misfolding and mutation.[3,4] Accumulation of damaged proteins beyond repair may not only lead to cytotoxicity but also trigger abnormal cellular status. The cell possesses many degradative pathways to help maintaining proteostasis in addition to controlling different physiological processes like the cell cycle, signaling, DNA transcription, repair and translation by downregulating their critical regulatory proteins. The main protein degradation pathways are ubiquitin (Ub)-based proteasome-mediated pathways and lysosomal-mediated pathways.[5] The discovery and study of both systems were of a great importance in understanding different regulatory process in the cell, which has been appreciated with two Nobel Prizes. In 2004, Aaron Ciechanover, Avram Hershko, and Irwin Rose received the Nobel Prize in Chemistry for the discovery of Ub-mediated protein degradation, and in 2016, Yoshinori Ohsumi was awarded the Nobel Prize in Medicine and Physiology for his discoveries of mechanisms for autophagy.[6]

1.1.1 Proteasome Biology

Ubiquitin proteasome pathway (UPP) is responsible for the degradation of damaged and short-lived regulatory proteins as well as most of the slower breakdown of the bulk of cellular proteins. UPP is expressed in all eukaryotic cells.[7] Proteasome may be classified into 20S proteasome and 26S proteasome in relation to the dependence on Ub/ Adenosine triphosphate (ATP) proteolytic mechanism.[8] The 26S proteasome is the cornerstone of the UPP of protein degradation. It is a multicatalytic protease expressed in the cytoplasm and nucleus of all eukaryotic cells. The 26S proteasome is a multisubunit complex that consists of two main components, 20S catalytic core and 19S regulatory complex (Figure 1.1). The 28 subunits of the 20S proteasome catalytic core comprise two groups of 7α subunits in the two end rings and two groups of 7ß subunits in the two central rings, which form a highly conserved cylindrical structure of four rings. α-Subunits at the end rings serve as a gate where proteins get access to the proteolytic site. $ß_1$, $ß_2$ and $ß_5$ in the inner rings are the active

peptidase sites responsible for caspase-like (C-L) activity (cleavage after acidic residue), trypsin-like (T-L) activity (cleavage after basic residue), and chymotrypsin-like (CT-L) activity (cleavage after hydrophobic residue), respectively.[9-11] The CT-L site was considered to have the most important role in protein breakdown,[12,13] however, it was demonstrated later that C-L and T-L activities are also essential in the protein degradation process.[14] The hydroxyl group of the *N*-terminal threonine of these active sites act as a nucleophile that attacks and cleaves the peptide bonds of target proteins.[15,16]The regulatory particle (RP) 19S is composed of two subcomplexes, a lid which is made of nine subunits (Rpn3, 5-9, 11, 12, and 15) and a base associated with the core that comprises ten subunits including six ATPases (four Rpn1, 2, 10, 13, and Rpt 1-6).[17] RP is an ATP-dependent activator that is responsible for the recognition of polyubiquitylated proteins, deubiquitylation and recycling of polyubiquitin chains, proteins' unfolding and translocation of unfolded proteins into the interior of S20 core particle for degradation.[8]

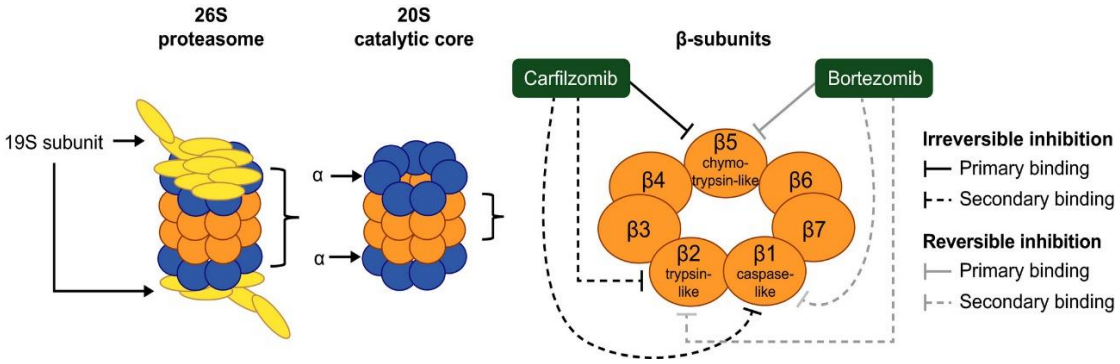


Figure 1.1: Structure and components of the 26S proteasome and the location of the active sites in the 20S core. (From Yong *et al*. 2018)[18]

Ub-dependent process consists of two key steps: target protein ubiquitination, and protein degradation (Figure 1.2).

Ub, a small protein composed of 76 amino acids, is attached to a target protein as a recognition sign in a process called ubiquitination. This process involves three classes of enzymes, identified as ubiquitin-activating enzymes (E1), ubiquitin-transferring enzymes (E2) and ubiquitin ligases (E3, E4).[19-22] Ubiquitination starts when an E1 enzyme activate an Ub molecule in a process powered by ATP. The activated Ub is then passed on to the E2 enzyme, which in turn presents ubiquitin to E3. E3 catalyzes the covalent attachment of ubiquitin to the target protein, which is specifi0 rrrrrdcally bound to E3.[8] In this process, the target protein may receive one ubiquitin (monoubiquitination) or multiple ubiquitin molecules (multiubiquitination) that is catalyzed by E4 ligases resulting in Ub chain elongation.[22]

Rpn10 and Rpn13 subunits in the base of RP functions as Ub receptors which recognize and capture ubiquitinated target proteins to deliver them to the proteasome.[23,24] Subsequently, Ub domain is removed by deubiquitinating enzymes in the lid of RP.[25,26] Additionally, the

substrates are unfolded by Rpt 1-6, hence they can pass through the narrow opening at the center of the α-rings that are gated by the *N*-terminal tails.[27,28]

Once in the central cavity, the carbonyl carbon of a peptide bond in the substrate suffers a nucleophilic attack by the oxygen atom on the side chain of the N-terminal threonine (Thr1Oγ) of the respective catalytically active subunit.[12,29] The resulting oligopeptides' length ranges from 3 to 12 amino acids, though they may reach 25 amino acids.[30]

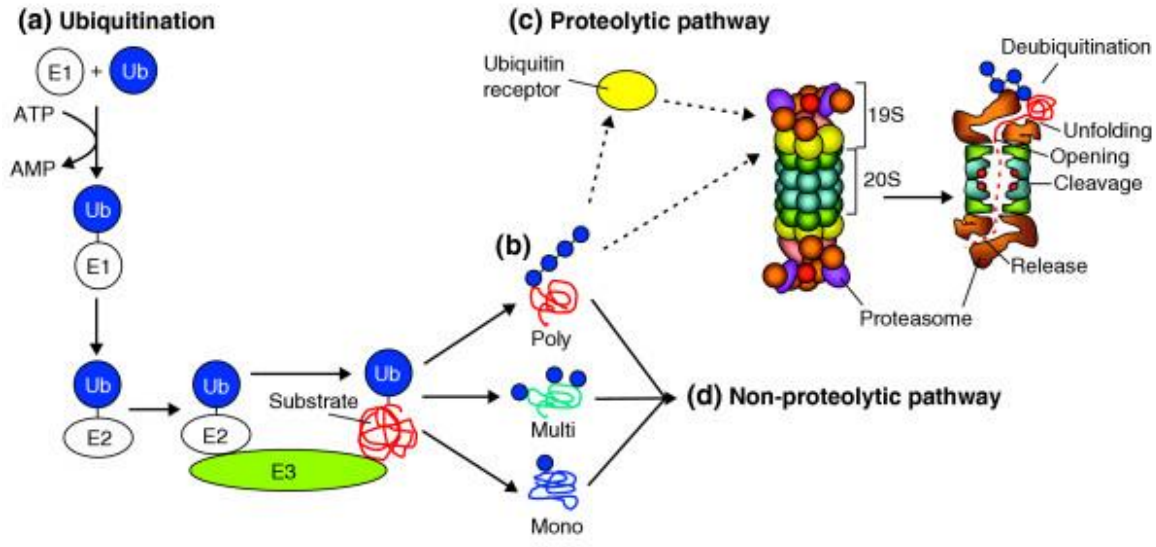


Figure 1.2: Ubiquitin-mediated protein degradation. (from Kaiser et al. 2005)[31]

Nevertheless, the 19S regulatory particle may be dissociated from 20S core particle, mostly under oxidative stress, where the dissociated 20S proteasome mediates proteolysis of oxygen-damaged proteins, mutated, or natural unfolded proteins.[8]

1.1.2 Proteasome as a possible therapeutic target

Medical interest in the proteasome as a target for drug discovery has been growing in the last two decades due to the remarkable clinical success of the first proteasome inhibitor, bortezomib (Velcade™) after being approved by the Food and Drug Association (FDA) in 2003 for the treatment of refractory multiple myeloma.[32]

The proteasome is essential in the regulation of cells apoptosis as it is involved in the regulation of many apoptosis-inducing protein levels.[33,34] Many malignant cells were found to have elevated proteasome activity,[35-38] where the proteasome downregulates some pro-apoptotic proteins protecting the cell from apoptosis.[39] Consequently, proteasome inhibition may result in the accumulation of apoptosis-inducing factors, leading to cell death.[40] Additionally, proteasome inhibition causes the accumulation of damaged proteins in the cell, which may increase the sensitivity of malignant cells to chemo- or radiotherapy as it was found in many studies.[41-44] Interestingly, many types of cancer cells were found to be more

affected by proteasome inhibition than normal cells.[41-44] Moreover impairment of angiogenesis in the tumors was showed to result from the inhibition of the proteasome, which would reduce tumors growth and metastasis.[45-47] Altogether, these findings have turned proteasome inhibition as a promising strategy to treat cancer.

Deregulation or failure of proteolytic activity of the proteasome seems to contribute to other diseases, including immune-related diseases,[48-50] cardiomyopathies,[51-55] and neurodegenerative diseases.[56-58] UPP has been shown to have a crucial role in cardiac cells survival and functions. Likewise, UPP has been found to play a key role in the regulation of ß-adrenergic signaling pathway in the heart.[59] There has been a large body of evidence indicating the involvement of proteasome system dysfunction in numerous heart diseases including ischemia, reperfusion, atherosclerosis, hypertrophy, heart failure, and cardiomyopathies.[55] Buildup of prohypertrophic and proapoptotic factors in addition to accumulation of oxidized proteins in cardiac cells are invoked by UPP function impairment.[60] The growing interest of UPP role in the heart is due to the unexpected serious cardiac side effects that were caused by proteasome inhibitors. Interestingly, short term bortezomib use seems to have a cardioprotective effect during ischemia/reperfusion injury and cardiac hypertrophy. Nevertheless, long term treatment with proteasome inhibitors is associated with significant cardiac toxicity.[60]

Many neurodegenerative diseases such as Alzheimer's and Parkinson disease among others are characterized by the aggregation of misfolded proteins. This could indicate an impairment of proteostasis and proteasome function. Consequently, the role of the UPP in this type of diseases has received significant interest in a wide body of studies. [61-66]

Proteasome inhibition demonstrated to produce a reduction in inflammation and immune response.[67] This effect is expected due to the essential role of proteasome in antigen processing and presentation in addition to its involvement in the signaling cascades in the immune cells.[68] Immunoproteasome, which is the proteasome expressed in the immune cells, was found to have a crucial impact in proinflammatory diseases where was shown to be inappropriately expressed.[69-72]

Given this considerable abundance of studies on the involvement of the proteasome in many diseases, it is clear that modulation of proteasome activity could make a potential treatment strategy for different conditions along with cancer. Hence, proteasome inhibition could be used to induce apoptosis in solid and hematologic cancer and to induce an immunosuppressant effect in autoimmune diseases, whereas proteasome-activating compounds could be useful for other diseases such as neurodegeneration.

1.1.3 Proteasome inhibitors

Inhibition of the proteasome has emerged as an advanced strategy following the preclinical and subsequent clinical development of bortezomib. Numerous proteasome inhibitors have been described, many of which interfere directly with the proteolytic activity of the 20S core particle, primarily the CT-L site. These inhibitors bind either reversibly or irreversibly to the active sites in the core particle, and they display varying levels of specificity for the 26S proteasome. Nowadays, only three inhibitors are approved to be used clinically, bortezomib, carfilzomib, and ixazomib for multiple myeloma or mantle-cell lymphoma (Figure 1.3).[73] All the three approved inhibitors are currently being investigated in several clinical trials as a single agent or combined with other agents in treatment regimens against multiple types of solid and hematological malignancies in addition to some autoimmune and inflammatory conditions.[74]

Five main classes of proteasome inhibitors can be identified based on their chemical structure and active moiety: peptide aldehydes, peptide boronates, peptide vinyl sulfones, peptide epoxyketones, and ß-lactone. Other compounds that display a wide variety of scaffolds of core structures and pharmacophores constitute a miscellaneous class of inhibitors.

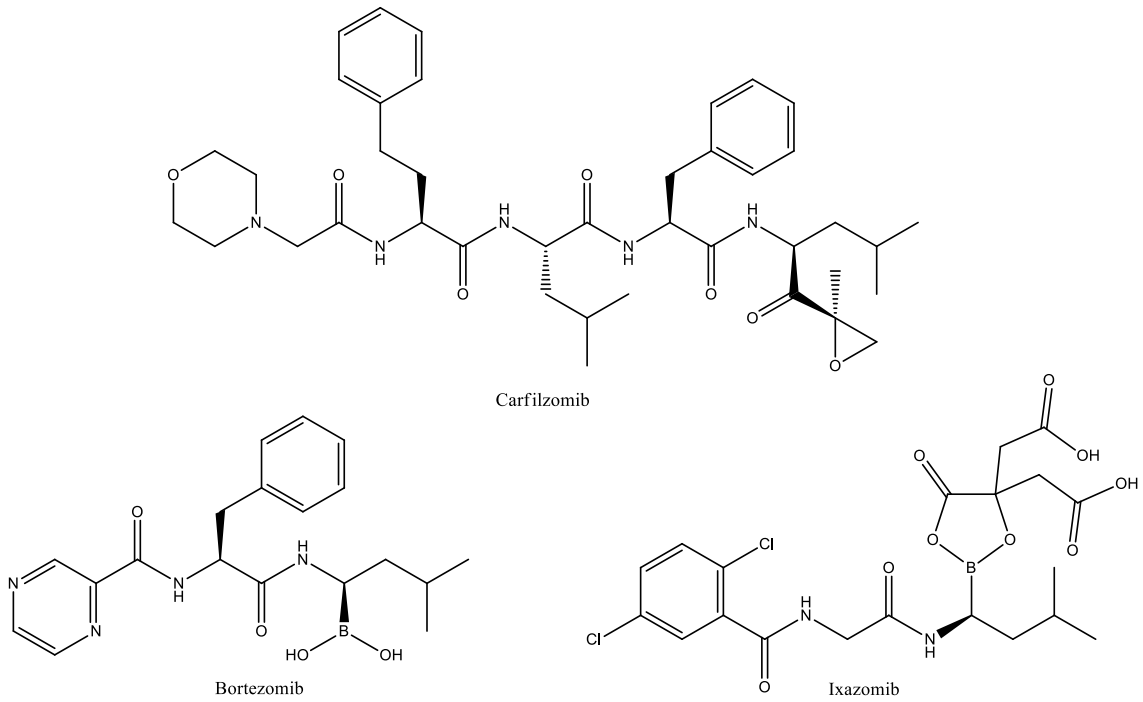


Figure 1.3: Proteasome inhibitors approved for the clinical use.

1.1.3.1 Peptide aldehydes

Peptide aldehydes are reversible proteasome inhibitors that were the first class to be developed and they are still widely used in research.[75,76] In addition to CT-L site inhibition, these compounds can inhibit cysteine and serine proteases, which lowers their selectivity as

20S proteasome inhibitors. Peptide aldehydes have stability problems as they are rapidly oxidized into inactive acids *in vivo* and did not show systemic activity in treated mice.[77] MG132 (carbobenzoxyl-*L*-leucyl-*L*-leucyl-*L*-leucinal) is one of the most potent peptide aldehydes (Figure 1.4). It is a hydrophobic tripeptide aldehyde extracted from a Chinese medicinal plant.[78]

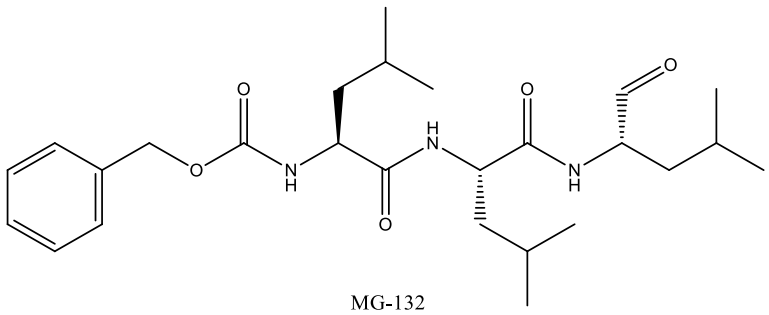


Figure 1.4: MG132 (carbobenzoxyl-*L*-leucyl-*L*-leucyl-*L*-leucinal)

1.1.3.2 Peptide boronates

Peptide boronates are reversible proteasome inhibitors that target mainly CT-L and C-L sites with minimal effect on T-L activity.[79] However, the boronate-proteasome complexes have very slow dissociation rates due to the formation of covalent bonds.[80] The inhibitors in this class are characterized by being more potent, metabolically more stable, and have higher selectivity than the corresponding peptide aldehydes.[81] Thus, the peptide boronates arose as better drug candidates than other classes of proteasome inhibitors. Bortezomib, a dipeptide boronic acid analog, binds covalently to the catalytic threonine residue in the active site of the 20S proteasome *via* its boron atom.[80] It is indicated as a first-line treatment of previously untreated or relapsed mantle cell lymphoma and multiple myeloma. Bortezomib is usually administered intravenously. However, some clinical issues have emerged after treatment with bortezomib such as several side effects[82] in addition to development of intrinsic and acquired resistance.[83] Ixazomib is the first orally bioavailable and the most recent approved proteasome inhibitor.[84] Ixazomib showed to produce apoptosis in bortezomib-resistant multiple myeloma cells in in addition to lower incidence of peripheral neuropathy.[85]

1.1.3.3 Peptide vinyl sulfones

Peptide containing a *C*-terminal vinyl sulfone moiety are irreversible proteasome inhibitors that form a covalent bond with proteolytically active subunits of the proteasome. These compounds inhibit mainly CT-L activity and to a less extent C-L and T-L activities.[86] However, these compounds are less potent than aldehydes and have similar limitation of selectivity.[86,87] These compounds are mainly used in research. ZLVS (*Z*-Leu-Leu-Leu-vs), a vinyl sulfone

analogue of MG132, and NLVS are examples of vinyl sulfones that act as a proteasome inhibitors (Figure 1.5).[86]

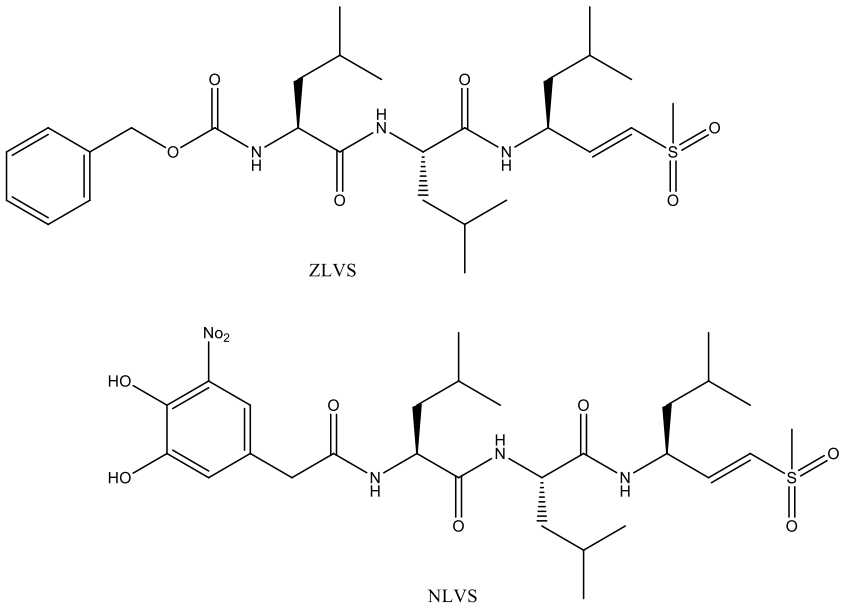


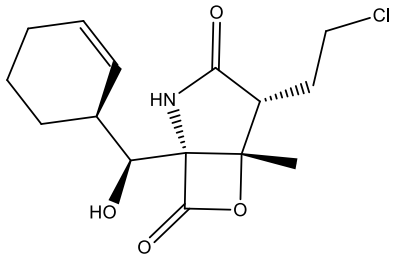
Figure 1.5: peptide vinyl sulfone inhibitors ZLVS and NLVS.

1.1.3.4 Peptide epoxyketones

Carfilzomib (Figure 1.3) was the second proteasome inhibitor to be approved after bortezomib for the treatment of relapsed and refractory multiple myeloma.[88] It is an irreversible inhibitor developed from epoxomicin natural product that contains an epoxyketone group that binds covalently and selectively to the proteasome.[89] The main objectives of developing carfilzomib were to tackle the resistance issues and the side effects arisen with bortezomib. Nevertheless, various side effects are associated with this drug and it is susceptible to resistance development in multiple myeloma.[90]

1.1.3.5 ß-lactone

Marizomib (Figure 1.6) is a non-peptide natural product containing a ß-lactone-γ-lactam bicyclic ring.[91] It is an orally active[92] and a very potent irreversible inhibitor of the three activities of the proteasome CT-L, C-L , and T-L.[93,94] The use of marizomib is currently being investigated in a wide range of hematological and solid malignancies.



Marizomib

Figure 1.6: Chemical structure of marizomib.

1.1.3.6 Miscellaneous

In addition to these main classes, many natural and synthetic non-peptidic compounds have demonstrated inhibitory potency against the proteasome.[95,96] However, these identified inhibitors are still less potent than the approved drugs. We based our work on Maccari *et al*. study that identified 2-thioxoimidazolidin-4-one derivatives (Figure 1.7) as novel proteasome inhibitors.[97]

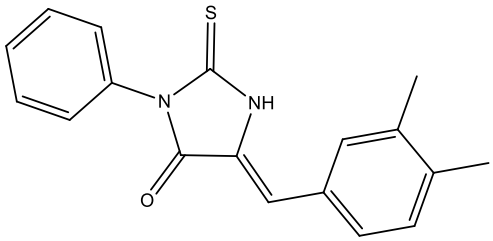


Figure 1.7: (5Z)-5-arylidene-3-aryl-2-thioxoimidazolidin-4-ones.

1.1.4 Objectives

In the last years, our research group has been widely involved in the synthesis of (thio)barbituric acid derivatives. This class of compounds has attracted the attention due to its wide range of biological activities in addition to their classical use related to CNS diseases. Most of the studies of our group have been focusing mainly on developing (thio)barbiturates derivatives as xanthine oxidase (XO) inhibitors, antioxidants, antibacterial and anti-proliferative agents.[98,99]

Many studies, in addition to the ones conducted by our group,[98,100] have reported antiproliferative activity of (thio)barbituric-containing compounds in different cancer cell lines.[101–103] Based on these observations and considering the structural similarity of thiobarbituric acid and 2-thioxoimidazolidin-4-one systems, we hypothesized that arylidene barbiturate and thiobarbiturate derivatives may inhibit the proteasome system. Hence, this

study may lead to discovery of a novel class of proteasome inhibitors with potential anticancer interest.

In the present study, we describe the synthesis and *in vitro* biological characterization of 5-substituted (thio)barbiturates. The effect of these compounds was investigated as proteasome inhibitors and XO inhibitors using enzymatic assays. Likewise, their cytotoxicity effect against normal human dermal fibroblasts (NHDF), Prostate cancer line (PC-3), and colon cancer cell line (Caco-2) was explored.

1.2 Experimental part

1.2.1 Chemistry

All commercially available solvents and starting materials in these syntheses were used without further purification. Solvents were dried with activated 4Å molecular sieves prior to use.

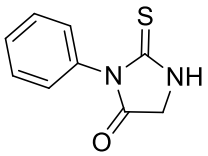
Phenylisothiocyanate, diethyl malonate, barbituric acid, thiobarbituric acid, ammonia, glycine, 4-methoxybenzaldehyde, 4-methylbenzaldehyde and diethyl ether were purchased from *Sigma Aldrich*. Hydrochloric acid 37%, sodium borohydride, HEPES, absolute ethanol and methanol were obtained from *Fisher Scientific*. Hexadeuterodimethyl sulfoxide (DMSO-d_6) were purchased from *Acros Organics*. Dichloromethane (DCM) and ethyl acetate were purchased from *Carlo Erba*. Petroleum ether and acetic acid (99-100%) were obtained from *Chem-Lab*. Benzaldehyde was purchased from *Merck Schuchardt*. Piperidine was purchased from *Alfa Aesar*. 1,3-Diphenyl thiobarbituric acid was previously synthesized by our research group.

Melting points were obtained on a Büchi melting point B-540 apparatus and are uncorrected. Thin Layer Chromatography (TLC) controls were carried out on commercial silica gel plates from Merck-Nagel 60 G/UV_{254} (0.2 mm) plates which were visualized by ultra-violet (UV) detection. The evaporation of the solvents was carried out using a R-215 rotator from Büchi.

^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz on a Brüker Avance III 400 MHz spectrometer respectively, using deuterated DMSO (DMSO-d_6) as a solvent. NMR spectra were analyzed and interpreted using Mestre nova 12.0.4 Lite. DMSO-d_6 signals were used as a reference for both ^{1}H and ^{13}C spectra. The chemical shift (δ) values are given in parts per million (ppm), and the coupling constants (*J)* are given in Hertz (Hz). The multiplicity of the signals is reported as s (singlet), d (doublet), dd (doublet of doublets), dt (doublet of triplets), t (triplet), td (triplet of doublets) and m (multiplet). The ^{1}H NMR of the synthesized 5-benzylidene(thio)barbiturate derivatives and (5Z)-5-(4-methoxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one are presented in Appendixes II-XIII.

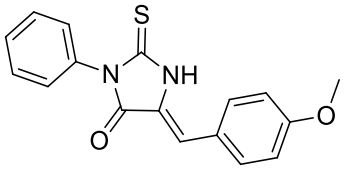
1.2.1.1 Synthesis

4-Phenyl-2-thioxoimidazolidin-4-one (1) [97]



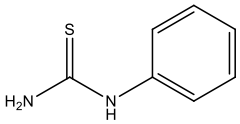
A mixture of phenylisothiocyanate (8.37 mmol, 1.11 g, 1 mL) and glycine (10.26 mmol, 770.6 mg) in hydroalcoholic solution (35% vol/vol) was stirred under reflux for 24 h. The solvent was evaporated under reduced pressure and the solid residue was washed with water and dried; Yield 63%; orange solid; mp 250 ºC;[97] ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.78 (s, 1H), 7.50 – 7.46 (m, 2H), 7.33 (dd, *J* = 8.4, 7.4 Hz, 2H), 7.15 – 7.10 (m, 1H), 4.29 (s, 1H).

(5*Z*)-5-(4-Methoxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one (2) [97]



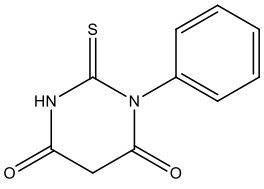
A mixture of 3-phenyl-2-thioxoimidazolidin-4-one (**1**) (1.54 mmol, 200 mg), 4-methoxybenzaldehyde (1.54 mmol, 193 µL) and piperidine (1.26 mmol,107 mg, 125 µL) in ethanol (12 ml) was refluxed for 4 hours. After cooling the reaction mixture, it was poured into water acidified with AcOH (pH 3-4). The resulting precipitate was obtained by filtration, washed with water and dried; Yield 71%; yellow solid; mp 234-237 ºC;[97] ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.52 (s, 1H), 7.83 (d, *J* = 8.8 Hz, 2H), 7.55 – 7.43 (m, 3H), 7.41 – 7.35 (m, 2H), 7.02 (d, *J* = 8.8 Hz, 2H), 6.67 (s, 1H), 3.83 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 178.0, 163.9, 160.4, 133.4, 132.4, 128.8, 128.7, 124.9, 124.5, 114.5, 113.5, 55.4.

Phenylthiourea 3 [99,104]



To a stirred solution of phenylisothiocyanate (16.7 mmol; 2.25 g, 2.04 mL) in DCM (20 mL) was added dropwise a solution of ammonia (31.3 mmol; 0.533 g; 6 ml) in methanol (20 mL) in an ice bath and then was stirred at room temperature. The reaction was followed by TLC (DCM) and completed in 24h. The resulting suspension was filtered and washed first with DCM and then with diethyl ether and then dried; Yield 84%; white crystals; mp 150–151 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (s, 1H), 7.44 (s, 2H), 7.40 (dd, *J* = 7.7, 1.2 Hz, 2H), 7.32 (dd, *J* = 8.6, 7.2 Hz, 2H), 7.11 (tt, *J* = 7.2, 1.2 Hz, 1H).

Phenylthiobarbituric acid ($4a$)[99]



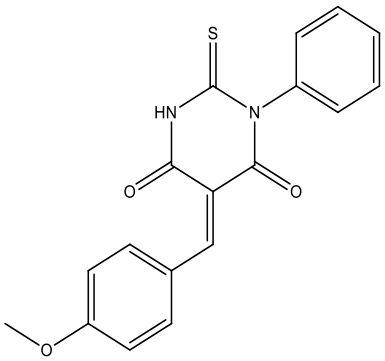
To a stirred mixture of molecular sieves and freshly prepared sodium ethoxide [by dissolving sodium (39.2 mmol; 0.9 g) in dried ethanol (10 mL)] was added diethyl malonate (39.2 mmol; 6.31 g) followed by phenylthiourea (**1**) (9.8 mmol; 1.5 g) and the resulting mixture was put to stir at reflux for 48 h. When the reaction was completed as indicated by TLC, the reaction mixture was diluted

with H_2O (50 mL). After removing the molecular sieves and evaporating most of the ethanol under reduced pressure, the residue was poured into cold water (50 mL), chilled and filtered. The aqueous layer was washed with diethyl ether (2 x 50 mL) to eliminate any unreacted diethyl malonate and then with chloroform (2 x 50 ml) to remove any unreacted phenylthiourea. The solid resulting from acidifying the aqueous layer with HCl was filtered off, washed with cold water and cold Et_2O, and dried; Yield 78%; white solid; mp 211-213 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.39 (s, 1H), 12.29 (s, 1H), 8.42 (d, *J* = 8.6 Hz, 2H), 8.27 (s, 1H), 7.08 (d, *J* = 8.6 Hz, 2H), 3.89 (s, 3H).

5-Benzylidenepyrimidines 6[105]

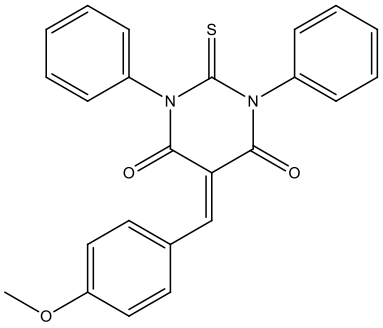
General procedure: a stirred mixture of respective (thio)barbituric acid **2** (1 mmol) and benzaldehyde (1 mmol or 3 mmol) in water was refluxed during 30 to 120 minutes. The resulting product was filtered and washed with water, ethanol and diethyl ether and then dried. To guarantee the use of analytically pure compounds in cellular assays, the products obtained were crystalized in ethyl acetate.

5-(4-methoxybenzyl)-1-phenylpyrimidine-2,4,6(1H,3H,5H)-trione (6a)



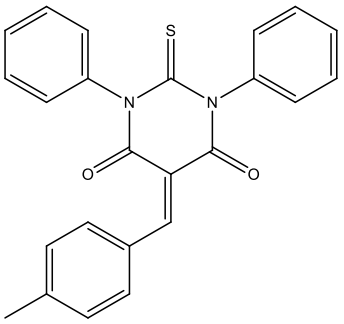
From 1-phenyl thiobarbituric acid (**4a**) (0.45 mmol, 0.10 g) and 4-methoxybenzaldehyde (**5a)** (0.45 mmol; 0.062 g; 0.057 ml) for 2h. Yield 41%; yellow solid; (*E*/*Z*-mixture); mp 279-281 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.69 and 12.61 (s, 1H), 8.45 and 8.43 (s, 1H), 8.39 – 8.27 (m, 4H), 7.45 (dt, *J* = 7.7, 4.2 Hz, 4H), 7.28 (t, *J* = 7.4 Hz, 4H), 7.11 (d, *J* = 9.0 Hz, 3H), 7.07 – 7.02 (m, 3H), 3.90 and 3.86 (s, 3H).

5-(4-methoxybenzylidene)-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (6b)

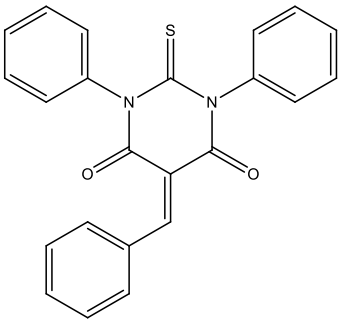


From 1,3-diphenyl thiobarbituric acid (**4b**) (0.67 mmol; 0.2 g) and 4-methoxybenzaldehyde (**5a)** (0.67 mmol; 0.092 g; 0.084 ml) for 2h. Yield 84%; orange solid; mp 277-279 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42 (s, 1H), 8.36 (d, *J* = 8.5 Hz, 2H), 7.47 (t, *J* = 7.7 Hz, 4H), 7.42 – 7.26 (m, 6H), 7.07 (d, *J* = 8.5 Hz, 2H), 3.87 (s, 3H).

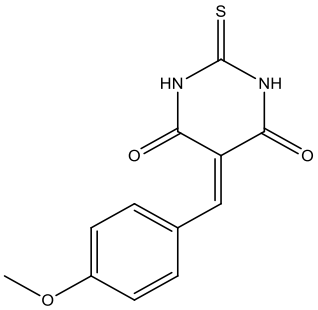
5-(4-methylbenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (6c) From 1,3-diphenyl thiobarbituric acid (**4b**) (0.67 mmol; 0.2 g) and 4-methylbenzaldehyde (**5b)** (0.67 mmol; 0.081g; 0.081 ml) for 2h. Yield 73%; orange solid; mp 294-296 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42 (s, 1H), 8.10 (d, *J* = 8.1 Hz, 2H), 7.51 – 7.42 (m, 4H), 7.42 – 7.37 (m, 2H), 7.35 – 7.28 (m, 6H), 2.38 (s, 3H).



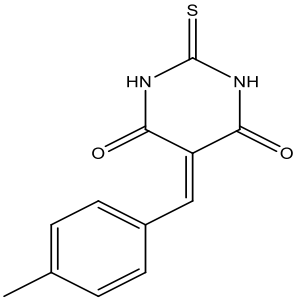
5-benzylidene-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (6d) From 1,3-diphenyl thiobarbituric acid (**4b**) (0.67 mmol; 0.2 g) benzaldehyde (**5c)** (0.67 mmol; 0.071 g; 0.068 ml) for 2h. Yield 67%; orange solid; mp 260-261 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (s, 1H), 8.08 (d, *J* = 7.7 Hz, 2H), 7.51 – 7.43 (m, 6H), 7.42 – 7.36 (m, 3H), 7.33 (d, *J* = 7.7 Hz, 4H).



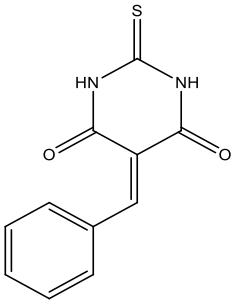
5-(4-methoxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (6e) From thiobarbituric acid (**4c**) (1.3 mmol; 0.2 g) and 4-methoxybenzaldehyde (**5a)** (1.3 mmol; 0.189 g; 0.165 ml) for 2h. Yield 83%; yellow solid; mp 315-318 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.39 (s, 1H), 12.29 (s, 1H), 8.42 (d, *J* = 8.6 Hz, 2H), 8.27 (s, 1H), 7.08 (d, *J* = 8.6 Hz, 2H), 3.89 (s, 3H).



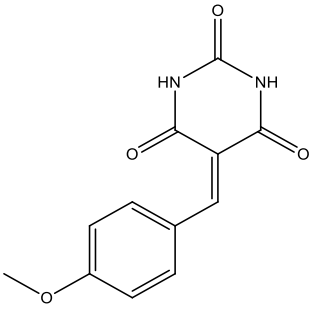
5-(4-methylbenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (6f) From thiobarbituric acid (**4c**) (1.3 mmol; 0.2 g) and 4-methylbenzaldehyde (**5b)** (1.3 mmol; 0.156 g; 0.158 ml) for 2h. Yield 86%; yellow solid; mp 323-324 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.43 (s, 1H), 12.32 (s, 1H), 8.26 (s, 1H), 8.15 (d, *J* = 8.3 Hz, 2H), 7.32 (d, *J* = 8.1 Hz, 2H), 2.39 (s, 3H).



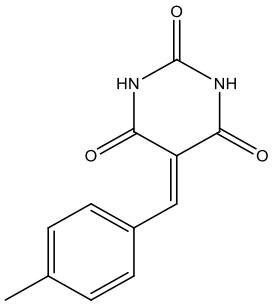
5-benzylidene-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (6g) From thiobarbituric acid (**4c**) (1.3 mmol; 0.2 g) and benzaldehyde (**5c)** (3.9 mmol; 0.413 g; 0.394 ml) for 40 minutes. Yield 68%; yellow solid; mp 282-283 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.46 (s, 1H), 12.34 (s, 1H), 8.29 (s, 1H), 8.13 (d, *J* = 7.7 Hz, 2H), 7.57 (t, *J* = 7.4 Hz, 1H), 7.48 (t, *J* = 7.6 Hz, 2H).



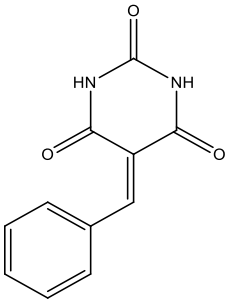
5-(4-methoxybenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (6h) From a mixture of barbituric acid (**4d**) (1.56 mmol; 0.2g) and 4-methoxybenzaldehyde (**5a)** (1.56 mmol; 0.212 g; 0.195 ml) for 2h. Yield 84%; yellow solid; mp 292-293 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.30 (s, 1H), 11.17 (s, 1H), 8.37 (d, *J* = 9.0 Hz, 2H), 8.25 (s,1H), 7.06 (d, *J* = 9.0 Hz, 2H), 3.87 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.9, 163.5, 162.2, 155.0, 150.2, 137.5, 125.2, 115.6, 114.0, 55.7.



5-(4-methylbenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (6i) From a mixture of barbituric acid (**4d)** (1.56 mmol; 0.2 g) and 4-methylbenzaldehyde (**5b)** (1.56 mmol; 0.188 g; 0.19 ml) for 2h. Yield 75%; yellow solid; mp 272-274 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.35 (s, 1H), 11.21 (s, 1H), 8.25 (s, 1H), 8.09 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 2.38 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.8, 161.9, 155.2, 150.4, 143.7, 134.1, 130.0, 129.0, 129.0, 117.9, 21.5.

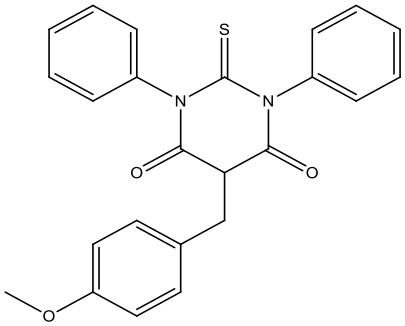


5-benzylidenepyrimidine-2,4,6(1H,3H,5H)-trione (6j) From a mixture of barbituric acid (**4d)** (1.56 mmol; 0.2 g) and benzaldehyde (**5c)** (1.56 mmol; 0.165 g; 0.158 ml) for 2h. Yield 50%; white solid; mp 260-263 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.39 (s, 1H), 11.23 (s, 1H), 8.28 (s, 1H), 8.08 (d, *J* = 7.3 Hz, 2H), 7.54 (t, *J* = 7.3 Hz, 1H), 7.47 (t, *J* = 7.5 Hz, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.4, 161.6, 154.7, 150.2, 133.1, 132.7, 132.2, 128.1, 119.1.

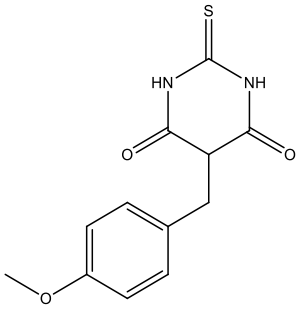


5-Benzylpyrimidines 8

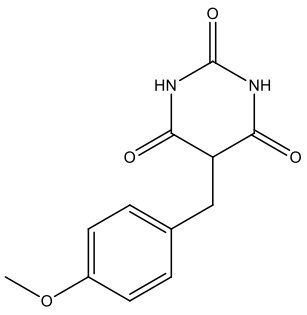
General procedure: Little portions of sodium borohydride (3 mmol) were added to a stirred solution of 5-benzylidenepyrimidines **6** (1 mmol) in ethanol (15 mL). The reaction was monitored by TLC (ethyl acetate) and completed after 4 h at room temperature. The solvent was evaporated under reduced pressure and water (10 mL) was added to the solid residue to form a suspension, which was acidified with 1 M aqueous hydrochloric acid (pH 5). The product was filtered and recrystallized from methanol.



5-(4-methoxybenzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (8b) From sodium borohydride (1.44 mmol, 18.25 mg) and 5-(4-methoxybenzylidene)-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (**6b)** (0.48 mmol, 200mg). Yield 70%; yellow solid; *mp 163-164* ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.41 (t, *J* = 7.5 Hz, 4H), 7.33 (d, *J* = 8.2 Hz, 2H), 7.27 – 7.07 (m, 5H), 7.03 - 6.73 (m, 3H), 3.72 (s, 3H), 3.59 (s, 2H).



5-(4-methoxybenzyl)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (8e) From sodium borohydride (2.28 mmol, 28.8 mg) and 5-(4-methoxybenzylidene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (**6e)** (0.76 mmol, 200 mg). Yield 50%; white solid; mp 150-152 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.02 (s, 2H), 7.07 (d, *J* = 8.1 Hz, 2H), 6.79 (d, *J* = 8.2 Hz, 2H), 3.69 (s, 3H), 3.51 (s, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.1, 160.3, 157.4, 132.3, 128.9, 113.5, 94.8, 55.0, 26.0.



5-(4-methoxybenzyl)pyrimidine-2,4,6(1H,3H,5H)-trione (8h) From sodium borohydride (2.43 mmol, 30.7 mg) and 5-(4-methoxybenzylidene) pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (**6h**) (0.81 mmol, 200 mg). Yield 49%; white solid; mp 204-207 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.16 (s, 2H), 6.99 (d, *J* = 8.1 Hz, 2H), 6.81 (d, *J* = 8.1 Hz, 2H), 3.81 (t, *J* = 4.7 Hz, 1H), 3.69 (s, 3H), 3.19 (d, *J* = 4.6 Hz, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.1, 158.1, 150.6, 130.0, 128.9, 113.7, 55.0, 49.6, 32.8.

1.2.2 Biological evaluation

Stock solutions (10 mM) in DMSO were prepared for all assayed compounds and stored at 4 ºC to be used in all the biological assays.

1.2.2.1 Xanthine oxidase assay

1.2.2.1.1 Preparation of sample solutions

The screening for the inhibition of xanthine oxidase was performed at concentrations of 10 and 100 μM of the assayed compounds. From the stock solutions of the assayed compounds were prepared diluted solutions in 50 mM dihydrogen phosphate buffer (pH 7.4) before each experiment. The xanthine (Sigma Aldrich) stock solution (10 mM) was prepared in a 25 mM aqueous NaOH solution. XO enzyme (40 U/10 mL, Sigma Aldrich) was diluted in 50 mM dihydrogen phosphate buffer (pH 7.4) to obtain a 0.1 U/mL solution. The final concentration of DMSO in each well was always guaranteed to be ≤ 1% to avoid significant interference with the enzyme activity.

1.2.2.1.2 Experimental procedure

XO activity was assessed by quantifying the uric acid formation produced from the oxidation of xanthine according to the method of Figueiredo *et al*.[106]

A 96-well plate was used. 50 μL of test solution and 50 μL of a XO bovine serum suspension (0.1 U/mL) were added in each well for a pre-incubation at 37 ºC for 5 min. The addition of 150 μL of xanthine solution (0.42 mM) commenced the enzymatic reaction and then the plate was incubated at 37 ºC during 10 min.

Absorbance was recorded at 295 nm at every minute for ten minutes, with 20 s of a slow stirring before each reading. Dihydrogen phosphate buffer (50 mM, pH 7.4) was used as negative control and allopurinol as a positive control. In order to discount the absorbance of each compound at this wavelength, a blank sample containing 50 mL of test solution and 200 mL of buffer was performed. The essays were performed in triplicate. Enzyme inhibitory percentage for each compound was calculated using the following formula:

$$\% \text{ of inhibition} = [1 - (Abs_{sample} - Abs_{blank\ of\ sample}) / Abs_{negative\ control}] \times 100$$

1.2.2.2 Proteasome inhibition assay

To evaluate proteasome inhibitory activity of the chosen compounds, the proteasome-Glo™ 3-substrate system was employed. The three distinct proteolytic activities associated with the proteasome, CT-L, T-L, and C-L were measured by monitoring the cleavage of luminogenic

substrates, Suc-LLVY-aminoluciferin, Z-LRR-aminoluciferin and Z-nLPnLD-aminoluciferin, respectively. These Substrates cleavage results in emission of “glow-type” luminescent signal, which was recorded and used for calculating the potency of the compounds. The preliminary screening for the inhibition of the three proteolytic activities of proteasome was carried out at 10 and 100 µM concentrations of all assayed compounds and 0.5 µg/ml of purified proteasome enzyme 20S. SpectraMax® luminometer was used and all assays were performed in white-walled multiwall plates. Cleavage of the substrates by the proteasome

1.2.2.2.1 Preparation of sample solutions

The Proteasome-Glo™ Reagents of the three proteolytic activities were prepared as prescribed in the protocol of the assay[107]. Human purified 20S proteasome (1 mg/ml) was diluted in 10mM HEPES pH 7.6 to obtain the concentration of (2 µg/ml) to be used during the assays. Stock solutions were stored at 4° C. From the stock solutions of the assayed compounds were prepared diluted solutions in 10 mM HEPES buffer pH 7.6 before each experiment.

1.2.2.2.2 Experimental procedure

To each well were added 25 µL of proteasome solution (2 µg/ml) to obtain a final concentration of 0.5 µg/ml in a total volume of 100 µL in each well. Then, a 25 µL from the diluted solutions of the assayed compounds was added followed by 50 µL of Proteasome-Glo™ Reagent. For each plate, two wells of blank were defined in which 50 µL of Proteasome-Glo™ Reagent and 50 µL of 10 mM HEPES were added to each of them. Four wells were prepared in each plate for the positive control were in each well 50 µL of Proteasome-Glo™ Reagent, 25 µL 10 mM HEPES and 25 µL purified proteasome enzyme were added. The plates were always placed on ice during the preparation and addition of the solutions. The contents of wells were gently mixed for 30 seconds in the SpectraMax® luminometer before initiating the readings. The temperature in the luminometer was guaranteed to be 37 ºC before inserting the plates. For both the initial screening and the concentration-response studies, a kinetic read type was chosen. Data was collected over 40 minutes with readings taken at 5 minutes intervals for the initial screening. On the other hand, data was collected over 70 minutes with readings taken at 5 minutes intervals for the concentration-response studies. The essays were performed in duplicate. After subtracting the value f mean relative luminescence units (RLU) of blank wells, percentual enzyme activity in the presence of test compounds was calculated using the following formula:

Proteasome (CT-L, C-L or T-L) activity (%) = [(mean RLU(sample 20-40 min) / mean RLU (positive control 20-40 min)] × 100

1.2.2.3 Cell proliferation assay and cell viability

1.2.2.3.1 Preparation of sample solutions

Solutions of the tested compounds were prepared by their adequate dilutions in the complete culture medium before each experiment. The maximum DMSO concentration in each well was ≤ 1% (vol/vol), which guarantees no significant interference on cell proliferation.

1.2.2.3.2 Cells cultures

The cell lines used in this study were NHDF, PC-3, and Caco-2 and all were obtained from American Type Culture Collection (ATCC). All cell lines were maintained at 37 ºC in a humidified atmosphere containing 5% CO_2. NHDF was cultured in RPMI 1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) (Merck), *L*-glutamine (2 mM) (Sigma Aldrich), HEPES (10 mM), sodium pyruvate (1 mM) (Sigma Aldrich), and 1% antibiotic/antimycotic (10,000 units/mL penicillin, 10 mg/mL streptomycin and 25 mg/mL amphotericin B). Caco-2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% antibiotic/antimycotic. PC-3 cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% of the antibiotic mixture of 10,000 U/mL penicillin G and 100mg/mL of streptomycin (Sp).

The cells used in the experiments were used in passages 12 to 14 (NHDF), passage 15 (Caco-2) and passage 17 (PC-3).

1.2.2.3.3 MTT assay

As a method of assessing cellular viability and proliferation following the addition of the tested compounds, the colorimetric MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical) assay was performed. Viable cells can metabolize MTT into formazan crystals that absorbs light at 570 nm, therefore, cell proliferation can be evaluated by quantifying the formed formazan crystals.[108]

NHDF, PC-3, Caco-2 cells were seeded at a density of (2×10^4 cells/mL) per well in 100 µL of complete medium in flat-bottom 96-well plates. After incubating NHDF and PC-3 for 48 h and Caco-2 for 72 h at 37 ºC for adherence, the culture medium was removed and 100 µL of tested compounds solutions at various concentrations were added and then the plates were incubated for 72 h at 37 ºC. Untreated cells were used as negative control for both cell lines. In the NHDF assay, fluorouracil (5-FU) as positive control.

Each concentration of all compounds was tested in quadruplicate in two independent assays for all the assays. At the end of the incubation time, the medium was discarded, and the cells were washed with phosphate buffer saline (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM and

KH_2PO_4 1.8 mM). Cells were then incubated with 100 µL of 0.5 mg/ml MTT in incomplete culture medium for 4 hours under culture conditions. Then, the medium-containing MTT was removed and formazan crystals were dissolved with DMSO followed by optical density readings at 570 nm by a BIO-RAD xMark™ microplate spectrophotometer. The absorbance of the untreated cultures was set at 100% and the results are expressed as the percentage of surviving cells.

1.2.3 Statistics

The results (cell viability and enzyme inhibition) are expressed as mean values ± standard error. The difference between groups was considered statistically significant at $p < 0.05$ (Two-way ANOVA). The IC_{50} values were calculated by sigmoidal fitting analysis considering a 95% confidence interval. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel 2013 software.

1.2.4 Superposition *in silico* studies

ChemDraw Ultra 12.0 software was used to perform the 2D comparison between bortezomib and **9b**.

For the 3D superposition, multiple software programs were used. Firstly, Chem3D was operated to obtain 3D structures of both compounds and also for energy minimization of the structures employing MMFF94 as a calculation method. Then, the compilation of the 3D structures of the studied compounds in one file was accomplished using Discovery Studio Visualizer 16.1. Finally, the alignment of the 3D structures was achieved using Open3DALIGN software.

1.3 Results and discussion

1.3.1 Design and Synthesis

This section includes a discussion of the design of skeletal structures of 5-substituted (thio)barbiturates as possible inhibitors of the proteasome in addition to the synthesis of these different compounds.

1.3.1.1 Design

To design these non-peptide inhibitors, we analyzed the structure of (5Z)-5-arylidene-3-aryl-2-thioxoimidazolidin-4-ones that were showed to have an inhibitory activity of proteasome[97] and the structure of bortezomib. (5Z)-5-(4-Methoxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one was used as reference compound and it was considered the substitution of thioxoimidazolidin-4-one, a five-membered ring, by similar substituted barbituric and thiobarbituric acids, six-membered pyrimidine rings. The pyrazine ring

constitutes a part of the structure of some proteasome inhibitors, such as the well-known proteasome inhibitor, bortezomib. These structural modifications led to the design of a series of 5-benzylidene(thio)barbiturates **6**. In this regard, we modified the structure by varying substitutions at N1, N3 and in the aromatic ring coupled to C5-position, as well as by replacing sulfur atom by oxygen at C2 (Figure 1.8).

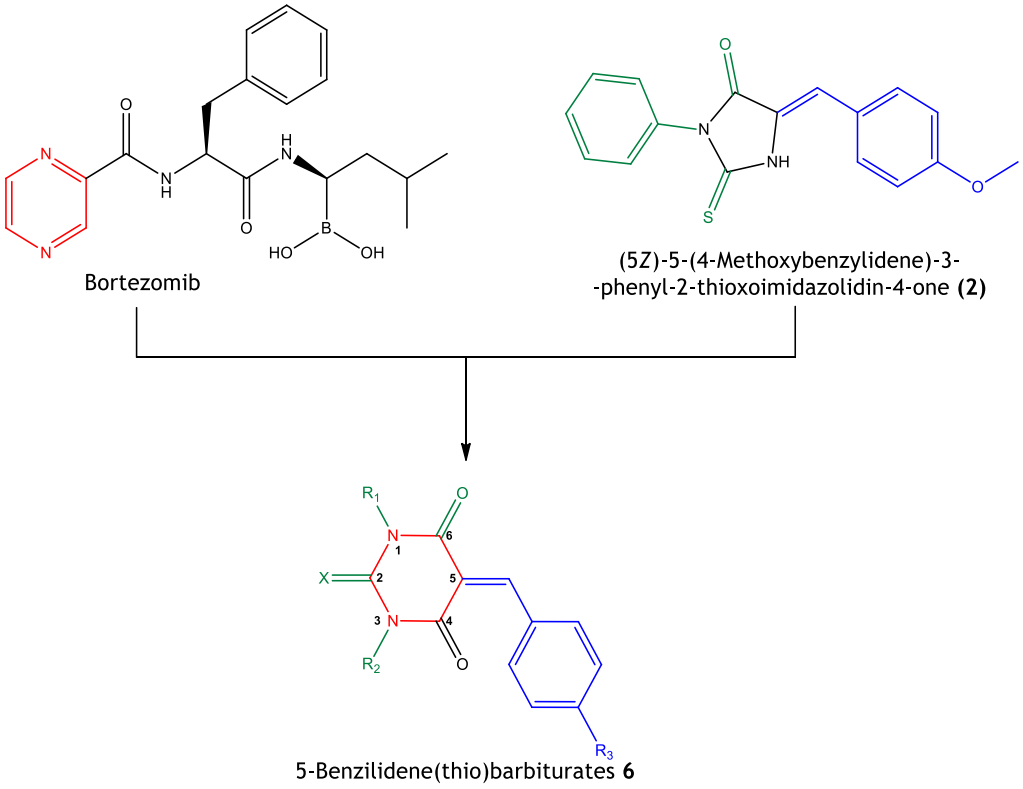


Figure 1.8: The design of 5-benzylidene(thio)barbiturates **6**.

Our first focus was to evaluate the effect of the substitutions at the aromatic ring coupled to the C5 position of (thio)barbiturate scaffold. These substitutions included methoxy group, based on our hit compound, hydrogen, to understand the importance of the presence of methoxy group at this position, and methyl group which is a weaker electron donor than methoxy. The second modification involved N1- and N3-substitutions, where phenyl and diphenyl analogs in addition to unsubstituted thiobarbituric acid compounds were generated. This would help in the determination of the effect of partially substituted, fully substituted, and free NH compounds. Moreover, we planned to synthesize barbituric acid analogs to evaluate if the replacement at the C2 position of sulfur by oxygen in such scaffold would help enhance the activity. Finally, we screened previously synthesized compounds in our lab with similar chemical structures to our studied compounds to include in the biological studies. The arylidene in C5 position in the selected compounds was substituted with hydrazinylethylidene, phenylhydrazinylethylidene and phenylaminomethylene. The main goal of choosing these compounds was to increase the spacing between the aromatic ring and barbituric scaffold and evaluate its effect that it may have on the biological activity.

1.3.1.2 Chemistry

1.3.1.2.1 Precursors

Phenylthiobarbituric acid was synthesized as a precursor for the synthesis of 5-benzylidenepyrimidines in two steps (Figure 1.9). Firstly, phenylthiourea (**3**) was prepared by reacting phenylisothiocyanate in DCM with ammonia which was mixed with ethanol according to the previous described method by our group[99]. This procedure ensures the formation of the product as a precipitate which is easy to isolate by simple filtration with high degree of yield (84%) and purity. Then, phenylthiourea (**3**) was employed in a condensation reaction with diethyl malonate in the presence of sodium ethoxide to obtain phenylthiobarbituric acid (**4a**).[99]

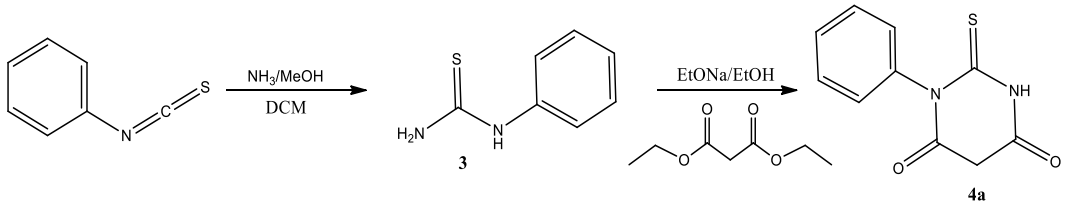


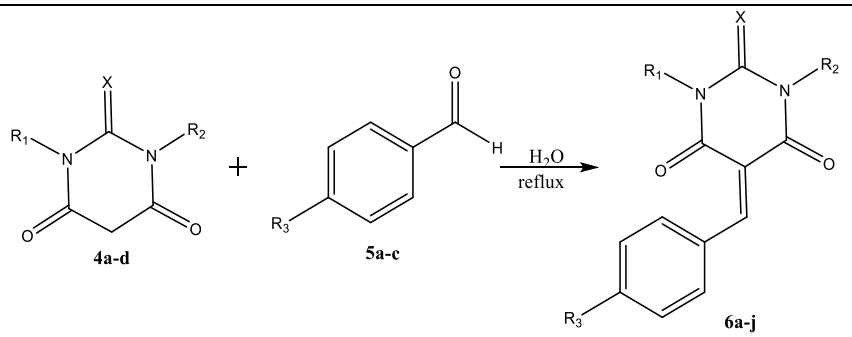
Figure 1.9: Synthesis of phenylthiobarbituric acid (**4a**).

1.3.1.2.2 5-Benzylidene(thio)barbiturates

A series of 5-benzylidene(thio)barbiturates derivatives **6a**-**6j** were easily prepared by a Knoevenagel condensation of a (thio)barbituric acid **(4)** and a benzaldehyde **(5)** under reflux condition using water as a solvent (Table 1.1).

Preparing 5-benzylidene(thio)barbiturates derivatives **6a**-**6j** under these conditions is considered eco-friendly synthesis due to the use of water as a solvent, which is environmentally benign and nonhazardous solvent providing clean processing and pollution prevention.[98] Moreover, no catalyst was needed for the reaction to occur meaning fewer residues and lower costs. Besides, the insoluble pursued compounds were precipitated throughout the reaction, yielding essentially a pure final compound and requiring simple washing after filtration. These conditions not only provided rapid reaction times but also moderate to high products yield (41– 86%). (Table 1.1)

Table 1.1: 5-Benzilidene(thio)barbiturate derivatives.



6	X	R_1	R_2	R_3	Yield (%)
6a	S	Ph	H	OMe	41
6b	S	Ph	Ph	OMe	84
6c	S	Ph	Ph	Me	73
6d	S	Ph	Ph	H	67
6e	S	H	H	OMe	83
6f	S	H	H	Me	86
6g	S	H	H	H	68
6h	O	H	H	OMe	84
6i	O	H	H	Me	75
6j	O	H	H	H	50

While it was noticed that the presence of the electron-donor methoxy group in C4 of the benzaldehyde may contribute in some cases to higher yields, the functional substitution of oxygen by sulfur at C-2 of the original barbituric acid stem didn't seem to have a great influence on the yield (Table 1.1).

The products were analyzed and characterized by proton nuclear magnetic resonance (^{1}H NMR). Compounds 2, **6h-**j, **8e,** and **8h** were analyzed by Carbon-13 nuclear magnetic resonance (^{13}C NMR). The formation of 5-benzylidene(thio)barbiturates was confirmed by the presence of the characteristic methylene ^{1}H NMR singlet in the region between 8.25 and 8.46 ppm (Appendix I). Asymmetric 5-benzylidene-phenylthiobarbiturate (**6a**) was a promising candidate because of the relatively similar structure with our reference compound (**2**). However, NMR studies demonstrated the formation of a mixture of two diastereomers *E* and *Z* although the synthesis of compound (**2**) yielded only the *(Z)* isomer that was proven to have an inhibitory activity of the proteasome. Thus, we opted for the exclusion of (**6a**) from the biological studies.

Although most of the compounds **6** were found to be pure, it was noticed that compound **(6g) has** residual impurities of the respective bis-thiobarbiturate (with the characteristic signal between 5.90 and 6.27 ppm) as well as benzaldehyde (can be seen at 9.88 – 10.3 ppm) which increased after repeated washing with ethanol and diethyl ether. This was also noticed when some of NMR tubes were heated to promote the dissolution of products in DMSO-d_6 as well as when some of the tubes were not prepared at the same day of NMR test. Among all the synthesized compounds, the most affected ones by this situation where the products of syntheses that involved thiobarbituric acid and unsubstituted benzaldehyde. In most of these cases, the proportion of 5,5-dibenzyl(thio)barbiturates to the respective benzaldehyde was 1:1 which led us to suspect of degradation of the targeted compounds. This observation stimulated us to perform a study to assess the stability of the synthesized compounds **6b-j** in solution to explore the possibility of advancing with the same to biological evaluation were the tested compounds must be dissolved in aqueous solutions. For this, the tested compounds were dissolved in DMSO-d_6. To better approximate to the conditions of the enzymatic and cytotoxicity assays the temperature of the study was set to 37 ℃ and the ^{1}H NMR analysis was undertaken after 24 h, and 48 h which are common incubation times followed in cytotoxicity studies. Under the conditions of this study after 24h, it was evident that all thiobarbituric derivatives **6b-g** degraded to variable extents into thiobarbituric acid and the respective aldehyde where the resulted thiobarbituric acid attacks the exocyclic double bond of another molecule of 5-benzylidenethiobarbiturate to form 5,5-dibenzyl(thio)barbiturates (Figure 1.10). DMSO-d_6 contains low residual water content that could promote the degradation process and the formation of the double addition product.

The degradation seemed to increase with time as the signal's intensity of the aldehyde and 5,5-dibenzylthiobarbiturates augmented along the study time. Yet, barbituric derivatives **6a-c** seemed to be stable along the time (Appendix II-XIII). These results suggest that the oxygen atom contributes somehow to higher stability of the exocyclic double bond in solution. Additionally, the reference compound **2** was also stable along the time of the study.

The reversibility of Knoevenagel condensation of barbituric acid with 4-substituted benzaldehydes was explored before by Kulchat *et al*. where they reported the cleavage of the exocyclic double bond of 5-benzylidene barbiturates by retro-Knoevenagel process in DMSO.[109] They noticed that the stability of 5-benzylidene barbiturates increased with electron-donating substituent at C4 of the aromatic ring. Additionally, the presence of a base catalyst accelerated the reaction of the formation as well as the retro-Knoevenagel process.[109] Moreover, many studies have reported the formation of double addition products where second barbituric acid moiety attack the exocyclic double bond in Knoevenagel products.[110,111] One study explored the reaction conditions that promote the selectivity of the reaction between (thio)barbituric acid and various benzaldehydes.[112] It confirmed the effect of *para*-substituent at the aromatic ring where electron-withdrawing groups increase the likelihood of

dimers formation. It also suggested that selecting benzaldehydes with electron-donating substituent at *para*-position and performing the reaction in water without catalyst at 100 ºC is the most favorable way to synthesize 5-benzylidene (thio)barbiturates selectively. Although most of the previous studies reported the degradation of 5-benzylidene barbiturates or the formation of dimers of barbituric derivatives, our study showed that only 5-benzylidene thiobarbiturates degrades in solution while 5-benzylidene barbiturates remain stable.

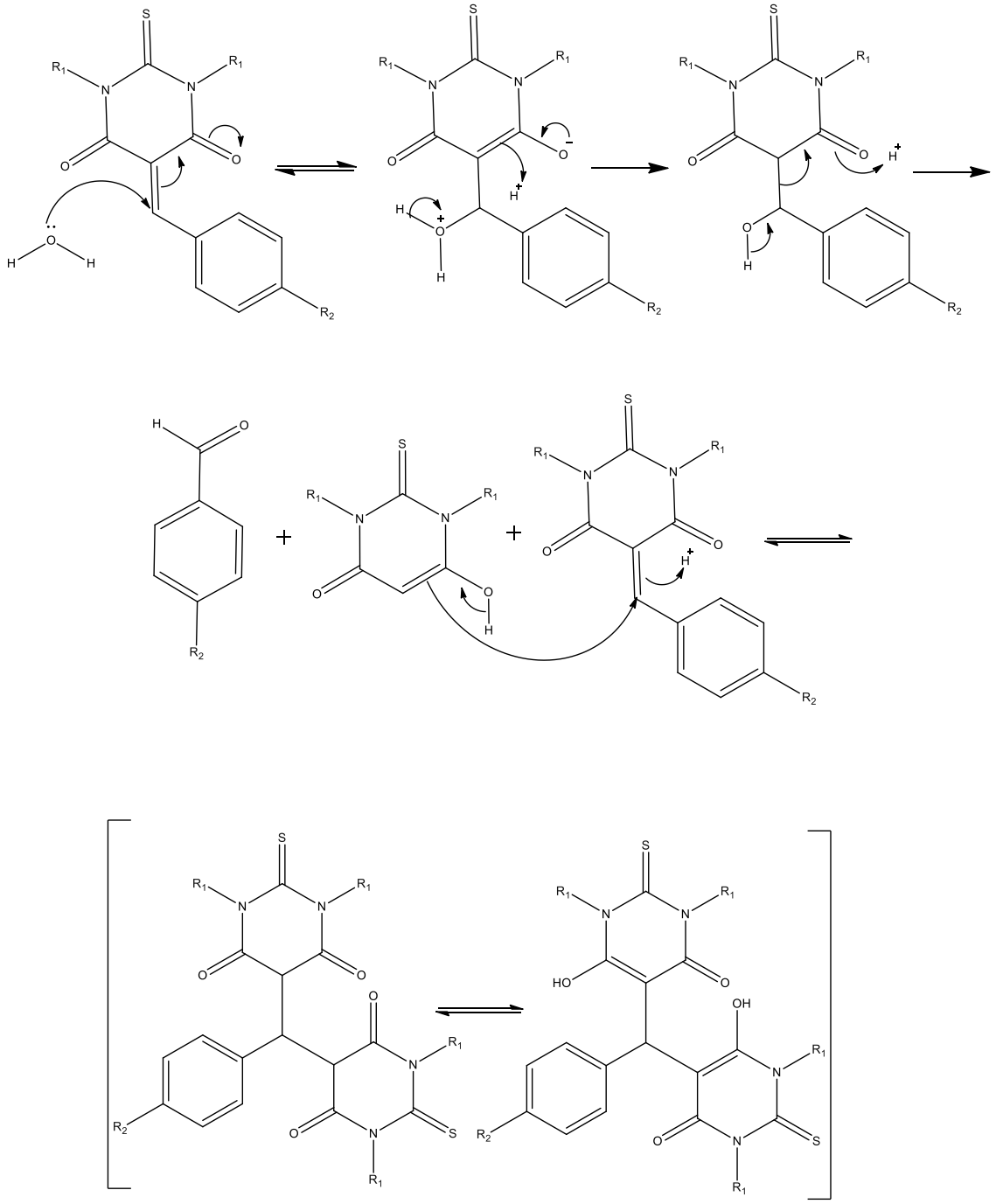


Figure 1.10: The possible mechanism of the degradation of 5-benzilidenethiobarbiturates **6** and the formation of the double add products.

In light of the results of the stability study, we decided to exclude compounds **6b-g** from the biological studies due to their solution instability. Instead, we sought to synthesize selectively the dimers of the compounds with methoxy substituent at C4 of the aromatic ring **6b**, **6e**, and **6h**. These compounds were chosen due to the similarity to our reference compound **2**.

Since the exocyclic double bond of 5-benzylidenethiobarbiturates seemed to be the target of nucleophilic attack in solution as previously mentioned, a reduction of this double bond was proposed as an attempt to increase the stability of the synthesized compounds in solution.

1.3.1.2.3 Bis-(thio)barbiturates synthesis attempts

Many attempts of synthesis were made to obtain the dimers employing a series of reaction conditions. In the first attempts, the reactions were carried out in ethanol at room temperature and at reflux without catalyst simulating a followed procedure to synthesis similar double addition products in our lab. The reaction was repeated in the same conditions with pyridine as a catalyst. Other solvents were used such as tetrahydrofuran (THF) and acetic acid at room temperature in addition to one attempt carried out in a mixture of acetic acid with sodium acetate at reflux[112]. Nevertheless, none of the conditions seemed to shift the reaction towards full formation of dimer without the formation of arylidene. In all the reaction we performed, we obtained a mixture of both compounds (Figure 1.11). In attempt to stabilize the formed dimers, dipentylamine was added to the reaction mixture in water which would form salt with the dimers[113], yet, we couldn't obtain solely the target product.

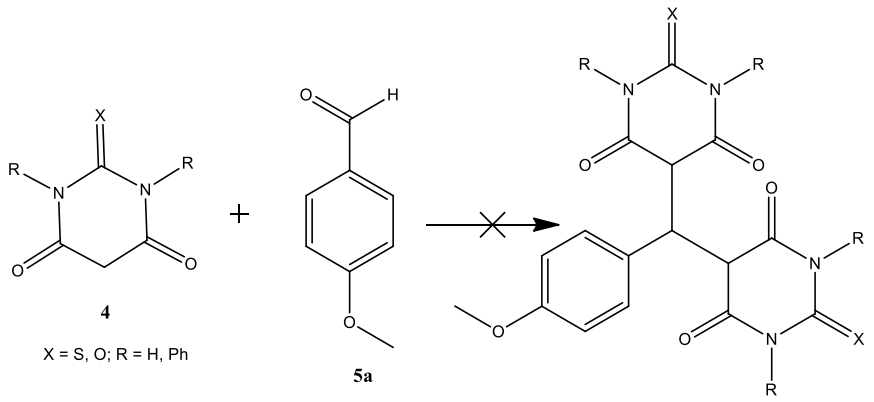


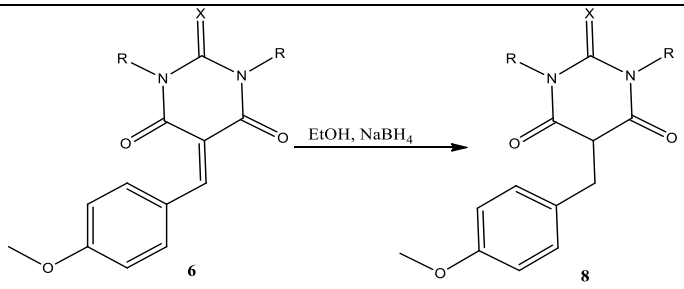
Figure 1.11: Attempts to synthesis bis-(thio)barbiturates **7.**

1.3.1.2.4 5-Benzyl(thio)barbiturates

To implement a comparison pattern in later biological assays, compounds **6b**, **6e** and **6h** were chosen to be reduced. This would permit a better understanding of the difference in effect, if

exists, between barbituric derivatives and thiobarbituric derivatives in biological studies. The reduction reaction was achieved at room temperature by treating the compounds with $NaBH_4$ in ethanol according to the previously described method.[114] The desired compounds **8b**, **8e**, and **8h** were obtained in yields range between 49% and 70%. (table 1.2)

Table 1.2: 5-Benzyl(thio)barbiturates **8.**



8	X	R_1	R_2	Yield (%)
8b	S	Ph	OMe	70
8e	S	H	OMe	50
8h	O	H	OMe	49

The formation of these compounds was verified by the disappearance of the ^{1}H NMR singlet in the region between 8.27-8.42 ppm (the characteristic methylene ^{1}H NMR singlet of 5-benzyledenebarbiturates) and the presence of a triplet and doublet signals corresponding to the methine and methylene in adjacent carbons. (Appendix I)

1.3.2 Biological evaluation

In this section the experimental results obtained in the XO inhibitory activity assay, proteasome inhibitory activity assay and evaluation of cytotoxicity activity of the synthesized 5-substituted (thio)barbiturates **6h-j**, **8b, e, h,** and compounds **9a-c**, **10** and **11** are presented and discussed (Table 1.3).

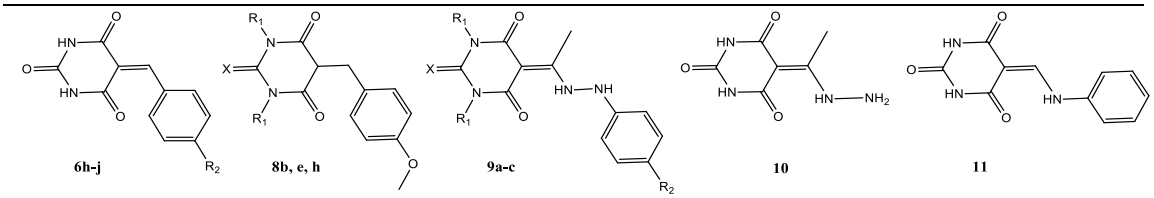
1.3.2.1 *In vitro* evaluation of proteasome inhibitory activity

The compounds **6h**-j, **8b**, **8h**, **8e**, **9a-c**, **10** and **11** underwent a preliminary screen on each proteolytic subunit at 10 and 100 μM (Figure 1.12). Bortezomib was used as a positive control only at concentration of 10 μM and it inhibited all three proteasome activities indiscriminately but, as expected, T-L activity was the least affected activity.

The assayed arylidenes **6h, 6i,** and **6j** exhibited inhibitory activity only against T-L activity at both concentrations and they seemed to be more potent than the reference compound **2,** which also inhibited this activity in contrast to the other two activities. Among the three 5-benzyl(thio)barbiturates synthesized **8b, 8e,** and **8h,** only **8e** exhibited some inhibitory

activity at 100 μM against CT-L and T-L activities which when compared to **8h** demonstrate that possibly the substitution of O by S may have affected the inhibitory activity of the compound. Considering the results of **6h** and **8h,** the exocyclic double bond may be necessary for the inhibition of T-L activity as the inhibitory potency diminished for the reduced compound **8h.**

Table 1.3: Compounds included in biological evaluation



Compound	X	R_1	R_2
6h	O	-	OCH_3
6i	O	-	CH_3
6j	O	-	H
8b	S	C_6H_5	-
8e	S	H	-
8h	O	H	-
9a	O	H	H
9b	O	H	NO_2
9c	S	C_6H_5	H
10	-	-	-
11	-	-	-

Interestingly, reference compound **2** seemed to have a very low inhibitory potency against CT-L and T-L activities and it showed no activity toward C-L activity although it was reported to have higher activity toward the three proteolytic activities in the study conducted by Maccari *et al.*[97] This discrepancy may be potentially due to the different enzymatic system used in the assays and also may be related to assay sensitivity considering that the signal-to-noise ratio in our screening was low and the differing time courses of the assays.

Compound **10** from the selected previously synthesized compounds only should inhibitory potency against T-L activity at 10 and 100 μM. However, compounds **9a, 9c,** and **11** demonstrated no significant inhibition for the three proteolytic activities.

Compounds **9b** have demonstrated to be the most potent inhibitor among all the tested compounds as it inhibited the three activities at both concentrations. However, it seemed to have less inhibitory potency than bortezomib toward CT-L and C-L activities and similar potency against T-L activity. A structure similarity was visually verified, hence, a two and three-dimensional superposition studies were performed (Figures 1.13 and 1.14). Although bortezomib has the two additional side chains that **9b** does not possess, the barbituric ring

lies in the same position as the pyrimidine ring of bortezomib. Besides, the nitro group is situated in the same place as the boronic acid. Nevertheless, it is not guaranteed that it interacts in this way as this all is only an assumption.

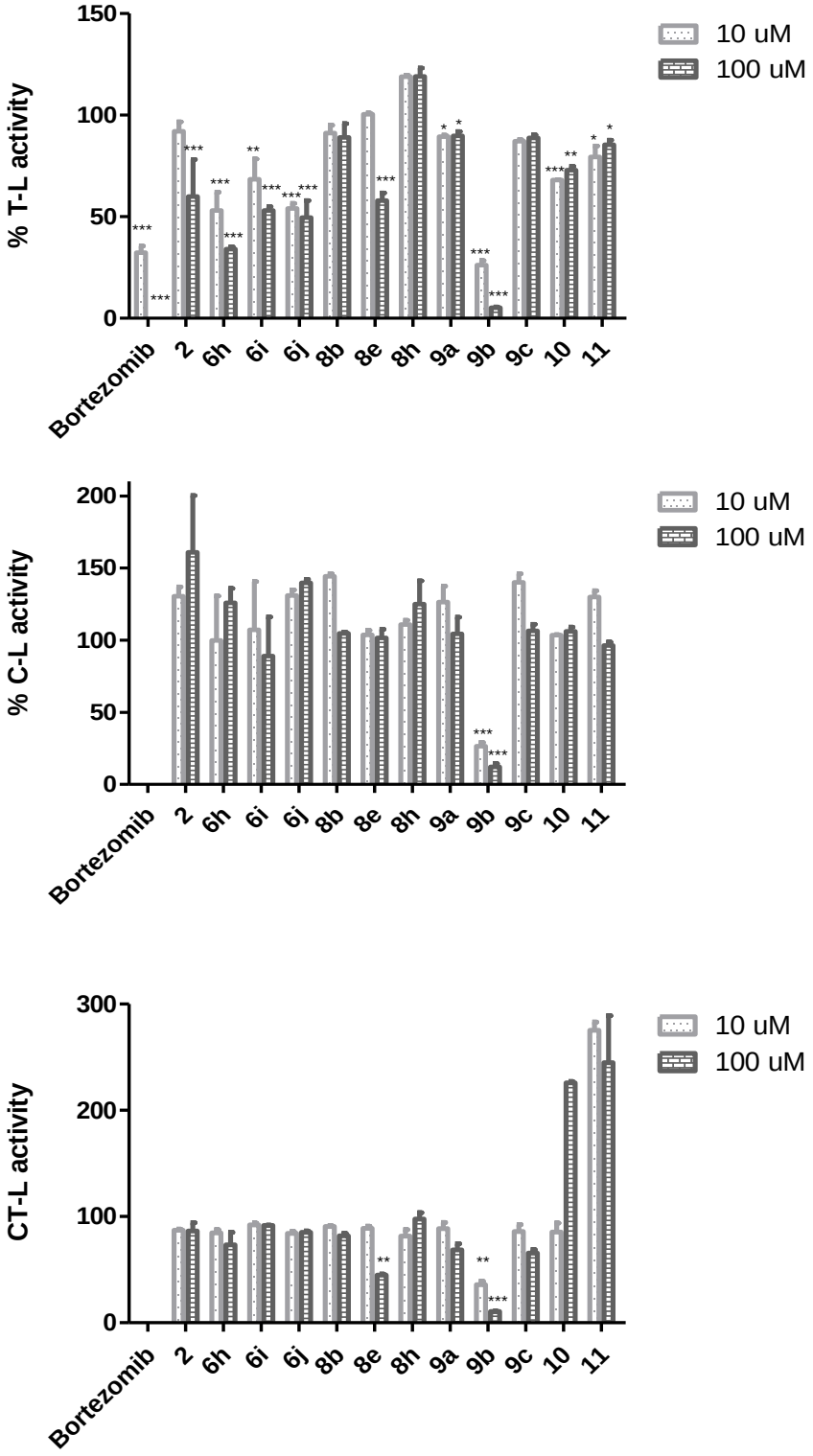


Figure 1.12: *In vitro* evaluation of proteasome inhibitory activity of compounds **2, 6h-j, 8b,e,h, 9a-c, 10, 11** and bortezomib against the three proteolytic activities C-L, T-L, and CT-L. Results are expressed as mean values ± standard error of the mean (SEM). Differences were considered statistically significant if *p < 0.05, **p < 0.01, ***p < 0.001 versus negative control.

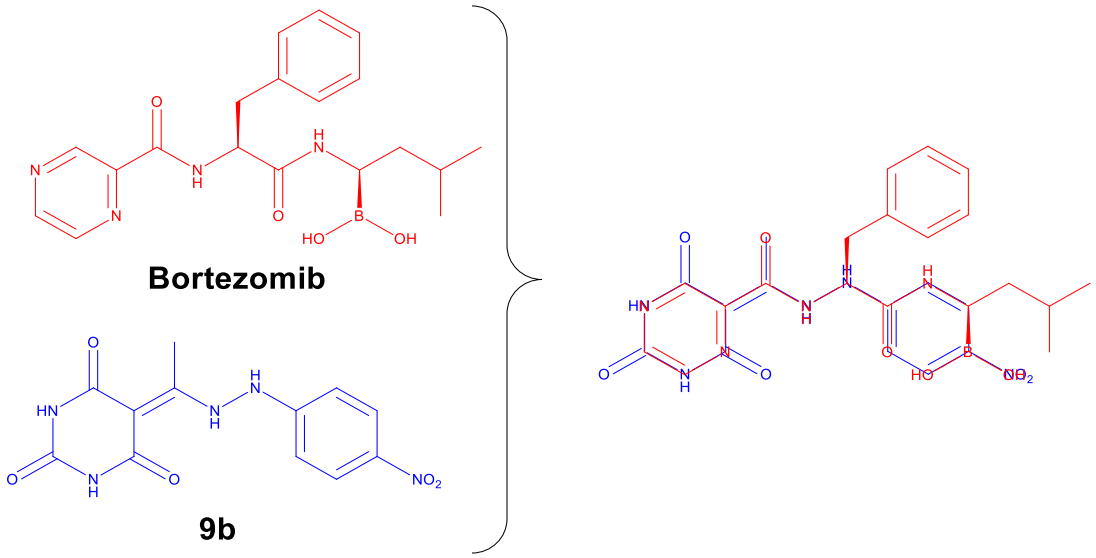


Figure 1.13: Two-dimensional superposition of bortezomib and **9b** to explore structural similarity between both.

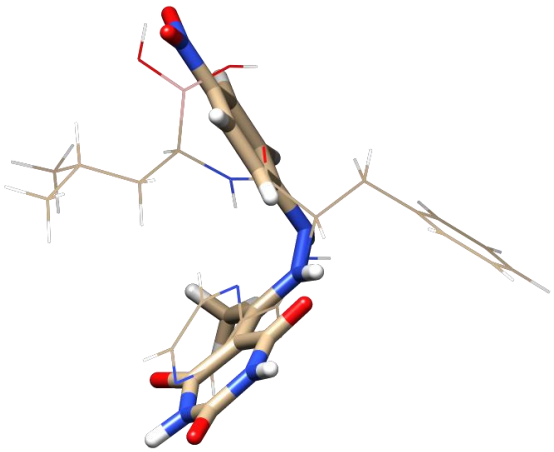


Figure 1.14: Three-dimensional superposition of bortezomib and **9b** to explore structural similarity between both.

Since **9b** displayed the most promising inhibitory potency, concentration-response curves for **9b** and bortezomib were constructed and IC_{50} for both compounds were calculated. For **9b** concentration-response curves, various concentrations were used (0.3, 1, 3, 10, and 33 μM). However, different concentrations set of bortezomib were used for the different proteolytic activities according to the known potency of the bortezomib against each activity. While 3.3, 10, 33.3, 100, 333.3, and 1000 nM set was used for C-L activity, 1, 3.3, 10, 33.3, 100, and 333.3 nM were used for CT-L activity and 33.3, 100, 333.3, 1000, 3333, and 10000 nM were used for T-L activity. Compound **9b** (Table 1.4) presented interesting IC_{50} against the three activities, yet, bortezomib continues to be far more potent inhibitor for CT-L activity.

Table 1.4: Estimated IC_{50} values (μM) for the inhibition of the different proteolytic activity of proteasome of **9b** and bortezomib

Activity type	T-L	C-L	CT-L
9b	4.26 ± 1.35	4.08 ± 1.28	6.66 ± 1.25
Bortezomib	2.38 ± 0.001	0.1 ± 0.001	0.01 ± 0.001

1.3.2.2 *In vitro* evaluation of xanthine oxidase inhibitory activity

Previous works by our group[98,100] and others[115] have demonstrated that many compounds containing (thio)barbiturates scaffold can possess XO inhibitory activity. Inhibition of XO would compromise the selectivity of the synthesized compound toward proteasome, therefore, an *in vitro* evaluation of XO inhibitory activity was conducted. The spectrophotometric assay was carried out under aerobic conditions using xanthine as the substrate and the commercial drug allopurinol as positive control. As the method is based on the transformation of xanthine to uric acid by the enzyme, the formation of uric acid was measured at 295 nm as described in the literature.[116,117] A screening test at 10 and 100 μM concentration of all the studied compounds was performed.

Any of the test compounds markedly inhibited the enzyme at 10 μM (Figure 1.15). At the concentration of 100 μM some compounds inhibited the enzyme to some extent, however the most potent among all the compounds **6h** inhibited not more than 38% of the activity of XO. We observed that the reduction of the exocyclic double bond in compound **6h** to give compound **8h** have led to a marked decrease of inhibitory potency. Moreover, replacement of the oxygen (**8h**) by sulfur atom (**8e**) in the six-membered ring seemed to reduce the inhibitory potency.

The fact that **9b** inhibited the enzyme to a very low extent could be advantageous as it demonstrates a possible selectivity for the proteasome (Figure 1.15).

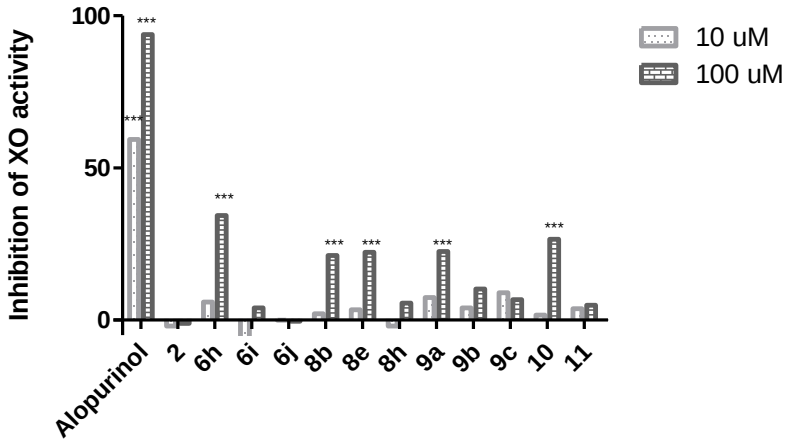


Figure 1.15: *In vitro* XO inhibitory activity of compounds **2, 6h-j, 8b,e,h, 9a-c, 10, 11** and allopurinol. Results are expressed as average values ± SEM. Differences were considered statistically significant if *p < 0.05, **p < 0.01, ***p < 0.001 versus negative control.

1.3.2.3 Cytotoxicity studies in human cells

Considering the relevant results observed in the proteasome inhibition assay, the cytotoxicity of **9b** in comparison to bortezomib was evaluated in NHDF. Additionally, the antiproliferative potential and selectivity of these compounds in Caco-2 cells as well as in PC-3 were also assessed in this study.

1.3.2.3.1 Cytotoxicity in NHDF

To determine whether the tested compounds are toxic on healthy cells, we treated normal human dermal fibroblast cells (NHDF) for 72 h with each compound at two concentrations, 10 and 100 μM. The cells were then subjected to MTT viability assay which quantifies the number of metabolically active cells.[118]

Initially, a screening assay at 10 and 100 μM of all the tested was performed. The results of this screening are demonstrated in Figure 1.16. Cells treated with 5-FU were used as the positive control. Untreated cells were used as the negative control and it was assumed as the 100% of cell viability.

Most of the compounds were not significantly cytotoxic for NHDF cells at 10 μM (Figure 1.16). However, compounds **9a** and **9c** at 100 μM displayed similar cytotoxicity to 5-FU. **9b, 10** and **11** demonstrated some cytotoxicity at concentration of 100 μM but it was inferior to the effect of 5-FU.

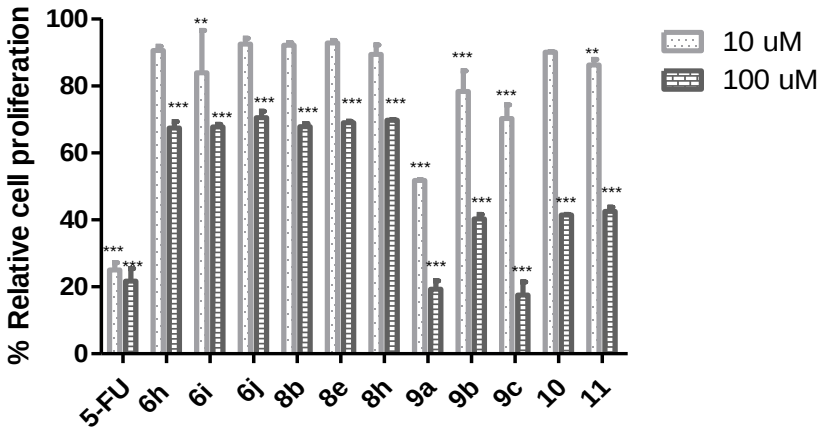


Figure 1.16: *In vitro* cytotoxic effect of compounds **2, 6h-j, 8b,e,h, 9a-c, 10, 11** and 5-FU in normal human dermal fibroblasts (NHDF). Results are expressed as average values ± SEM. Differences were considered statistically significant if *p < 0.05, **p < 0.01, ***p < 0.001 versus negative control.

Likewise, the IC_{50} (the concentration that provokes half the maximum effect in the cells) of **9b** and bortezomib were determined by constructing concentration-response curves for both. Accordingly, the cells were exposed to several concentrations of **9b (**1, 10, 33.33, 66.66, 100, and 200 μM **)** and bortezomib (0.1, 1, 2.5, 5, 10, and 50 nM) for 72h. IC_{50} of both compounds

were calculated using a nonlinear regression fit model with a 95% confidence interval. The results are presented in Table 1.5. Compound **9b** cytotoxicity on NHDF is markedly inferior to the cytotoxicity of bortezomib which can be interesting considering the potential future use of **9b** as a proteasome inhibitor. On the other hand, this also can be justified by the fact that although **9b** inhibited the different activities of proteasome to an interesting extent, it is still less potent than bortezomib as shown earlier.

Table 1.5: : Estimated IC_{50} values (μM) for cytotoxicity of **9b** and bortezomib in NHDF

9b	**78.91 ± 1.09**
Bortezomib	0.006 ± 0.001

1.3.2.3.2 Cytotoxicity effect in PC-3 and Caco-2 cells

Cytotoxic effects of **9b** and bortezomib in Caco-2 and PC-3 cells were determined by the well-established MTT cell proliferation assay. Both cell lines were exposed to **9b** and bortezomib at different concentrations during a period of 72 h. Untreated cells were used as the negative control.

The results (Figure 1.17) demonstrated that Caco-2 cells are more sensitive to bortezomib than PC-3. In fact, bortezomib inhibited cells proliferation significantly at all concentration by the same extent which seems to be the maximum inhibitory percentage that bortezomib can produce since it didn't suppress 100% of cells viability even with the highest tested concentration 10 μM. On the other hand, **9b** seemed to be less potent than bortezomib and affected Caco-2 cell viability markedly only at 100 μM concentration. Similarly, PC-3 proliferation was only inhibited significantly at 100 μM concentration. Although bortezomib was less potent against PC-3 cells, the viability of these cells was significantly decreased to 64, 46, 40, 29, 28 and 24% with 0.01, 0.033, 0.1, 0.333, 1, and 10 μM concentrations, respectively.

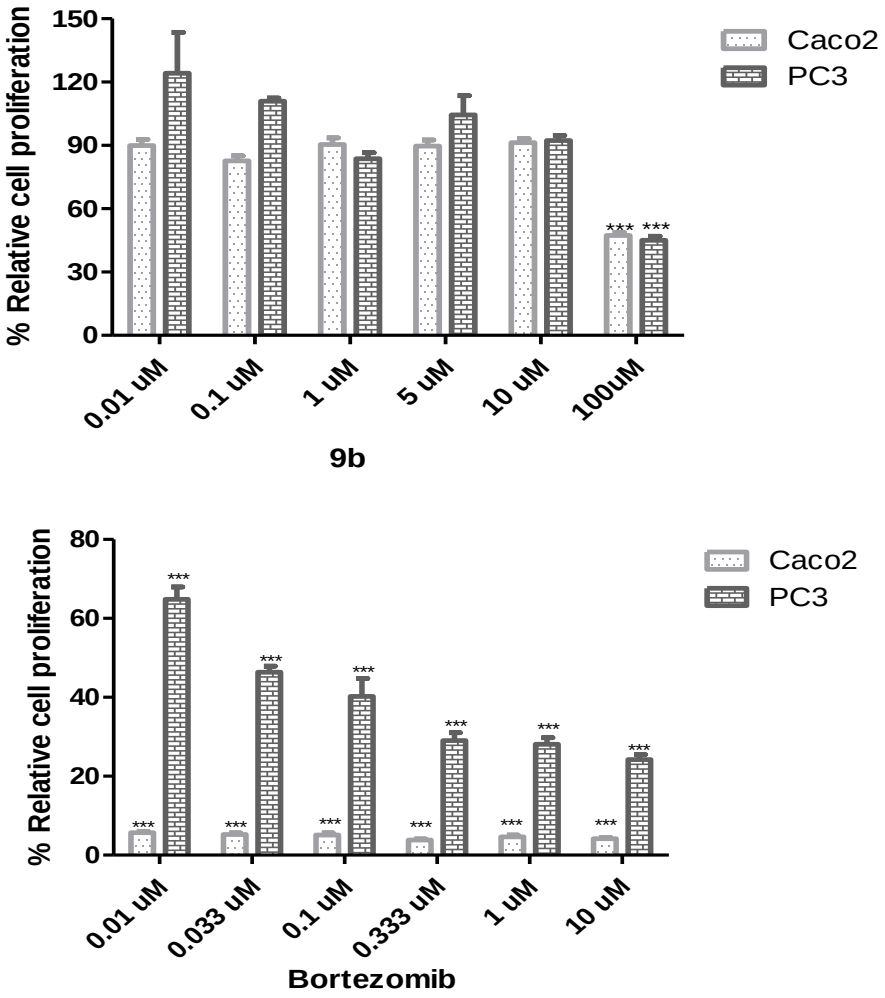


Figure 1.17: *In vitro* cytotoxic effect of **9b** and bortezomib in PC-3 and Caco-2 cell. All the data were statistically analyzed using the Two-way ANOVA. Results are expressed as average values ± SEM. Differences were considered significantly, if *p < 0.05, **p < 0.01, ***p < 0.001 versus negative control.

1.4 Conclusion and future perspectives

A series of 5-substituted barbiturate and thiobarbiturate derivatives were synthesized to be later evaluated as proteasome inhibitors. Due to the degradation of the 5-benzylidene thiobarbituric derivatives and the formation of bis-thiobarbiturates in aqueous solutions, these compounds were excluded from the biological evaluation. Selectively synthesis of the bis-(thio)barbiturates was not possible via the experimented conditions. However, reduction of the exocyclic double bond of 5-benzylidene (thio)barbiturates was possible as an attempt to increase the stability of the synthesized compounds.

Biological evaluation studies showed interesting results regarding proteasome inhibitory capacity of 5-[1-[2-(4-nitrophenyl)hydrazinyl]ethylidene]barbiturate, **9b**. In addition, this compound does not inhibit xanthine oxidase. Although **9b** showed cytotoxicity against healthy and cancer cell lines, it was less potent than bortezomib in both cases.

As future work, we propose studies of cytotoxicity in Jurkat cells, a leukemic T cell line. Moreover, molecular docking studies can be performed to predict the mode of binding and binding affinity of **9b** with the proteasome, also considering structural alterations that can increase this interaction.

On the other hand, it will be equally important to synthesize further derivatives of these compounds by varying the substituents on their molecular skeleton in order to optimize the biological results obtained. For example, we suggest the synthesis of 5-substituted (thio)barbiturates with a boronic acid group at the C4 position of the aromatic ring.

Chapter 2 - Community Pharmacy

2.1 Introduction

Community pharmacists play a key role in providing health care as medicines experts. The pharmacist is highly trained in the field of health and medicines, particularly, in the use of medicines, their interactions, and their composition. Pharmacists receive special training for dealing with patients and drugs in a hospital or community pharmacy in order to guarantee the rational use of the medicine as well as the well-being of the patients. Interpretation and validation of the prescriptions in addition to the delivery of medicines are the main responsibilities of community pharmacists. Pharmacists are not only the first but also the last point of contact with the patients in the health system. The majority of the population goes first to a pharmacy to seek treatment for their common health problems. Depending on the level presented, and always following protocols of pharmaceutical advice, the pharmacist may dispense non-prescription medicines, along with non-pharmacological treatment measures, or refer the patient for medical consultation. On the other hand, when the patient is accompanied by a medical prescription, the pharmacist will be the last health care professional to clarify any persisted doubts regarding patient's diagnosis such as how to take the medication or regarding non-pharmacological measures that may aid in their recovery.

The curricular internship in community pharmacy is essential for the consolidation and application of the theoretical knowledge acquired during the study period. It will also help in the growth at the professional level as the first contact with the reality of the profession and the labor market.

This report aims to describe the acquired knowledge and experience during the curricular internship, as well as the characterization of the pharmacy and all the functions performed by the pharmacist. The curriculum internship was held at the Holon Covilhã pharmacy under the supervision of pharmacist Patrícia Pais during the period from 22nd of January and 6th of April, 2018.

2.2 Holon Group

Holon Group is a national network of independent and autonomous pharmacies gathered by the brand name and shares a common vision, mission, and values. It consists of 391 pharmacies throughout the country and the number is increasing constantly. The group aims to optimize the services offered by the community pharmacy that drive quality and efficiency in pharmaceutical care. Holon pharmacies offer a proactive and personalized patient counseling, strong communication with its customers, differentiated products and innovative

services carried out by qualified pharmacists and other healthcare professionals who undertake continuous education in different areas. The group also sought to promote good health practices and rational use of medicines in the community which is reflected in its mission: "We turn a medical prescription into more quality of life, in any city, anywhere in the country."[119]

2.3 General characterization of Holon Covilhã pharmacy

2.3.1 Localization

The pharmacy is located in Alameda Pêro da Covilhã, n.º 31 R/C, Covilhã council, Castelo Branco district. Its strategic location next to Pêro da Covilhã Hospital, which is the main member of Cova da Beira hospital center, contributes greatly to its success and explains the variable population it serves. Additionally, being in the entrance of the city turns it into a destination for the travelers and tourists passing by the city.

2.3.2 Working Hours

Holon Covilhã pharmacy opens to the public from 8h in the morning until mid-night (24h) all the days of the week from Monday to Sunday including holidays complying with the Ordinance no. 277/2012, of the 12thof September, [120] amended by Ordinance no.14/2013, of the 11th of January[121] that defines the minimum weekly operating hours for Community pharmacies. Once a week, the pharmacy works for 24 hours fulfilling the shifts schedule that defines on-duty pharmacy in the municipality of Covilhã. The early-mentioned schedule follows a rotational regime and is elaborated by the Regional Health Administration (ARS). According to point 1 of article 6 of Ordinance no. 277/2012, of September 12, this information should be displayed in a visible manner.[120]

2.3.3 Outer space characterization

In accordance with Decree-Law no. 307/2007, of the 31st August,[122] Article 28 amended by Decree-Law no.171/2012 of 1st August[123] and the Manual of Good Pharmaceutical Practices for Community Pharmacy (BPF),[124] the pharmacy is properly identified by a green cross, which identifies the space as a pharmacy. The pharmacy also is identified by Illuminated exterior sign with a very characteristic image which indicates alongside with the organized interior without any type of products advertising that the pharmacy belongs to Holon group.

Furthermore, it exhibits announcements informing the public about opening hours, duty rotations, the identification of the chief pharmacist, the existence of a complaints book and video surveillance sign. Since the pharmacy premises must be easily identifiable as a healthcare facility and must reflect the professional nature of pharmacy, it is also identified

be a luminous green cross. The access for disabled people is guaranteed, as well as a discharge port for night's shifts. It is also important to mention the strongpoint of the outer space of the pharmacy which is the free spacious parking that facilities the access to the pharmacy.

2.3.4 Interior space characterization

The interior space of the pharmacy is divided into front-office and back-office. Both comply with the Deliberation No. 1502/2014, of the 3rd July on the facilities and equipment of the pharmacy. In addition to all the mandatory divisions, the interior space also has some optional areas.[125]

The front office is the area with public access where the pharmacy's staff get in direct contact with the customers. It consists of: the dispensary area, three counseling rooms and sanitary facilities for public use.

The dispensary area is spacious, air-conditioned, properly ventilated with adequate lighting and always kept clean. It contains five dispensing Counters with no barrier between pharmacy staff and patient, to ensure good communication on medicines instructions provided during the supply process, in addition to being individualized to guarantee the privacy a differentiation of the service. One of the counters has been made available for sitting down position for people with disabilities or customers with some motor limitations.[126] In this area, the shelves are organized in a functional categorization of various OTC medications and other health products. The shelves are grouped in several sets: family medicine, first aid, dermocosmetics, sexuality, seasonal products, baby-mama, veterinary products, oral hygiene, feet and legs, orthopedics, medical devices, and highlights. There is also a bench for people to take a rest while waiting to be assisted and a table with some games for children.

The counseling rooms permit a private discussion between the pharmacist and patient and/or their caregiver about issues related to their medical therapy or general health. The first one serves for measurement of blood pressure and biochemical parameters; the other three are used for consultations of different services such as diabetic foot, dermopharmacy, pedology, nutrition and administration of injectables.

The back-office is the operational or managerial area where the administrative tasks are executed. It contains the pharmacist chief's office, laboratory, dressing rooms, bathrooms for employees and a space for workshops and presentations that are organized in the pharmacy. Additionally, it is the area where orders checking, approval and intake occurred. This area has many divisions according to the status of the received products: products to be introduced, products to be stored and products to be returned. Most of the medicines are stored in the Robot which serves as a large storage area which is computer-controlled with a touch-screen interface. The robot has five output ports, three behind the dispensing

counters, one behind the sitting counter and an emergency exit with priority over the other ports. The existence of the robot reduces the daily workload by decreasing the effort spent on distributive tasks which increases the time the pharmacist has to interact with the patient. This contributes to improved and more personalized counselling as a result of providing the pharmacist with more time with patients.

There are also shelves for storage of customers' reserves and other sets of shelves for storage of products that do not fit in the robot.

On the wall, two boards are placed where discount details, shift schedule, useful contact, cleaning schedule and other information related to ongoing campaigns are affixed.

A pharmaceutical refrigerator with temperature record system is used to store thermolabile medicines such as insulin or vaccines.

The laboratory is equipped with all required laboratory materials. However, it is only used for Individualized Medication Preparation whereas all the compounded medicines are sent to be prepared in Diamantino pharmacy in Fundão. The laboratory contains a cupboard where all the obligatory bibliography for consultation when necessary are kept in hard copies as well as digital formats such as Portuguese Pharmacopoeia, Prontuário Terapêutico, Formulário Galénico Português, antibiotherapy manuals and pharmacognosy among others.[122,124]

The pharmacy is equipped with surveillance cameras, fire extinguishers and other necessary means to respond to potential emergency situations.[124]

2.3.5 Human resources

Excellent services are not only guaranteed by the best equipment and the best physical structure at the pharmacy, but also by highly qualified employees. The FCH team provides high-quality services, always with the aim of promoting health, the well-being of customers, and the efficacy and safety of medicines. During my internship, the staff of the pharmacy was composed of the chief pharmacist, Dr. Pedro Diamantino, and other 7 pharmacists in addition to 3 pharmacy technicians. However, they do not work simultaneously since there are monthly-made shifts schedules to respond to the opening hours of the pharmacy.

At FHC there is a division of tasks where each employee has properly established functions and is given all the information and materials necessary to develop and fulfill their goals. In addition to the individualized tasks, the team also has common responsibilities, such as: dispensing of medicines and other health products; conducting biochemical tests; prescription conference; arranging and organizing all workspaces; provision of high-quality services on a daily basis and promote the safe and rational use of the medicinal product.

The team is a young team with a great desire to learn more and to develop their knowledge constantly. I was always able to feel the great spirit between the staff and their constant help to each other and to the trainees which contribute to better integration and training of trainees.

2.3.6 Informatic resources and general equipment

For efficient performance of the daily activities at the pharmacy, appropriate equipment and informatic resources are needed. The equipment available at FCH includes computers, telephones, printers, citizen card readers, scale with height meter and body mass index (BMI), blood pressure monitor, laboratory material, biochemical test apparatus, thermometers and all essential equipment for pharmaceutical activity. Laboratory materials are available in the pharmacy according to the law,[127] yet, they are not used since compounded medicines are not prepared in the pharmacy.

The computer software used at FHC is Sifarma2000. This software assists with the daily activity of the pharmacy such as dispensing medicines, stock control, inventory, receiving and managing orders, returns processing, and billing. Additionally, the sheet of each product in the software contains information on stock movements, purchases, sales, and prices. This feature facilitates inventory management and allows stock tracking.

Sifarma 2000 also provides quick access to scientific information that supports the pharmacist while dispensing a prescription such as, medicines qualitative and quantitative composition, indications, dosing, adverse reactions, precautions, interactions, and contraindications. It also grants pharmacotherapeutic follow-up of patients, through the creation of profile sheets in which personal data are recorded, reimbursement plans, results of determinations of biochemical parameters, history of medication dispensed and respective dosages, and warnings regarding the customer. These profile sheets are only created after obtaining a signed consent of the patient.

Through Google Drive, all Holon pharmacies and their employees have access to common documents, such as Quality Manuals and supplier lists among others. There is also a part of Google Drive with limited access only to pharmacy employees, where information such as prescription verification, workshops, cleaning registration, employees' registration, employees' workshops registration, quality targets and many others are found. Every member of the team has a Holon email with an individual account that gives access to different areas of Google Drive and Holon group website.

During my internship, I was able to learn a lot about Sifarma and to use it properly. Similarly, I was taught to use different equipment and got to use it on patients like blood pressure monitor and glucose meter.

2.4 Holon products

Holon group offers a wide range of affordable products under its trademark. These products include food supplements, bandages, and skin repair products, slimming products, dietary supplements, medical devices, baby care products, dermocosmetics, oral hygiene, and ophthalmic cleaning.

These products are very advantageous for the customers as they offer high-quality products at low prices compared to other equivalent products and they can only be found in Holon pharmacies. Each product has a technical file in Holon portal online, which is accessible for all the employees, setting out all the essential information for appropriate patient counselling.

2.5 Marketing and Communication

Holon group has a variety of strategies for communicating with customers, starting from the infrastructure and uniformity of white coats, including Holon Magazine, Holon TV, information leaflets, Muppies to promote the services that the pharmacy offer to the community, Holon Facebook page and merchandising products. The correct implementation of these strategies is important for the construction of the Holon brand. Thus, there is an assigned person in the team to take responsibility for this section, responsible Marketing, and Communication. The main activity is to monitor the compliance and adaptation of these guidelines according to the needs of the pharmacy. In Holon Pharmacy Portal there are media to assist in planning and implementing of marketing and communication strategies.

2.6 Provisioning and Storage of medicinal products

I spent the first month of my internship in the back-office. During this time, I was able to receive and store orders, perform returns and participate in expiry date controls. This contributed to better integration in the work environment and make my next step, attending costumers, easier since could familiarize myself with the commercial names of various medicines and review the active principles as well as therapeutic indications of many of them.

2.6.1 Suppliers Selection

Suppliers and wholesalers selection is an essential step that may influence the quality of the service provided by the pharmacy. It is not an easy task to choose the right supplier that will deliver the orders at the right time, with the right prices, and in high quality. Therefore, when choosing a supplier over another, many factors are taken into consideration like: portfolio of available products, the location of the warehouses, their applied prices and

discounts, the delivery schedule, quality of services delivered, flexibility for order changes in terms, and level of service (number of units ordered / number of units supplied).

Quality of the service is evaluated according to the frequency of problem occurrence, supplier's capacity, and rapidity to resolve them and the fulfillment of the schedule of deliveries.

It is beneficial to work with more than one supplier to ensure that out-of-stocks products at the preferred supplier can be obtained from the other supplier.

2.6.2 Stock management

Stock management is an important practice at community pharmacy to maintain a balance between the invested capital, the available space, and costumers' demands. Achieving this balance leads mainly to the efficient utilization of resources. Minimum and maximum stock levels should be established to avoid overstocking, which increase the risk of medicines expiring, low storage availability, and excessive stock costs. Additionally, the levels serve also to prevent understocking which lead to failure in fulfilling customers' needs.

Several aspects should be considered when defining stock and its level. Firstly, the community served by the pharmacy, prescription habits of the doctors of the region and the time of the year affect widely the decision on stock levels. Moreover, the storage capacity of the pharmacy and advertising campaigns also have an impact on the decision. Not to forget the fact that pharmacies are obliged to possess at least three medicines out of the cheapest of each homogeneous group which also affect the stock definition.[128] The previously mentioned factors are constantly under evaluation that leads to a regular adjustment of the stock.

Defining stocks can be done manually or automatically. The manual definition of minimum and maximum stock can be made according to product rotation, in case of exposed products, or to the number of packages that are sold monthly in case of unexposed products. On the other hand, the automatic definition of stocks is carried out by the computer software, Sifarma2000, which estimates the needs according to the rotation of the products and generates an automatic order. It can also be adapted to products with greater seasonal dependence. The minimum and maximum stocks are defined as per the sales of products and when the minimum stock is reached an order proposal is generated. When a customer requests a product that is out of stock or even if it does not make a part of the stock, the stock at other Holon pharmacies, which share the same owners, is checked and if the product is found in one of them, a loan is registered in Google Drive.

2.6.3 Ordering & Receiving Process

Orders can be created in several ways depending on their purpose and urgency. The daily orders, which are the most frequent orders in FHC, are made twice a day, one before lunch time and the other at night in a pre-defined hour with the supplier. This helps to ensure that the requested products can be delivered on the agreed time. Daily orders are made using Sifarma 2000 software. Sifarma2000 makes an order proposal the minimum and maximum level of each product and the existing stock in the pharmacy. The products and quantities recommended in the proposal are checked by one of the staffs to make adjustments when necessary, so the stock is always kept within the predetermined level. Moreover, adjustments are made to the quantities or products to be ordered if the sales are higher than expected or if there is some type of discount offered by another supplier.

If an unavailable product or medication is requested during dispensing, the pharmacist can place an instant order through sifarma2000 with estimated date and time for arrival. In this case, a reservation of the product is made in the name of the customer with associated contact which will be visible on Sifarma 2000 at the time of receiving it. The reservation could be paid or unpaid. Thus, in case of unpaid reservations, the reserved products have a specific stock to ensure that they will not be sold to another person and can remain in that stock for 15 days. In case of paid reservations, the products stay in the pharmacy until they are picked up by the customer or until they expire.

In relation to acquisition of products directly from laboratories, it is usually done when there is an economic advantage and it implies that a large volume of products is ordered. The products most often ordered this way are some OTCs, child care products, cosmetics, and seasonal products.

The orders are delivered to the pharmacy in plastic containers at the pre-defined times. Soon after receiving the orders, containers are opened, and delivery notes are checked. Likewise, the containers are checked for the presence of any medicines that require refrigeration, which are normally delivered in thermal containers, so they can be moved immediately to the to-be-received shelf in the fridge to be stored there until the order is entered into the system.

After initiating order reception in Sifarma2000, invoice’s number and value are registered. Afterward, products’ bar codes are scanned and the quantities, sales price to the pharmacy, retail price (PVP) and expiry date are recorded. Here, it is worth mentioning that OTCs and some other health products have their price calculated according to the pharmacy's margin since they do not have defined retail prices. At the end of order receiving, labels are printed with the price and respective bar code for OTCs and other health products that do not have a defined retail price. Performing a good receiving process is a vital component to ensure the correct management of the stocks and to facilitate countability process. Consequently, better

management of the pharmacy. Likewise, during the process, received products should be guaranteed to be in the required conditions of quality and safety, i.e. packaging conditions, no spills, expiration date, price in the correct packaging, etc.[124]

2.6.4 Billing process

Orders' invoices are sent in two or three copies, the original, the duplicate and if any, the triplicate. The original invoice is dated and signed with the employee's initials and is later kept in a drawer identified with the name of the supplier for later submission to the pharmacy accounting. The invoice, or delivery note, contains information regarding the products (including national code, trade name, dosage, pharmaceutical form, quantity/volume), number of units ordered and shipped, the existence of bonuses/discounts, PVF, PVP, and VAT. Furthermore, it comprises information identifying the pharmacy (name, address and tax number, type of customer, among others) and the supplier. Each delivery note is identified with an invoice number and a bar code, to facilitates the accounting work at the end of each month.

2.6.5 Product returns

Sometimes products must be returned to the supplier for not complying with the quality requirement of the pharmacy. This happens in cases like damaged products, expired products or with short validity and products that were not ordered. Besides the previously mentioned cases, medicinal products are returned to the laboratory when they have been withdrawn from the market.[129]

In product return process, a Return Note should be created by accessing the Returns Management area in the orders section of Sifarma 2000. The note is filled in with the name of the supplier, product to be returned, why it is returned, invoice price, invoice number, date and time for the product to be picked up and, in the comments, a more specific summary of the reason for the return. Three copies of this document are printed, all need to be dated, signed and stamped by the person in charge. Two copies are sent to the supplier while the third is held at the pharmacy and must be signed and stamped by the distributor with the date of collection of the product. Upon receiving the medicines and other health products, they are properly stored in their designated places. The storage should be done in a way that ensures proper preservation of the products and that they are easily found by pharmacy employees.

Each product's sheet in Sifarma2000 contains the location of the product to facilitate finding them.

2.6.6 Storage

The storage of all products is made based on the rule: "First-Expire, First-Out" (FEFO). This means that the products are stored in a way that products with shorter shelf-life are dispensed first. Products that do not have expiry dates are stored according to "First-In, First-Out" (FIFO) principle. FIFO principle implies that products to be placed to be dispensed first are the ones that have been in the pharmacy for a longer time.

Most of the Prescription drugs and OTC are stored in the robot, including psychotropic and syrups. During storage in the robot, it is necessary to record the expiry date of each product and to scan the bar code. The robot stores the products according to the FEFO principle. The robot allows smart control of the stock by optimizing storage area and reducing the effort that needs to be made by pharmacy employees for stock management.

Medicines that require to be stored at low temperatures are held in the refrigerator in alphabetical order.

Most non-medicines products are freely accessible to the public and they are normally labeled with their prices and bar codes. Products that do not fit in the public area are stored in the back-office in specified shelves.

Temperature and relative humidity in all the areas assigned to be used for products storage must be controlled to ensure that medicines and other health products are preserved under adequate conditions for their stability.

2.6.7 Control of expiry dates

Expiration dates control is one of the essential processes in stock management. This control ensures that all sold products have the necessary shelf life to be used. This begins at the moment of order receiving and the introduction of the products into the robot. Every month a list of products which expires within three months is printed using Sifarma2000. Subsequently, the data in the list are physically verified, by checking the stock of all the products mentioned in the list including those that are in the robot. When there are discrepancies, the corrected quantities and expiry dates are updated in Sifarma2000. Expired products are placed on a specific shelf and identified to be returned to the laboratory or supplier, or to be removed from the stock record in cases in which the laboratory does not accept the return. Products with sufficient validity to be used can be sold since the conditions of storage were always taken into account. These products are normally placed on a specific shelf in the back-office and have a priority to be sold. During my internship, I was able couple of times to perform this monthly task.

2.7 Compounded medicines

The compounded medicines to be prepared in the pharmacy are sparse and although the pharmacy has an equipped laboratory as the law stated, medicines compounding of Holon Covilhã pharmacy is accomplished in Diamantino pharmacy to reduce the costs of raw material acquisition.

I spent two days in Diamantino pharmacy to get the required training and prepare some compounded medicines. In the first day, I get the chance to attend a presentation about the procedures followed in the pharmacy to prepare compounded medicines.

We were introduced to the legislation that regulates the extemporaneous preparation in the pharmacy as well as the good practices that should be followed. Additionally, we were introduced to all the information sources that can be used during the preparations (Portuguese pharmacopeia, European Pharmacopeia, *Formulário galenico português*, Pharmaceutical Compounding Information Center (*Cimpi*). etc.). We were presented with all possible scenarios we could encounter in the pharmacy such as magistral formulas ("any medicinal product prepared in a pharmacy in accordance with a prescription for an individual patient"),[130] and officinal formulas ("any medicinal product which is prepared in a pharmacy in accordance with the prescriptions of a pharmacopoeia and is intended to be supplied directly to the patients"),[131]

In the second day, I had the opportunity to prepare topical sulfur ointment as a treatment for a patient with scabies. The patient had a prescription of the type "MM" (compounded medicines) with magistral formula. However, this compounded medicine does not have a preparation sheet in the *Formulario galenico portugues*. Thus, the pharmacist had to contact *Cimpi* to provide the best technic for its preparation. *Cimpi* sent all the bibliography needed for the preparation. The raw materials required were sulfur powder and solid paraffin. We did the preparation following the documents sent by *Cimpi* and according to the standards of Good Pharmaceutical Practice.[124,129]

Following the preparation, quality control should be performed by checking: organoleptic characteristics (color, odor, appearance as it should be homogeneous), quantity to be dispensed (variation less than 5%), and conformity with monograph definition of semi-solid preparations for local application. I was also able to calculate the price of the preparation according to Ordinance No. 769/2004 of the 1st of July.[132] The total price of the preparation is the sum of raw material costs, packaging material costs, and the fees multiplied by 1.3, plus VAT (6%)

The fees value is based on an F factor whose value is updated and published annually by the National Statistical Institute (INE).[132]

The preparations made are recorded on a excel sheet in the drive of the pharmacy with batch identification (consisting of a sequential number and the year of preparation which facilitates traceability), record of substances used and their batches, preparation protocol, information regarding customer and prescribing physician, the performed quality control, storage conditions, shelf-life and the price for public.

2.8 Pharmaceutical Dispensing and Counselling

Despite the various functions that are carried out in a Community Pharmacy, the principal activity of the pharmacist in community pharmacy is still dispensing medicines and patients' counselling. The role of the pharmacist in the dispensing process is of great importance since the pharmacist will probably be the last healthcare professional to interact with the patient before taking their medicine.

Regardless of whether the medicines are prescribed by a physician or not, it is the responsibility of the pharmacist to always deliver appropriate counselling to the patient, concerning the correct use of the medicines along with dosage, duration of treatment and important interactions or adverse effects when applied. This will help significantly in reducing the errors associated with medicines use.

Holon Pharmacies have a Manual of Attendance to ensure a uniform service in all the pharmacies, emphasizing the importance of a positive attitude and constant proactivity.

After a few weeks of observing Pharmaceutical dispensing and counselling of the pharmacists, I started attending patients at the counter always under the supervision of a pharmacist. The observation phase was crucial for me as it helped me to perform better attendance and it allowed me to figure out the best way to approach customers, to advise them on their medication, to counsel on non-prescription medicines and the best attitude in each situation.

2.8.1 Prescription Medicines

Prescription only medicines can only be dispensed in the pharmacy and with presenting a medical prescription, and this category includes medicines which, according to Decree-Law No 176/2006 of the 30th of August (Statute for Medicinal Products)[133] present at least one of the following characteristics:

- May constitute a risk to the health of the patient when used without medical supervision even when used for the correct indication;
- May constitute a risk for the health of the patient when used in considerable quantities for purposes other than those they are indicated for;
- Drugs containing substances, or preparations based on these substances, whose activity or adverse reactions require further investigation;

- Medicinal products for parenteral administration.

In Portugal three types of prescriptions are used by the National Health System (SNS): Paperless Electronic prescriptions, Materialized electronic prescriptions and Manual prescriptions. Additionally, there are Veterinary prescriptions which are not related to the national health system.

During the last few years, more specifically since April 1, 2016, the dematerialized electronic prescription has become compulsory for the entire SNS, by Order 2935-B / 2016, of the 25th February.[134]

The materialized electronic prescriptions are only temporary, functioning as a transition between manual prescriptions and dematerialized electronic prescriptions.[135] These prescriptions are printed according to the model approved by Order 15700/2012 of the 30th of November.[136] The model is divided into two parts: the first part corresponds to the medical prescription itself while the second part corresponds to the treatment guide that is kept with the patient.

Dematerialized electronic prescriptions, which represent the majority of prescriptions dispensed in the pharmacy, may be sent by message to the patient's phone that includes the number of the prescription, access and dispensing code, and code of the right of option. Patients may be provided with a printed treatment guide with all the precious codes and prescription number during the consultation if preferred by the patient. In occasional cases, manual prescriptions can be used according to article 8 of Administrative Rule no. 224/2015, of July 27th.[137] These cases can be:

- Failure of the computer system;
- Inadaptation of the prescriber;
- Prescription at home;
- Maximum 40 receipts per month.

Regardless of the type of prescription, all prescriptions must comply with certain criteria that are described in Administrative Rule no. 224/2015 of the 27th of July.[137] Prescriptions must include international common denomination of the active substance, the pharmaceutical form, the dose, the size of the package and the dosage regimen. However, some medicines may be prescribed by the brand name in case of the non-existence of a generic medicinal product in the market or when it is accompanied by one of the possible technical justifications which prevent the replacement of the prescribed medicinal product under the brand name:[137]

- Exception (a) Prescription of a medicinal product with a narrow therapeutic margin, according to information provided by INFARMED;
- Exception b) Established suspected, previously reported to INFARMED, of intolerance or adverse reaction to a drug with the same active substance but identified by another commercial name;
- Exception (c) Prescription of a medicinal product intended to ensure continuity of treatment with an estimated duration of more than 28 days.

Furthermore, all prescription must have prescription number, prescriber identification, place of prescription, date of prescription, validity, patients name, responsible financial entity, identification of special reimbursement regime ("R" for retired patients and "O" for patients who have some other special contribution regimen identified by a specific diploma when applicable), and handwritten signature of the prescribing physician in materialized electronic prescriptions and paper prescriptions.[135]

Additionally, each manual or materialized electronic prescription can only contain up to four different medicinal products drugs where the total number of prescribed packages should not exceed the limit of two per medicinal product. However, In the case of dematerialized prescriptions, a maximum limit of six packs per prescription can be prescribed with also a maximum of two packs of each medicinal product. When the prescribed medicinal products are in the form of Unit-Dose Packaging, up to four packs of the same medicinal product may be prescribed in each prescription.[137]

Regarding the validity of the prescriptions, there are two different situations in the case of paperless electronic prescriptions. These prescriptions may be either non-renewable, used mainly for short-term treatments, with a validity of one month from the prescription date or may be renewable, more common in chronic treatments, with a validity of six months. Nonetheless, these two types of prescriptions can arise in the same paperless electronic prescription, where the expiration dates are specified to each medicine prescribed. The materialized and manual prescriptions are generally valid for 30 days.[138]

All types of prescriptions should be carefully evaluated at the pharmacy counter, confirming whether all the mandatory parameters are well filled.

During my internship, I got the opportunity to dispense all types of prescriptions. Most of the dispensing I performed involved prescription-only medicines, and the pharmaceutical intervention was very important to educate patients and ensure adequate treatment. The clarification of any doubt of the customers is crucial to ensure the correct use of medicines and avoid any dosage error, medication changes and interactions with other medicines/foods/beverages. Additionally, it was important to educate patients on the most common adverse effects and how to deal with it. I was able also to prepare oral suspension of

several antibiotics for pediatric use. During the preparation of oral suspensions, care must be taken to avoid the creation of aggregates that may result in errors of dosage administration. Furthermore, the preparation procedure varies from suspension to suspension that is why it is always preferable to read the preparation instructions.

2.8.2 Reimbursement schemes

In Portugal, many of prescription-only medicines are not fully paid by patients where part of the price of these medicines are paid by the state. The main reimbursement entity is the national health system which has several subsystems. There is also the possibility that medicine price reimbursement may have complementarity with other organisms than the NHS, so the customer may thus benefit from two different organizations. Reimbursement by the State is made according to medicines' pharmacotherapeutic classification, or through a special regime for beneficiaries and special groups of customers or pathologies.[138]

The software assumes automatically the reimbursement system inserted in the prescription in case of electronic prescriptions. However, if the patient has more than one reimbursement system, manual selection of the second system and validation may be necessary by entering the patient's card number for the second reimbursement system. In this situation, it is necessary to take a photocopy of the prescription, hence, the original version can be sent to the principal organism while prescription photocopy attached to a photocopy of the beneficiary card of the can be sent to the complimentary organism.

In the case of manual prescriptions, it is necessary to select manually the reimbursement system in the software so that the reimbursement is assumed when dispensing the medicines. Depending on the reimbursement system selected, an identification number is issued that must be confirmed when prescriptions conference is performed.

For customers with insurance, the prescriptions have a different model than the regular one of the NHS and the reimbursement is 100% guaranteed by their insurance.

During my internship the majority of reimbursement were covered by the SNS, both in the general scheme (plan 01) and in exceptional schemes such as plan 45 (concerning legal diplomas), plan 46 (migrant worker), and plan 48 (pensioners), among others. Moreover, in Covilhã many of the elderly are covered by a specific reimbursement system for working in the Wool Industry.[139] This exceptional reimbursement scheme (SNS-LA) implies that the maximum value of the reimbursement of medicines corresponded to the reference price of the homogeneous group (average of the lowest five prices of the homogeneous group),[140] which means that the State only reimburse the full of the cheaper medicines, and if the customer chooses more expensive one, they would have to pay the difference between the value of the medicine they choose and the reference value.

However, Article 2 of the previously mentioned ordinance[140] has recently undergone an amendment by Ordinance 154/2018 of the 28th of May.[141] According to this amendment, the reimbursement of medicines became 100% of the product's PVP, regardless of the reference price of the homogeneous group. This means that the state will cover 100% of the product's PVP regardless of patient's choice of receiving the cheaper or a more expensive drug in the homogeneous group. This amendment entered into force after finishing my internship.

2.8.3 Prescription management and reimbursement process

An essential part of the correct management of the community pharmacy is the processing of prescriptions to be reimbursed by the respective organisms. Paperless electronic prescriptions are processed automatically when dispensed and all the information is sent automatically to the CCF (Invoice Conference Center) by Sifarma 2000, hence, it is not necessary to send any physical documentation. Yet, it is necessary at the end of each month to close the lots and issue invoices with the payment information referents to electronic prescriptions. However, manual and materialized electronic prescriptions need to be fully processed by the pharmacy's employees on a monthly basis.

All manual prescriptions must be verified daily by two different employees as these can be misinterpreted for misunderstanding the handwriting leading to dispensing of the wrong medications or wrong dosages. Thus, the possibility of detecting the error and contacting the patient before taking medicine is higher.

The prescriptions are verified to identify the prescriber, the prescription place and its visa sticker, patient's contribution subsystem type, doctor's signature, prescription's validity and absence of erasures. If the patient is a pensioner, the visa sticker of prescription place must be green. The signature of the patient, the signature of the dispensing employee, the date of dispensing and stamp of the pharmacy should be checked. Additionally, it should be confirmed that the medicines dispensed match the ones prescribed and comply with legal specifications such as the number of packages and size.

At the beginning of each month, the prescriptions and the documents necessary for the guarantee of reimbursement are sent to the respective reimbursement organizations. Until the 5th of each month, NHS prescriptions with plans (01, 48, LA, Etc.) are gathered by batches and accompanied by the respective batch Identification documents, record resumes, and billing documentation to be picked up by CTT that transport them to the CCF.

On the other hand, prescriptions covered by different reimbursement organisms should be sent to ANF (National Association of Pharmacies), usually until the 10th of each month, that take the responsibility of their distribution to the respective reimbursement organisms. In this case, the same documentation mentioned above should be sent with the addition of

supplementary receipts of paperless electronic prescriptions which should be duly signed by the customer and attached to a copy of the beneficiary card of that organism.

If any prescription or batch does not comply with the legal requirements, it will be returned to the pharmacy for necessary readjustment, so it can be resubmitted the following month. In case the pharmacy could not rectify the returned prescription/batch, reimbursement would not be made to the pharmacy. Thus, this process should be performed carefully for the pharmacy not to have any financial loss.

2.8.4 Non-Prescription Medicines

Any medicine that does not fit into any of the criteria of prescription-only medicines belongs to the group of non-prescription medicines. These medications can be dispensed without a prescription. However, it should only be dispensed for specific therapeutic indications and after an evaluation of patient's symptoms, clinical state and patient's regular medications. These specific therapeutic indications, such as flu and colds, headache and mild to moderate muscle aches and allergies, are included in the list of situations that can be self-medicated and should be dispensed under pharmaceutical counselling.[142]

After evaluating patient's case and selecting the most cost-effective drug for the situation, the pharmacist should counsel the patient regarding: medicine dose, mode of administration, precautions for use, adverse effects and interactions, and should inform the patient that in case of persistence and/or worsening of symptoms, a physician should be seen.

The Holon group has counselling protocols for this type of situations that help the standardization of pharmaceutical intervention. These protocols cover features of the health problem, pharmacological and non-pharmacological measures for its management, and cases where referring to a physician is necessary. These protocols were very useful during my internship.

The most common situations I faced during my internship were mainly cases of cold and flu (such as nasal congestion and dry or productive cough), gastrointestinal symptoms (such as heartburn, diarrhea, and constipation), muscle pain and allergies.

2.8.5 Psychotropic and Narcotic

Due to the characteristics of narcotic drugs and psychotropic drugs, their dispensation is strictly controlled. When dispensing a narcotic or psychotropic drug, the computer system presents some additional fields which must be duly filled out with: prescribing doctor's name and number of professional card, the full name, date of birth, address and citizen / ID card number of the purchaser, along with the name and address of the person for whom the medication is intended (when purchaser and the patient are not the same). It is also important to note that the person picking up the medicines should be at least 18 years old.[138]

When the dispensing process is completed, a document with all the information regarding the dispensing (medicine, prescription number, dispensing date, data collected from the customer and purchaser) is printed. This document is stored in a specific file, with a photocopy of the prescription in case of manual or electronic materialized prescriptions and is kept for 3 years.

Copies of the manual prescriptions along with the outflow logs (medicines and data of the acquirer) of Psychotropics and Narcotics shall be sent to INFARMED until the 8th of each month. Annually, a balance of Psychotropics and Narcotics inputs and outputs, as well as benzodiazepines, is sent to INFARMED.[138]

These drugs are not dispensed as frequent as other medicines however, I had the opportunity to dispense with some of these medicines like Palexia® (tapentadol), Ritalin® (methylphenidate), buprenorphine, and transdermal Fentanyl systems.

2.9 Projects and Pharmaceutical Intervention

Holon pharmacy believes in pharmacist's role as an educator and community health promoter, it lunches many workshops in the society targeting different groups. Moreover, it takes part in many national and international studies taking advantage of its variable population. Additionally, by carrying out these screenings and workshops, the population will recognize the services the pharmacy offers thus they could benefit from it and the pharmacy will invigorate its services.

In the 8th of February, a workshop in pedology has taken place for children and teenagers in school. They were presented to the science of pedology and what it covers in addition to the most common pathologies that may affect the feet (ex: worts, corns and calluses, tinea pedis, onychomycosis, ingrown toenails. etc.) and how they can be as well as the main treatment for each. Furthermore, the pedologist demonstrated the healthy structure of the sole of the feet, how it changes totally in people with flat feet and how it can be managed. In the end of the presentation, the doubts of the audience were cleared, and they were invited to have a pedology consul in the pharmacy in case there is any necessity.

In the 16th of February, we went to a nursery school with the dermopharmacist, Jessica, to make a presentation about Babe's skincare for the new moms. Jessica explained the differences between the skin of an adult and the skin of an infant. She taught the moms the correct way to bath their babes, the importance of skin hydration and sun protection. They also were explained how to manage the problems of atopic skin and the type of products they should use to reduce the discomfort the babe might have. Diaper rash management was also covered in the presentation. The mothers showed great interest in the presentations and all their questions were answered.

In the 22nd of February, another activity was held targeting pregnant women under the name "*Conversas com Barriguinhas*". In this activity, healthy nutrition habits during pregnancy were the main subject. The presentation was displayed by the nutritionist of the pharmacy, Daniela. During the presentation, Daniela corrected some common nutritional misconceptions among pregnant women and encouraged the adoption of healthy diets that could provide the pregnant with all the necessary nutritional elements for the fetus development. She also covered gestational diabetes in her presentations and diet modifications that should be done in such cases. Not only pregnant women attended this activity but also their partners who revealed perfect curiosity about the theme.

In addition to these activities, the pharmacy also carried out many screenings in which I took part.

Firstly, the first screening test took place in Liga dos Amigos do Bairro dos Penedos Altos in which participants underwent an assessment using a validated questionnaire to determine their risk of developing type 2 diabetes along with determining their glycemia, blood pressure, body index, and abdominal perimeter. The assessment aimed at identifying those at risk of developing the condition as well as patients with type 2 diabetes before they start to develop complications. Patients were alerted to potential risk factors to enable lifestyle modification.

Secondly, I attended arterial stiffness assessment follow up in the pharmacy that was held in the counseling room by the pharmacist Natália Craveiro. Arterial stiffness (AS) is measured by pulse wave velocity (PWV), which has been defined as the velocity at which the pulse wave travels a defined distance in the thoracic aorta.[143] Measuring the arterial stiffness enables to identify and treat the cardiovascular disease before it strikes and evaluate if a medication, change of lifestyle, or new dietary changes are improving cardiovascular health. The invited participants had already done an evaluation of arterial stiffness and the age of the arteries last year and were asked to do the evaluation this year to assess the changes in their lifestyle and how these changes have affected their state of arterial stiffness. In all participants, the clinical history and lifestyle habits were assessed using a standard questionnaire. Body weight, lean body mass, body fat percentage, height and waist circumference were measured, and the body mass index was calculated. The measurement of AS is made by a simple and non-invasive method, similar to that used to evaluate blood pressure. It can measure the blood pressure in the arm (Brachial Blood Pressure) and at the heart (Central Blood Pressure) in one simple step. An armband is placed on the arm and allows to obtain parameters related to the health of the arteries. Blood pressure (BP) is measured automatically in the first measurement which also serves for calibration purposes. After a one-minute interval, a second measurement is carried out to calculate the PWV. This test aids as a proactive approach focused on the search for subclinical AS in the general population. Some participants showed better results this year and some had worse, we suspected that this

is due to their changes in lifestyle habits. Patients with worse results were advised to show the test report to their family physician and were counseled on how they can recover the CV health by following healthy diets, doing exercises and quit smoking if it is the case.

Furthermore, to increase the awareness of the obstructive respiratory diseases in the population and to promote the early diagnosis of new cases, the pharmacy organized a spirometry testing day inviting smokers, ex-smokers and people with respiratory complaints to participate. Before carrying out the test each participant was verbally explained the objectives and methods of the study and was asked to sign a written consent for the pharmacy to have the personal data. Subsequently, Quality of Life questionnaire in addition to COPD Questionnaire (What is your degree of risk) were applied. The participants had to repeat the test at least 3 times to get productive curves and ensure that the results have high quality. After the test, the participants were given a report with their results with some observations of the pharmacist when necessary to show to their family physician. The activity helped to alert the smokers on how tobacco is affecting their lung functions. It also served to promote smoking cessation service and the follow -up of people with Chronic Respiratory Disease in the framework of the Holon Pharmaceutical Consultation.

Lastly, I had the opportunity to go to Santa Casa da Misericórdia nursing home to help the nurses with the preparation of the weekly medication for the elderly residing there. Each patient has a Weekly Pill Organizer which is divided into 5 compartments for each day and is filled according to the therapeutic plan of the patient. The preparation must be done once weekly, usually on Thursday. During the preparation we needed to be highly rigorous, particularly when splitting a tablet, and confirmation of the work was a must after filling each organizer. The experience was enlightening into the reality of elderly medications.

2.10 Holon Services

Many health services are provided by the pharmacy in addition to dispensing medicines. The main advantage of these services is accessibility and affordability with high quality.

In Holon Portal, there are manuals for each service that can be provided in the pharmacy. These manuals contain information about the human and technical resource to be employed, as well as the procedures to be carried out. The price varies from service to service and can be adapted according to the type of consultation developed for each case.

All customers attending one of the services are coded to allow an evaluation of their progress. After the first consultation, a Quality of Life questionnaire is filled by the customer and then repeated after 6 months to understand how the follow-up in each service has had an impact on their daily lives and is useful to evaluate the progress of the patient and the quality of the service

The main services available at FHC are described below.

2.10.1 Nutrition

This service is delivered by the nutritionist provided by the association of Holon pharmacies. The main goal of the service is to guide the customer into behavior modification, assuring a diet that covers their nutritional needs. It is thus a channel to enhance the state of health, for patients with chronic diseases in addition to people suffering from obesity. Considering the relationship between obesity and cardiovascular diseases, diabetes, dyslipidemia, respiratory diseases, osteoarticular diseases, among others, it is a very important service to promote weight loss and healthy eating habits to manage the associated pathologies.

There are two types of counselling session, first-time consultation, and follow-up consultation.

While the first-time consultation is designed to perform the primary assessment of the customer and to define the targets, follow-up consultation's main purpose to evaluate customer's progression in addition to adjusting the plan when necessary.

I was able to attend both types of counselling sessions of different type of customers with different cases during my internship.

2.10.2 Footcare Service

This service is carried out by a specialized podiatrist who has a strong clinical knowledge to provide a quality service in the prevention and treatment of the pathologies and other problems of the foot. The main feet problems that are normally treated in the pharmacy in these sessions are, dry skin and cracked heels, corns, calluses, ingrown toenails, physiognomic feet and toes changes in addition to skin infections, such as warts, verrucae, athletes' foot, fungal nail infections. This service is intended for any individual of any age.

I did not have the opportunity to attend any footcare consult but the specialized podiatrist of Holon pharmacy, Vera, presented me to the service.

2.10.3 Dose Administration Aid

It is a service directed to patients with difficulties in managing their own therapy, such as polymedicated patients, patients with complex therapeutic regimen and patients having difficulties in adhering to their therapy. Before taking advantage of the service, patients or their caregiver must be interviewed by the pharmacist that will be responsible for the preparation of their medication. This interview serves to get the patient's medical history and evaluate their medications. It is also important to evaluate patient's information about their cases and medications as well as the way they take their medicines.

The Dosset box is designed to fulfill patients need for a week. The rows of the box represent the days of the week while the column corresponds to times of the day (fasting, breakfast, lunch, dinner, bed). Each dossett box has a sequential batch to which all batches and validities of the medications used to prepare it are associated. Moreover, all dossett boxes are identified with the patient's name, contact of the pharmacy, pharmacist responsible for the preparation and the dates of each box to be used in (start and end date).

The stability of the drug can only be guaranteed for 28 days after opening removing it from its original packaging. Thus, most dossett boxes are made monthly, but some may be prepared with higher frequency if necessary.

One of the advantages of this service is allowing the patient to take the right medicine at the right time which may contribute to increased effectiveness and safety of medicines.

2.10.4 Injectable Medication Administration

This service includes administration of injectable drugs and vaccines that are not included in the national vaccination plan by a specialized pharmacist. The specialized pharmacist must have adequate training recognized by the Order of Pharmacists to be able to administer injectable drugs and vaccines.[144]

This activity is carried out in one of the counselling offices that contains all essential means for the treatment of an anaphylactic reaction (e.g. adrenaline pen, oxygen, facial masks, etc.), and that has all the necessary conditions for the safety of customers and pharmacists.[145]

At FHC, the pharmacists licensed to deliver this service are Dr. Patrícia Amaral, Dr. Patrícia Pais and Dr. Mafalda Silva.

After administering an injectable drug or a vaccine, evaluation questionnaire regarding allergies and possible previous anaphylactic reactions is completed with the patient, to which a photocopy of the prescription is attached. Likewise, it is necessary to register the name of the patient, date of birth, medicines / vaccines administered, lot, solvents used, route of administration and the pharmacist who administered it in Sifarma2000.

I had the opportunity to attend, with authorization from the patient, the administration of an injectable drug.

2.10.5 Pharmaceutical Consultation

Two types of Pharmaceutical consultation services are offered in the pharmacy, smoking cessation consultations and pharmacotherapeutic follow-up. Both services are offered by Dr. Patrícia Pais.

Pharmacotherapeutic follow-up is intended to evaluate patients' therapy and the outcomes of treatment aiming to achieve disease control and prevent complications and thereby improve the well-being of patients. The follow-up is based on the principles of Clinical Pharmacotherapy where patient adherence with the prescribed regimen is monitored, drug-related problems are addressed, and patients are counselled on the correct way to use their medicines. This service benefits mostly polymedicated patients and patients with chronic diseases among others.

During my internship, the pharmacist to deliver the service was getting the training to carry out this service.

Smoking cessation consultations were newly established service in the pharmacy and I was able to attend a training session on the matter. This service is offered to all smokers whenever they express interest in quitting smoking. The pharmacist provides the client with specialized support to help them during the process and will normally establish a personalized plan after evaluating smoking habits and health state of the client.

2.10.6 Diabetic Foot Service

Diabetic foot consultations are delivered monthly by a nurse. The target population of this service are patients suffering from diabetes type I or II. The main goals of the consultations are the prevention of neuropathy and peripheral arterial disease, foot ulceration, infection, and lower extremity amputation in diabetic patients. Normally several types of tests are done in the consultations:

- Sensitivity tests: to evaluate pressure perception using a Semmes-Weinstein 10g monofilament which, when applied to various areas of the feet and legs while the eyes are closed, gives a sense of the severity of their neuropathy;
- Vascular evaluation: where palpation of pulses is done together with an evaluation of temperature and the presence of edema to assess arterial irrigation;
- Evaluation of the Ankle-Brachial Pressure Index (IPTB): which is a non-invasive screening performed using a doppler in which a comparison of the pressure in the brachial pulse with the pressure of the ankle pulse is made. A large difference between the two values may result from severe peripheral arterial insufficiency.

One of the reasons that many patients stick to this service is getting their nails cut. This ensures that patients get a proper cut and careful monitoring of the health of their feet.

Foot problems among diabetic patients are quite common and often overlooked until they become quite serious, therefore this service is a chance to increase diabetic patients' quality of life.

2.10.7 Skincare Service

The service is provided by the pharmacist who has professional training in this area. It is designed to counsel patient on skincare products, evaluate skin type in terms of its oiliness, hydration, pore size, wrinkles and blemishes, perform hair assessment (hair density, the health of the hair strands, oiliness or the presence of dandruff in the scalp), and monitor some skin conditions (acne, psoriasis, etc.), with subsequent counselling on the most suitable skincare products for the condition. Through evaluation sessions, it is possible to establish much more personalized advice directing the customer to the most appropriate management of the condition bearing in mind that many skin conditions can be perfectly managed using non-medicinal skincare products.

2.10.8 Health Check

The pharmacy offers through this service determination of biochemical parameters (total and LDL cholesterol, triglycerides, and blood glucose) along with physiological parameters (blood pressure). The measurements are carried out in one counselling room to provide the customers with more privacy. When necessary, patients are counselled regarding their values and are taught about the modification they may do with their lifestyle to improve their values.

Measured values are recorded on registration cards that are kept with the customer. When possible, the measured values should also be recorded on the customer's follow-up sheet in Sifarma 2000.

During my internship, I was able to regularly measure blood pressure for customers, as well as other biochemical parameters, especially blood glucose and total cholesterol.

2.11 Valormed

Valormed is a non-profitable waste management corporation that is mainly concerned with the collection and treatment of out-of-use medicines' waste and medicines empty packaging. It was created as a result of the collective efforts of pharmaceutical industries, distributors, and pharmacies.[146]

The pharmacist plays a key role in society in promoting the correct elimination of medicines waste in society by encouraging pharmacy's customers to recycle expired or unused medicines by discarding them Valormed's container in the pharmacy.

FHC has Valormed containers in the dispensary area (in the front-office) and in one of counselling room where customers can dispose of their unused or expired medicines, empty packages, and any medicine related waste. When these containers are full, they are weighed, then their weight is recorded on the container along with the pharmacy's code to be

collected by distributors. These containers are then transported to sorting centers to be sent subsequently for recycling or incineration.

2.12 Conclusion

Although this internship was only for three months, it was of great value for me as it introduced me to the reality of community pharmacy. It made me realize that not only theoretical knowledge is important to be a good pharmacist, but also practical experience and direct contact with patients. Additionally, I understood that finishing the university does not mean that we do not have to study anymore, as healthcare professionals we will be always in a continues learning journey. Most importantly, during this internship, I appreciated more the fact that the role of a pharmacist is not just dispensing but also it has great importance in the lives of many patients seeking advice at the pharmacy.

The internship was at the same time an opportunity for personal enhancement since it helped in improving my human and communicative capacities.

It was also a privilege to be able to be trained by all the employees at the pharmacy and work with them. They were very supportive during my entire placement and helped me which facilitated my integration into the team and made me enjoy the whole experience.

Chapter 3 - Hospital Pharmacy

3.1 Introduction

As part of the requirements for the degree of Integrated Master in Pharmaceutical Sciences from the University of Beira Interior, I have completed my hospital pharmacy Internship program at The Barts Heart Centre in St Bartholomew's Hospital, a teaching hospital based in London, UK. The training was xecuted in the frame of The European Union's Erasmus+ programme and took place in the period from the 11th of June to the 1st of September. The whole internship was guided by my main supervisor and contact person, Paul Wright, the Lead Cardiac Pharmacist at Barts. The fact that I spent most of the time in Cardiology wards is reflected in my report. Additionally, I had the opportunity to spend a week in Barts cancer centre. I was also able to spend a day with a clinical trials pharmacist who took me through the clinical trials department. Moreover, I spent sometimes in dispensary and had the opportunity to carry out some activities there. I collaborated in analysing data and preparing a report of pharmacists' contributions on the wards, in addition to audit data collection. I also helped in collecting data for a study on the use of Warfarin and Novel Oral Anticoagulants (NOACs) in treatment of Left Ventricle thrombus.

This report is organized based on my three-month internship and focuses mainly on the activity I observed or performed myself. It is divided into four main subchapters, The Barts Heart Centre, Oncology, Clinical trials, and Dispensary.

3.2 The Barts Heart Centre

The Barts Heart Centre is one of the largest cardiovascular centres in the UK and Europe. It provides specialist care for 250 cardiac beds, 58 critical care beds, 10 theatres, 10 catheter labs, magnetic resonance imaging (MRI) and computed tomography CT scans.[147] Wards are distributed in 6 floors as follow:

- The first floor is divided into 1C and 1E (intensive care) and 1D (high dependency unit). I was able to observe the pharmacist while working on these wards for a day. The differences of these critical wards from a regular ward were easily recognizable from the type of regular medications given to patients while on ward to the routes of administration taking into consideration that many patients were either unconscious or receiving dialysis;
- The third floor consists of 3A East, an intervention ward where heart attack patients are admitted, 3A West, an Electrophysiology ward, 3D, an intervention and electrophysiology ward which is a 3A East stepdown and where inter-hospital transfer patients are admitted, and finally 3C Radial lounge, an elective

intervention ward. I spent most of the time of my internship in the third flood moving between these wards;

- The fourth floor is a thoracic ward in which all patients who undergo thoracic surgery are allocated;
- The sixth floor contains 6A, another intensive care ward, and 6D a heart failure, cardiomyopathy, transcatheter aortic valve implantation (TAVI), and grown-up congenital heart (GUCH) problems ward. The cases of infective endocarditis (IE) were mainly found on 6D, hence I could do the therapeutic drug monitoring on antibiotics on this ward.

3.2.1 Clinical pharmacist's activities on the ward

Hospital pharmacists are the medicines experts in the multidisciplinary team that is constituted of doctors, nurses, and other healthcare professionals. Pharmacist with all healthcare professionals work together on a daily basis to provide the best care for patients. Depending on the type of the patients and the ward, the main interventions a clinical pharmacist include:

- Participation in wards daily rounds;
- Drug history taking and medicines reconciliation;
- Daily patients' clinical and medications profile review which involves prescriptions assessment to ensure that prescriptions are legal, safe, effective, and appropriate in addition to identifying and resolving any medication discrepancies;
- Delivering information, advice, and recommendations to other healthcare professionals regarding medicines selection and use such as dose amendments, choosing antibiotic and duration of treatment, and formulation adaptation;
- Ensure that sufficient supplies of all medicines are available;
- Counselling patient or caregiver on medicines use with a focus on high-risk medications;
- Therapeutic drug monitoring;
- Discharge prescriptions checking and writing.

In addition to pharmacists, all wards have pharmacy technicians and sometimes pre-registration pharmacists providing and assisting in ward-based medicines management services. Many activities can be assumed by pharmacy technicians and pre-registration pharmacists, including, documenting drug histories and full medicines reconciliation, Patients' Own Drugs (POD) checking, and ordering and supplying medication. Additionally, pre-registration pharmacists can undertake patient counselling.

I was able to perform myself different activities on the wards which I will be explaining in detail below.

3.2.2 Drug chart review

One of the main activities of the clinical pharmacist on the ward is to review patients' medications and their clinical condition. Drug charts for inpatients are examined on a daily basis to ensure the appropriateness of prescribed medications. Drug charts used for inpatients contain the following sections: once only medications (e.g. loading doses, pre-medication), drug allergies, venous thromboembolism prophylaxis, regular prescriptions (which has three subsections: Diabetes medication, anti-infectives and regular medicines), as required medications, infusion prescriptions, and drug history. It is the responsibility of the clinical pharmacist to check all these sections to ensure that the patient is receiving the most adequate treatment. Drug chart is normally reviewed in conjugation with patient's clinical record to:

- Ensure that all necessary and appropriate medicines are prescribed with clear instructions of when to be started and how to be administered;
- Ensure the safety of treatment regimen and checking drug profile for medication duplication, interactions, and incompatibilities;
- Ensure that the correct doses are prescribed according to patient condition, age, hepatic and renal function;
- Ensure suitability of dosage schedule;
- Evaluate the therapeutic outcome in comparison with the therapeutic goal;
- Evaluate disease progression and consider changes in therapy when required;
- Consider monitoring if necessary;
- Ensure that all doses are administered by checking the medication administration record.

Drug chart review by a pharmacist is an essential process to ensure the provision of the best treatment regimen to patients in addition to prevent or minimize drug-related problems, which may lead to morbidity and in some cases to mortality, by identifying them early in the treatment.

3.2.3 Drug history and medicine reconciliation

When a patient first admitted to the hospital obtaining an accurate drug history is essential to perform medication reconciliation. Normally, it is the responsibility of the pharmacist or a trained pharmacy technician to interview the patient and collect all the information needed. An accurate medication history is a crucial tool to prevent prescribers from inadvertently making improper decisions about the patient's treatment. It is also important to prevent the harm that may be caused if current medicines are excluded or prescribed at the incorrect dose for the patient, or if previously discontinued medicines are restarted.

Drug history consists of all medicines (prescribed and purchased) a patient was taking prior to their admission to the hospital with the doses, frequency of administration and the indications. Patients should be asked distinctly about the use of injections, eye drops, patches, creams, or inhalers since some patients may not consider these as medicines. Dietary supplements and herbal medicines consumption should be documented as well, considering that these may also provoke adverse drug reaction or interact with medicines initiated on admission.

Additionally, details of any allergies or previous side effects to medicines (or excipients) must be recorded.

Various sources can be considered when obtaining drug histories, such as patient, patient's own medication, relatives/caregiver, repeated prescriptions, recent hospital discharge summary, community pharmacy, and summary care record. Preferably, at least two reliable sources should be used.

Whenever it is possible, patients should be encouraged to bring their medicines with them into the hospital to facilitate medicines reconciliation and avoid missing doses of any regular medication. When the patients bring their own medicines, it is important to check the information and the instructions on the labels along with verifying expiry dates for all the boxes.

Furthermore, patients are normally asked about their medication stock at home to meet their needs when they are to be discharged. Drug histories are usually documented on the back side of drug chart which must be signed with the pharmacist initials and dated after obtaining all the necessary information. When a patient has a documented allergy to a medicine, an alert sticker must be affixed to the front page of the drug chart in a way to be visible whenever the drug chart is to be used or edited.

In case any amendment to inpatient prescription is required, the medical team is informed to ensure that the patient's medicines are properly prescribed. Any modifications, omissions or additions to patient's regular medicine must be clearly documented. This would help the pharmacist writing the discharge letter to the patient's general practitioner (GP) to provide accurate information regarding all the changes made to a patient's medicines during their admission.

During my internship, I was able to determine a discrepancy during medicine reconciliation of a patient admitted to have a procedure in the cath lab. The patient confirmed that he was on an anticoagulant which was verified to be edoxaban by POD and lipid-lowering medication in addition to other chronic medicines. When checking the PODs, I noticed that patient did not have any lipid-lowering medicines. Moreover, he had rivaroxaban, another anticoagulant

which he confirmed that he is taking as a lipid lowering agent. After checking the label on rivaroxaban, I found out that it was mistakenly labelled as rosuvastatin. Then, I checked patient's record and confirmed that he should be taking rosuvastatin and rivaroxaban was not prescribed for him at all. I explained to the patient that he should not be taking two anticoagulants at the same time and that he was given the wrong medicine in the pharmacy. I reported the situation for the pharmacy in charge of the ward and for the doctor that was going to perform the procedure. The procedure was rescheduled as the patient should not have been taking anticoagulant prior to the procedure. Additionally, a report of the case was submitted in the Incident Reporting Software of the hospital. This experience was a good example of how important it is to talk with the patient and check their PODs when taking drug histories.

3.2.4 Warfarin counselling

Warfarin is perhaps the most widely studied oral anticoagulant. It has been used as the main agent for the prevention and treatment of venous thromboembolism (VTE) and embolic stroke for a long period of time.[148] Warfarin acts as an anticoagulant by interfering with the hepatic synthesis of vitamin K-dependent clotting factors II, VII, IX, and X, in addition to proteins C and S.[149] It is indicated for the prevention and treatment of systemic embolic complications (e.g., stroke) linked to atrial fibrillation; prophylaxis and treatment of pulmonary embolism; post-myocardial infarction (MI) to lower the risk of thromboembolic events after an MI event and as a prophylaxis and treatment of thromboembolic complications associated with prosthetic cardiac valve replacement.[149] Since the internship has taken place for most of the time in the cardiac ward, I have encountered a plenty of patients either commencing treatment with warfarin or were already on warfarin before admission. When initiating warfarin therapy, the main concern is to accomplish a reasonable balance between reaching therapeutic INR (International Normalized Ratio) in a timely manner to prevent thrombotic events and avoiding warfarin-related haemorrhage. Patients' knowledge regarding their warfarin treatment is a fundamental component for higher therapy adherence and INR control which leads to an optimal outcome. Therefore, any patient to be started on warfarin needs to be provided with comprehensive verbal and written education before their discharge by a pharmacist.

During the internship, I was signed off to perform warfarin counselling and had the opportunity to do it for different types of patients with several clinical conditions. Firstly, the patient is provided with the yellow oral anticoagulation therapy pack. It includes a warfarin information booklet, an alert card, anticoagulant record booklet with additional spare sheets. During the consultation, the pharmacist goes through the information booklet with the patient, ensuring that the points below are covered:

- Indication;

- Expected duration of treatment;
- Basic mood of action;
- Monitoring: what is INR (measures how long it takes for the blood to clot); INR target range based upon their indication; frequency of monitoring (which is normally once or twice a week and then tends to decrease once INR is stabilized);
- How to take it and dosing: once a day, preferably in the evening, at the same time each day, dose adjustment is made according to INR levels to maintain it within the narrow therapeutic window. To facilitate making up the prescribed dose, warfarin tablets are colour-coded as follows: 0.5mg = white, 1mg = brown, 3mg = blue, 5mg = pink;
- What to do if dose missed: take as soon as possible if in the same day but never double the dose to catch up if the next dose is due. Make a note in the yellow anticoagulation record book;
- What to do if take too many: contact the anticoagulation Clinic/Doctor;
- Intercurrent illness/new antibiotics: inform the prescriber about taking warfarin and the anticoagulation clinic. More frequent INR testing may be necessary;
- Side effects: get bruises easily, heavy or prolonged bleeding;
- Bleeding complications and what to do in event of bleeding: paying attention and to seek urgent medical attention when noticing one of next warning signs: blood in stool or urine, nose bleeds (persisting for >20mins), coughing or vomiting blood, massive or spontaneous bruising, excessive menstrual blood loss, unusual severe headaches and wounds that last longer than 20 minutes to stop bleeding;
- Surgical procedures (including dental procedures): it is always important to inform healthcare professionals about taking warfarin;
- Potential drug interactions and over the counter (OTC) medicines: warfarin may interact with many medicines / herbal preparations; hence, patient should always inform doctor/dentist/pharmacist that they are on warfarin if commencing new medicines or buying OTC medicines to avoid possible drug interactions and duplication with other anticoagulants. Additionally, the patient should avoid any aspirin containing preparation and non-steroidal anti-inflammatory medications unless prescribed by a doctor. Paracetamol may be used for pain relief with Warfarin for pain;
- Dietary restriction: INR may be influenced by the amount of vitamin K in diet, therefore, the ingestion of dietary vitamin K should be consistent while taking warfarin. Cranberry and grapefruit juices must be avoided.
- Alcohol intake: it is recommended to avoid excess alcohol consumption and binge drinking due to the risk of associated injuries and chronic liver disease (which may affect coagulation factor synthesis);
- Contraception and pregnancy: women on warfarin should use reliable contraception as warfarin is known to be teratogenic;

- Hormone replacement therapy: oestrogen-containing preparations should be avoided in case of women with deep vein thrombosis/pulmonary embolism (DVT/PE), due to the augmented thrombotic risk. Alternatives to be discussed with the consultant;
- Hobbies and leisure activities: contact sports and other risky sports are better to be avoided due to augmented risk of injury, bruising/bleeding.

Before finalizing the counselling, patient's questions and concerns should be addressed and any doubts should be clarified.

3.2.5 Novel Oral Anticoagulants counselling

Nowadays, non-vitamin K antagonist oral anticoagulants (or novel oral anticoagulants- NOACs) are widely prescribed for the treatment of new DVT and PE, prevention of stroke and systemic embolism in non-valvular atrial fibrillation (AF) with one or more risk factors, and as prophylaxis of VTE following hip and knee replacement.[150] NOACs include apixaban (Eliquis™), dabigatran (Pradaxa™), edoxaban (Lixiana™) and rivaroxaban (Xarelto™). apixaban, rivaroxaban, and edoxaban are factor Xa inhibitors while dabigatran is direct thrombin inhibitor.[150] Just like warfarin, counselling on NOACs should be provided when they are prescribed. I was also signed off to perform NOACs counselling, therefore I was able to provide a lot of patients receiving different NOACS personalized counselling.

The following points should be covered in the counselling:

- Indication;
- Expected duration of treatment;
- Basic mode of action;
- Administration instructions: once daily in case of rivaroxaban and edoxaban at the same time each day, and twice daily for dabigatran and apixaban. Apixaban, edoxaban and dabigatran can be taken with or without food whereas rivaroxaban should be taken with food as food improves its absorption;
- Missed doses: advice not to neglect doses. But if it happens the action will depend on the NOAC; Dabigatran: a missed dose can be taken up to 6 hours before the next due dose, otherwise, it should be omitted. Rivaroxaban, edoxaban, and apixaban: the forgotten dose to be taken as soon as the patient remembers it. For all of them double dose can't be taken to make up the forgotten dose;
- Side effects: get bruises easily, heavy or prolonged bleeding;
- Bleeding complications: paying attention and to seek urgent medical attention when noticing one of next warning signs: blood in stool or urine, nose bleeds (persisting for >20mins), coughing or vomiting blood, massive or spontaneous

bruising, excessive menstrual blood loss, unusual severe headaches and wounds that last longer than 20 minutes to stop bleeding;

- Monitoring with NOACs: although regular blood tests are not necessary with NOACs, some blood tests to assess kidney function, liver function and a full blood count need to be done before starting the treatment. These tests should be repeated at least once yearly;
- Other medicines: other medicines may interact with NOACs. It is essential that the patient let their doctor or pharmacist know that they are taking a NOAC before start taking any other prescribed or over-the-counter medicines (including vitamins and herbal supplements);
- Alcohol – Alcohol may increase the anticoagulation effect. The patient should be notified to avoid heavy drinking while taking a NOAC. Tiny amounts (1–2 standard drinks per day) should not cause problems.
- Medical and dental procedures: it is necessary to inform healthcare professionals about taking NOACS before any planned procedure.
- Hobbies and leisure activities: contact sports and other risky sports are better to be avoided due to the augmented risk of injury, bruising/bleeding.
- Pregnancy and breastfeeding: apixaban, edoxaban, and rivaroxaban are advised to be avoided during pregnancy and breastfeeding. However, dabigatran should be avoided during breastfeeding and pregnancy unless essential.

The checklists for warfarin and rivaroxaban (as an example for NOACs) counselling can be found in the appendix XIV.

3.2.6 Discharge counselling

Once the discharge decision is made, the pharmacist must check all the medications they need to take home and help in writing the discharge letter to be sent to the patient's GP. Afterward, the patient or their caregiver is counselled on the newly prescribed medicines.

The counselling session usually covers the following points: the name and indication of the medicine, how it works, the dose to be taken, administration instruction and cautions, duration of drug therapy, therapeutic contraindications, interaction, the most relevant side effects and the actions to be made if they occur. Furthermore, suitable storage, and the action to be taken in the event of a missed dose should also be covered. Afterwards, the patient's or their caregiver's understanding of the use and the schedule of the drug to be taken are evaluated. The differences between pre-admission and discharge regimens are highlighted to avoid any confusion and to improve patient's adherence to the new regimens. The main medications to be counselled on at are normally antiplatelet (such as Aspirin, Clopidogrel), beta blocker, angiotensin-converting enzyme (ACE) inhibitors, glyceryl trinitrate (GTN) spray, statins, and diuretics such as spironolactone.

3.2.7 Clinics

A range of outpatient cardiology clinics operate in the hospital where pharmacists play a vital role. The clinics are managed by specialist nurses, pharmacists, and consultants. Some of the clinics are pharmacist-led clinics where consultant pharmacist interviews patients, evaluates their clinical status and medications, and may modify their doses or prescribe new ones when necessary. I was able to attend three of the main clinics at Barts where I could shadow the pharmacists during their work which I am describing below.

3.2.7.1 Hypertension clinic

It is a pharmacist-led clinic established to manage referred patients with difficult-to-control blood pressure or resistant hypertension. Patients attending this clinic may also be unable to tolerate one or more antihypertensive drugs, or the usually recommended antihypertensive are contraindicated in their cases. Patients are seen first by a nurse who measures their blood pressure, weight, height, and body mass index before forwarding them to the consultant pharmacist. The consultant pharmacist interviews the patient and assesses the BP of the past months, life Style (Salt Restriction, diet, exercise), comorbidities, patient's recent clinical analyses, all the medications the patient is taking, and any side effect or intolerability for any medicine. The pharmacist may make changes to the patient's medication and prescribe new drugs or alter the doses of prescribed drugs. If in doubt, the pharmacist may discuss the case and medication changes with a consultant cardiologist who is normally in the next room. After reaching a decision, the patient is counselled on the changes made to the therapy and given an appointment to follow up.

3.2.7.2 Anticoagulation clinic

Anticoagulation clinic delivers specialized monitoring and medication adjustment service for patients on anticoagulants, particularly warfarin. Specialist nurses and trained Pharmacists share the responsibility in managing the clinic. Outpatients frequent the clinic to get close monitoring of their anticoagulation therapy. It is usually monitored by testing patient's INR level. INR value should fall in the target range specified for each patient. If the INR is too low or too high, the pharmacist investigates the possible cause for the deviation and tracks INR results over multiple visits. After evaluating all the information provided by the patient regarding any possible changes in lifestyle, disease state, new medicines or missed doses in addition to INR readings, the pharmacist determines the adjustment needed to be done on the dose of the anticoagulant. Patients are seen in the Clinic once a week initially when using warfarin. Then, they may be checked less frequently when their INR results are stabilized within the specified range. During the visit, patients are counselled if necessary and all their doubts are cleared.

3.2.7.3 Pre-admission clinic

A pharmacist is integrated into a multidisciplinary team of a doctor and a nurse in the pre-admission clinic where elective angioplasty patients are seen before their procedures. All patients at the pre-admission clinic are seen by a pharmacist who is responsible for documenting an accurate drug history then transcribing it on the drug chart and discharge prescriptions to prevent delayed discharge on the day of the procedure. The pharmacist assesses the need to suspend certain medicines prior to the procedure (e.g. anticoagulants) or to omit their doses in the morning of the procedure (e.g. oral antidiabetics and oral diuretics). Loading doses of clopidogrel and/or aspirin are given to patients if required. Patients who are incapable of tolerating aspirin or clopidogrel are detected and then questioned to determine if antiplatelets are inappropriate or if the addition of a proton pump inhibitor could resolve the problem and reduce gastric irritation. Patients with coronary heart disease are advised to take: ACE inhibitors, beta-blocker and statin as secondary prevention. If a patient is not taking one or more of these classes, the pharmacist liaises with medical staff to optimize patient therapy by prescribing the missing medicines. At the end of the appointment, the pharmacist counsels the patients on any alterations made to their medications and all the achieved optimization are relayed to their GPs. Additionally, patients are asked to bring in their medicines on their admission which would help in case they need to spend the night in the hospital after their procedure.

3.2.8 Patients' profiles

Herein I present profiles of patients I followed during the time I spent on the wards.

3.2.8.1 Case 1: Non-ST-segment elevation myocardial infarction

An 82 years old gentleman, 45.5 kg, was admitted with epigastric pain, associated with nausea and shortness of breath, similar to the pain of a previous MI he had. The pain was continuous (until GTN given), ECG showed lateral ST depressions and blood test showed a rise in troponin (NSTEMI).

Observation on admission: BP: 185/96, HR: 60, SpO_2: 98%

Chest: Bilateral basal crackles.

Mild pedal oedema

Past medical history: The patient has had a 6-month history of unstable angina and orthopnoea. He has a background of double bypass surgery 2009, hypercholesterolemia, hypertension, heart failure, postural hypotension, atrioventricular nodal reentrant tachycardia ablation 2013 and coeliac disease. On reviewing the history, the patient has mentioned a 6-month history of weight loss and anorexia in addition to a history of constipation and a vague history of blood in stools.

Social History: Ex-smoker, lives independently, but not very active

Drug History: Drug allergies/serious adverse drug reactions: Erythromycin, Penicillin.
Medications used before admission are presented in Table 3.1.

Table 3.1: Drug Therapy Before Admission

Medication	Indication
Lansoprazole 30 mg OD Gaviscon 10 ml ON	Gastroprotection/Acid reflux
Fludrocortisone 100 ug BD	Postural hypotension
Cyanocobalamin 50 ug OD Ferrous Fumarate 210 mg BD Folic Acid 5 mg OD	Anaemia
GTN spray	Angina
Pravastatin 20 mg OD	Hypercholesterolemia
Furosemide 20 mg OD	HF
Aspirin 75 mg OD	CVD
Adcal D3 750/200 BD	

Significant Investigations: The patient underwent angiogram which showed patent grafts with severe proximal left anterior descending coronary artery (LAD) disease and chronic total occlusion in the proximal right coronary artery (RCA). It was decided not to proceed with Percutaneous Coronary Intervention (PCI) and to manage the case with medication.
Echocardiogram: revealed severely impaired systolic function (LVEF = 30-35%); moderate mitral regurgitation; mild to moderate aortic regurgitation.
Additionally, some clinical analyses were performed. The results are presented in Table 3.2.

Table 3.2: Lab Results

Date	11/8	13/8	14/8	15/8	Normal value
Troponin	82 ng/L	44 ng/L	50 ng/L	-	-
Creatinine (Cr)	102 μmol/L	99 μmol/L	131 μmol/L	95 μmol/L	59-104 μmol/L
Cr clearance	32 mL/min	33 mL/min	25 mL/min	34 mL/min	>90 mL/min
Haematocrit	39	40	42	34	0.4-0.5
haemoglobin	122 g/L	128 g/L	133 g/L	112 g/L	130-170 g/L
K+	5.0 mmol/L	4.6 mmol/L	4.4 mmol/L	3.6 mmol/L	3.5-5.3 mmol/L
Na+	133 mmol/L	139 mmol/L	135 mmol/L	139 mmol/L	133-146 mmol/L
BP	170 mmHg	143/63 mmHg	112/56 mmHg	128/61 mmHg	<140/90 mmHg
HR	75 b/min	57 b/min	61 b/min	60 b/min	

The primary focus of the management is to provide supportive care and pain relief during the acute attack and to prevent further cardiac events and death. Patients with NSTEMI should be given treatments to minimalize their cardiovascular risk. The importance of lifestyle changes, especially smoking cessation, should be emphasized. All patient with NSTEMI will be started on five main drugs: dual antiplatelet therapy, beta-blocker, ACE inhibitors, statins, and GTN spray for symptoms relief.[151]

The dual antiplatelet therapy (DAPT) initiation is recommended to prevent atherothrombotic events following an acute coronary syndrome with elevated cardiac biomarkers.[151] Treatment should be started following the acute coronary event, within 24 hours after admission to the hospital. The usual duration of DAPT is 12 months. Aspirin should be combined either with clopidogrel or ticagrelor based on patient GRACE and CRUSADE scores for the 12 months. Afterward, aspirin should be continued lifelong in a dose of 75 mg daily for secondary prevention.[151]

GRACE score estimates mortality risk within six months for patients with acute coronary syndrome using eight factors: age, renal function, heart rate, systolic blood pressure, congestive heart failure, ST-segment deviation, cardiac arrest, and elevated cardiac biomarkers.[152]

CRUSADE score predicts patient's chance of having an in-hospital major bleeding considering baseline patient characteristics (sex, history of diabetes, peripheral vascular disease), observation on admission (heart rate, systolic blood pressure, signs of chronic heart failure), and admission laboratory values (haematocrit, calculated creatinine clearance).[153]

According to CRUSADE score, the patient has a high risk of in-hospital bleeding. GRACE score estimates that the patient has a 16% mortality risk within 6 months to 3 years which is considered as high. Bearing this in mind, clopidogrel is the safest option for the patient in combination with low dose aspirin since ticagrelor is more potent and it is associated with higher bleeding risk.[151]

Beta-blockers and ACE inhibitors were prescribed after acute coronary syndrome in aim to reduce cardiac workload and to improve the healing process and exercise tolerance in addition to symptoms relief. They are also known to prevent to some degree cardiac remodelling and decrease mortality in the long-term.[151]

High-intensity statin should also be given as secondary prevention considering that it reduces cardiovascular disease events and total mortality regardless of the original cholesterol concentration.[154]

Clinical Course: During admission, the patient has had functional symptoms, one of which was pre-syncope. These symptoms were probably occurring due to longstanding autonomic dysfunction and the use of ramipril. Additionally, he experienced diarrhoea due to gluten intake in the hospital despite his celiac disease. After ramipril discontinuation and restarting the patient on a gluten-free diet, symptoms have started to settle.

Patient's medication during admission and on discharge are presented in Tables 3.3 and 3.4.

Table 3.3: Medications Received During Admission

DRUG	DOSE	IND	COMMENTS
Atorvastatin	*80 mg – 40 mg*	ACS	Started on 80 mg but then was reduced to 40 mg considering Patient's age and weight.
Aspirin	75 mg OD	ACS	
Clopidogrel	75 mg OD	ACS	
Bisoprolol	1.25 mg OD	ACS	
Ramipril	1.25 then 2.5 mg OD	ACS	Was given for 3 days then stopped due to postural hypotension.
Fludrocortisone	100 ug BD	Postural hypotension	
Furosemide	20 mg OD	HF	Patient wasn't taking it because it makes him feel dizzy, it was stopped.
Eplerenone	25 mg then 12.5 mg OD	HF/ LVD	Stopped as patient suffers postural drops and is on fludrocortisone.
Cyanocobalamin	50 ug OD	Anaemia	Drug history
Ferrus fumarate	210 mg BD	Anaemia	Drug history
Folic acid	5 mg OD	Anaemia	Drug history
Lansoprazole	30 mg OD		Was stopped as patient had diarrhoea and switched by ranitidine
Ranitidine	150 mg BD	Gastroprotection	
Enoxaparine	40 mg then 20 mg OD	VTE prophylaxis	The dose was reduced as the patient is underweight
Adcal D3	750/200 BD	Drug history	
Lactulose	10 ml PRN	Constipation	
Gaviscon	10 ml PRN	Acid reflux	
GTN spray	PRN	Angina	
Paracetamol	1 g PRN	Pain/fever	
ondansetron	4-8 mg PRN	Nausea/vomiting	

Table 3.4: Medications on Discharge

Medication	Indication	Counselling
Fludrocortisone 100 µg BD (oral)	postural hypotension	*Drug history*
Folic acid 5 mg OD (oral)	Anaemia	*Drug history*
Cyanocobalamin 50 µg OD (oral)	Anaemia	*Drug history*
Ferrous fumarate 210 mg BD (oral)	Anaemia	*Drug history*
Bisoprolol 1.25 mg (oral)	Secondary prevention for ACS	*NEW* GP to up-titrate to maximum tolerated dose, within 4-6 weeks of discharge.
Clopidogrel 75 mg OD (oral)	ACS	*NEW* to continue for 12 months
Ranitidine 150 mg BD (oral)	Gastroprotection/Acid reflux	*NEW* replaced lansoprazole when patient experienced diarrhoea.
Lactulose 10 ml BD (oral)	Constipation	*Drug history* Not required during admission as patient experienced some diarrhoea. To only use at home if/when needed for constipation
GTN spray 400 µg /dose 1-2 puffs PRN (sublingual)	ACS	*Drug history* To be used under the tongue when required for chest pain. To be used in sitting position.
Paracetamol 1 g QID (oral)	Pain management	To be used when required for pain relief. Replaced co-codamol due to increased risk of constipation with codeine, considering coeliac disease.
Adcal D3 1500 mg/400 units BD (oral)		*Drug history*
Aspirin 75 mg OD dispersible (oral)	ACS	*Drug history* Lifelong for secondary prevention (CVD)

3.2.8.2 Case 2: ST-segment elevation myocardial infarction

A 47 years old male, 71 Kg, was admitted to hospital with sudden onset chest pain at 02:00 on the 5th of August with nausea, vomiting, dizziness and feeling sweaty. The patient attended Newham hospital at 02:30. He was found to have inferior ischemic changes with ongoing chest pain, thus he was loaded with aspirin and clopidogrel and transferred to Bart's hospital where he was sent directly to cath lab.

Observation on admission: BP: 111/78, HR: 56, SpO_2: 98%

ECG showed inferior ST-elevation and the patient was diagnosed with ST-elevation myocardial infarction (STEMI). STEMI indicates that a total occlusion in one of the coronary arteries happened and that caused infarction in the muscle supplied by the affected artery.[155] The

damaged myocytes release troponin in the bloodstream leading to a significant rise in serum troponin which is considered a diagnostic marker in addition to other tests.[155]

Past medical history: Ex-smoker and nil past medical history and nil family history.

Drug History: Nil regular drug.

Drug allergies/serious adverse drug reactions: No known drug allergy.

Significant Investigations:

Patient has had an angiogram which revealed dominant, severe mid RCA disease, consequently, the patient had PCI with a drug-eluting stent to mid-RCA. An ECHO was performed on the 5th of August and it showed that the LV systolic function is normal (EF 55-60 %) with moderate to severe mitral regurgitation and moderate tricuspid regurgitation.

Results of clinical analyses are presented in Table 3.5.

Table 3.5: Lab Results

Date	5/8	7/8	8/8	Normal value
Troponin	147 ng/L	-	-	-
Creatinine	80 µmol/L	79 µmol/L	83 µmol/L	59-104
Cr clearance	101 mL/min	103 mL/min	98 mL/min	>90 mL/min
Haematocrit	0.40	0.38	0.39	0.4-0.5
Haemoglobin	130 g/L	122 g/L	123 g/L	130-170
K+	4.0 mmol/L	3.8 mmol/L	4.8 mmol/L	3.5-5.3 mmol/L
Na+	138 mmol/L	140 mmol/L	139 mmol/L	133-146 mmol/L
BP	111/78 mmHg	108/60 mmHg	120/77 mmHg	<140/90 mmHg
HR	90-120 bpm	63 bpm	66 bpm	

When managing STEMI in hospital, the main goals are to deliver supportive care and pain relief, to promote reperfusion and to decrease mortality. Oxygen, nitrates, and diamorphine or morphine are normally used for initial support and pain relief; aspirin and PCI or thrombolytics help in stimulating reperfusion.[151]

For long-term management, the use of DAPT, beta-blockers, ACE inhibitors, and statins improve survival and help to reduce mortality risk following STEMI.[155] These drugs should ideally be initiated shortly after the event. DAPT is an essential part of the treatment to prevent any clot formation in the stent and to help keep the vessel open. All patients, in whom they are not contra-indicated, should receive aspirin indefinitely at a dose of 75 mg daily.[155] The addition of clopidogrel or ticagrelor depends on CRUSADE score, the risk of in-hospital bleeding, given that GRACE score would be high for all STEMI patient. The patient has a low-risk score, that is a low-risk of in-hospital bleeding, subsequently, he was started on ticagrelor 90 mg BD for 12 months. When the patient has started to have the runs of AF, rivaroxaban 15 mg OD was prescribed for stroke prevention. Consequently, ticagrelor was switched to clopidogrel 75 mg OD to reduce the risk of bleeding from combining an anticoagulant with a potent antiplatelet. The combination of low-dose rivaroxaban with

aspirin and clopidogrel is licensed for the prevention of atherothrombotic events following STEMI.[151] Beta-blockers and ACE inhibitor should be given to all patients as secondary prevention for the reduction of mortality risk. Statins help in preventing recurrent cardiovascular events.[151,155]

Clinical Course: Post procedure, the patient had runs of AF, therefore, he was loaded with 3.75 mg of bisoprolol and monitored then started on rivaroxaban 15 mg for a month. The dose of rivaroxaban was intended to be increased when aspirin stopped. He also had an episode of bradycardia and hypotension on 5/8/18 with sinus rhythm but with negative p-waves, no evidence of dissociation. Ramipril and beta blockers were withheld at that time in addition to giving the patient some fluids which led to the improvement of patient's BP and Heart rate.

Patient's medication during admission and on discharge are presented in Tables 3.6 and 3.7.

Table 3.6: Medications Received During Admission

Medication	Dose	Indication	Comments
Atorvastatin	80 mg	ACS	
Aspirin	75 mg	ACS + PCI with DES	
Clopidogrel	75 mg	ACS + PCI with DES	
Ramipril	1.25 then 2.5 mg (up-titration)	ACS	Was held for a day when patient had hypotension
Bisoprolol	1.5 then 5 mg OD (up-titration)	ACS	Was held on the day when patient had bradycardia and hypotension
Lansoprazole	30 mg	Gastroprotection	Combined with dual antiplatelet therapy for gastroprotection.
Rivaroxaban	15 mg OD	AF	For a month.
GTN spray	PRN	Angina	
Paracetamol	1 g PRN	Pain/fever	
Oramorph	5 mg PRN	Pain relief	
Metoclopramide	10 mg PRN	Nausea/vomiting	

Table 3.7: Medications on Discharge

Medication	Indication	Monitoring	Counselling
Aspirin disp 75 mg OD	STEMI / DES	Bleeding	For 1 month until 05/09/18
Bisoprolol (oral) 5 mg OD	Post-STEMI	HR, HTN	GP to up-titrate to maximum tolerated dose, within 4-6 weeks of discharge, in order to maximize patient's secondary prevention
Ramipril (oral) 2.5 mg OD	Post-STEMI	HTN, renal function	GP to up-titrate to maximum tolerated dose, within 4-6 weeks of discharge, to maximize patient's secondary prevention
Atorvastatin (oral) 80 mg OD	Post-STEMI optimization of secondary prevention therapy	Liver enzymes.	Secondary prevention after ACS.
Lansoprazole (oral) 30 mg OD	Gastric protection while on dual antiplatelet therapy plus anticoagulation	Serum Na+	Gastroprotection with dual antiplatelet therapy
Clopidogrel (oral) 75 mg OD	STEMI / DES	Bleeding	To continue for 12 months until August 2019
Rivaroxaban (oral) 15 mg OD	AF	Bleeding Renal function	Dose to be increased to 20 mg daily in 1 month when aspirin stops. Patient was fully counselled prior to discharge
GTN (sublingual)spray 400 µg/dose 1-2 spray PRN	ACS		To be used under the tongue when required for chest pain. To be used in sitting position.

3.2.9 Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) is the clinical practice of determining drugs' concentration in the blood that aims to enhance drug efficacy and reduce the toxicity of drugs with a narrow therapeutic window.[156] Monitoring drug concentration is useful to maintain drug concentrations within a target range, thereby optimizing individual dosage regimens. For some drugs, TDM is applied without necessarily quantifying serum drug concentration. It rather involves measuring one of the biological parameters that are affected directly by the administration of the drug or by monitoring the development of any toxicity symptoms. For most of the medicines, it is unjustified to employ TDM. However, it is utilized mostly for monitoring drugs with narrow therapeutic ranges, drugs with significant pharmacokinetic variability, and drugs that are known to cause serious adverse effects. A drug should satisfy certain criteria to be appropriate for therapeutic drug monitoring, such as a relationship between plasma concentrations and the clinical outcome and a verified target concentration range. TDM commences when the drug is prescribed and incorporates establishing an initial dosage regimen suitable for the clinical condition and the patient characteristics such as age, weight, organs function, and concomitant drug therapy. Many factors need to be borne in mind when interpreting concentration measurements like the blood specimen collection time in relation to drug administration time, previously administered doses, the desired clinical targets, and patient reaction to the drug.

In Bart's hospital, many drugs are monitored when they are first giving to patients. Since my placement mostly took place in Cardiology, I had the opportunity to perform TDM of three drugs for three different patients, warfarin by measuring INR, vancomycin, and gentamicin.

3.2.9.1 Case 1: Vancomycin

A 72 years old male was transferred from another hospital for further investigation on mitral valve infective endocarditis and a possible re-do mitral and tricuspid valve repair surgery as a result of severe regurgitation. The blood culture in the previous hospital was positive for *Staphylococcal epidermidis* thus he was commenced on an antibiotic regimen for 6 weeks (started 28/06; finish date 09/08) consisteing of rifampicin 600 mg BD, vancomycin 1 g BD and gentamicin 60 mg BD. However, the microorganism was found to be resistant to gentamicin but susceptible to vancomycin and rifampicin which led to withdrawing gentamicin. His inpatient stay was complicated by pulmonary oedema, anaemia secondary to valvular haemolysis, hyponatraemia, and recurrent sepsis. His case was discussed at infective endocarditis MDT meeting, all agreed that re-do surgery would carry excessive risk, therefore, medical therapy with a complete course of antibiotics were recommended.
Past Medical History: Benign prostatic hyperplasia, hypertension, chronic obstructive pulmonary disease, severe pulmonary hypertension, osteoarthritis, MV and TV repair, anaemia, degenerative spondylolisthesis, right lacunar infarct, and chronic low sodium.

Social History: Mobilizes with stick, independent, no alcohol, ex-smoker, ex-musician/rockstar.

Drug History: The patient does not have any known drug allergy.

Patient's treatment regimen before and during admission are detailed in Tables 3.8 and 3.9 in addition to drugs stopped during admission that are presented in table 3.10.

Table 3.8: Drug Therapy Before Admission

Medication	Indication
Tiotropium inhaler OD Carbocysteine 750 mg TDS Salbutamol nebs 2.5 mg QDS Tiotropium 18 mcg OD	COPD GOLD stage 3
Amiodarone 200 mg OD Rivaroxaban 20 mg OD	Atrial Fibrillation
Bumetanide 1 mg BD Ramipril 3.75 mg OD	HF
Vitamin B12 Thiamine	Anaemia
Nicotine patches 14 mg OD	NRT
Promethazine 25 mg ON	Hypnotic
Atorvastatin 40 mg ON	Hypercholesterolemia

Table 3.9: Drug Therapy During Admission

Medication	Indication
Carbocysteine 750 mg TDS Salbutamol nebs 2.5 mg QDS Tiotropium inhaler 18 µg OD	COPD GOLD stage 3
Bisoprolol 7.5 mg Digoxin 62.5 µg	Atrial Fibrillation and tachycardia/deteriorating heart function
Furosemide 20 mg BD Hydralazine 50 mg BD	HF
Vitamin B12 Thiamine	Anaemia
Nicotine patches 14 mg od	NRT
Enoxaparin 80 mg OD	(1.5 mg/kg for AF switched from Rivaroxaban)
Isosorbide mononitrate 10 mg BD	Pulmonary hypertension
Tolvaptan 15 mg once a week.	Hyponatraemia
Zopiclone 7.5 mg ON PRN	Hypnotic
Atorvastatin 40 mg ON	Hypercholesterolemia
GTN Spray PRN	Symptoms relief

Table 3.10: Medications Stopped During Admission

Medication	Indication
Rivaroxaban 20 mg OD	Due to interaction with rifampicin (started on enoxaparin)
Ramipril 2.5 mg OD Bumetanide 1 mg BD Promethazine 25 mg ON	Hyponatraemia/Hypovolaemia
Amiodaron 200 mg OD	Atrial flutter-switched to digoxin

Vancomycin TDM:

Vancomycin is a glycopeptide antibiotic that exhibits bactericidal activity against aerobic and anaerobic Gram-positive bacteria. The first doses of vancomycin should be based on body weight, while maintenance dose amendments should be based on serum-vancomycin concentrations and depend on the age and the renal function.[151]

When the patient came in, his creatinine clearance was 55 mL/min and according to the guideline he should be on 750 mg BD,[151] but he was on 1g from the previous hospital. All patients necessitate serum-vancomycin determination that normally starts on the second day of treatment, immediately before the next dose.[151] Target trough level for the patient was 15-20 mg/L. The first trough level was taken before giving the patient the evening dose and it came back a little high (22.3 mg/L), therefore it was necessary to reduce the dose to 750 mg BD.

Frequency of monitoring is influenced by the clinical condition and response to treatment. Regular monitoring should be performed in high-dose therapy and longer-term use, particularly in patients with impaired renal function, hearing problems, or concurrent use of nephrotoxic or ototoxic drugs. Since the patient kidney function was fluctuating and he is 72 years old, his renal function, hepatic function, and clinical status were monitored closely in addition to vancomycin level.

Table 3.11: Vancomycin Trough Levels

Date	2/7	4/7	5/7	7/7	10/7	12/7	13/7	16/7	17/8	18/7	19/7	21/7	24/7
Dose	1 g BD	750 BD	500 BD	500 BD	500 750	750 BD	950 1 g	1 g 750	750 BD	750 600	600 BD	750 500	750 -
Trough level	22.3	21.4	21.0	15.7	12.1	25.2	14.8	24.1	22.0	21.1	20.8	17.9	16.5

This patient was not discharged yet when I finished my internship, therefore, his medications on discharge are not mentioned here.

3.2.9.2 Case 2: Gentamicin and Warfarin

A 25 years old non-smoker male, 78 kg, developed symptoms of fever and headache at the end of June this year. The patient presented to Barnet hospital and initially treated via ambulatory care with co-amoxiclav after positive blood culture (*Streptococcus sanguinis*) and urine culture (enterococcus). He was initially recovered well but he was readmitted on 17th July with a return of symptoms. The patient did not have any past medical issues and stated that he had travelled to Moscow in June, but he did not travel outside of Europe.

On examination, the patient was found to have a soft diastolic murmur. A transthoracic echocardiogram demonstrated severe aortic regurgitation (AR). When a transoesophageal echocardiogram was performed, the atrial valve was found to be bicuspid with very small vegetation on the posterior cusp, severe AR, dilated left ventricular, and impaired systolic function (43%). All of which indicated subacute infective endocarditis of the bicuspid aortic valve with a very small and dense/calcific vegetation attached to the posterior cusp. The bicuspid aortic valve is a congenital heart problem in which two of the three leaflets of the aortic valve fuse to form one leaflet.[157] It is associated with aortic stenosis, aortic regurgitation, and infective endocarditis.[157] In this patient case, the doctors decided that he needed an urgent valve replacement in addition to treatment with antibiotics.

Drug History**:** The patient did not have any known drug allergy and he had not been taking any regular medication. He was started on gentamicin 80 mg twice a day in the first hospital with piperacillin/tazobactam (Tozacin™) that was stopped and switched to amoxicillin when he arrived at Bart's.

Medication prescribed pre-operation:

Ramipril 1.25 mg once a day in addition to bisoprolol 2.5 mg once a day to reduce the workload on the heart by reducing arterial pressure, preload and afterload as well as decreasing left ventricular remodelling;

Gentamicin IV 240 mg OD (see dose calculation below) with amoxicillin IV 2 g OD in four-week regimen to treat infective endocarditis.

Medication prescribed post-operation:

Ferrous sulphate 200 mg three times a day to treat the drop in his haemoglobin and haematocrit after the surgery;

Warfarin post mechanical valve replacement for lifelong;

Enoxaparin 40 mg BD as a bridging therapy until warfarin reaches a therapeutic level as determined via the INR.

Paracetamol 1 g QDS for pain relief in addition to codeine 60 mg QDS;

Senna 15 mg BD and lactulose 15 mg BD for post-operation constipation;

Aspirin 75 mg combined with warfarin for reduction in thromboembolism events following mechanical valve replacement;

Lansoprazole 30 mg OD for gastro protection.

Gentamicin TDM:

Gentamicin is a broad-spectrum aminoglycoside that has bactericidal activity against mostly aerobic gram-negative bacteria and some gram-positive bacteria. It inhibits bacterial protein synthesis by binding to 30S and 50S ribosomal subunits.[158] When combined with ß-lactam antibiotics, gentamicin works synergistically to enhance their activity against streptococci, hence, BNF recommends the use of this combination in treating endocarditis which was followed in the case of this patient.[151]

The objective of gentamicin treatment is to reach an initial high peak concentration to kill the microorganism but permit the concentration to drop to a low (trough) level between doses to prevent accumulation and potential toxicity. Gentamicin dose for infective endocarditis is 3 mg/kg OD and it could be modified according to creatinine clearance since it is cleared by the kidneys, and it is therefore important that renal function is assessed before treatment commencement.[151] The patient creatinine clearance on admission was 125ml/min, therefore, the dose he should receive is 240 mg OD. However, the patient was on 80 mg BD when he was admitted which was changed to 240 mg OD considering many studies which suggested that once-daily compared with multiple-daily gentamicin dosing may reduce the potential for toxicity due to washout period allowed by infrequent dosing in addition to the fact that high peaks are more effective in achieving bactericidal effect.[159] For obese patients, corrected body weight should be used for dosing calculation remembering that gentamicin is highly hydrophilic and is not distributed into adipose tissue.[151]

The major side effects of gentamicin are dose-dependent such as ototoxicity, which is irreversible, and nephrotoxicity.[158] As a result, monitoring trough plasma concentrations is important to ensure that no accumulation occurs.[151] Serum-gentamicin concentrations should be measured in all patients receiving parenteral aminoglycosides. Target trough level for once-daily regimen, which is the regimen of this patient, is < 1 mg/L and it should be measured just before the next dose is due.[151] The level was being repeated twice weekly since patient's renal function was stable. Patient trough level was always within the required range, hence, no dosing modification was needed. (Table 3.13)

The dose of aminoglycoside should be adjusted accordingly If renal function is reduced during treatment, but during the admission of the patient his renal function was stable, and he did not manifest any nephrotoxicity nor ototoxicity. (Table 3.13)

C-reactive protein serum level, which is a marker for inflammation in the body, was monitored as well. Throughout antibiotic treatment, patient's C-reactive protein level was dropping gradually which means the inflammation was improving. (Table 3.13)

Table 3.12: Monitored Parameters

Date	31/7	1/8	4/8	6/8	9/8
Gentamicin dose	240 mg OD	240 mg OD	240 mg OD	240 mg OD	Stopped
Pre-dose (trough) gentamicin level	0.5	0.3	0.3	0.2	-
Serum Creatinine	79 µmol/L	78 µmol/L	73 µmol/L	75 µmol/L	70 µmol/L
C-reactive protein	40 mg/L	98 mg/L	52 mg/L	26 mg/L	10 mg/L

Warfarin TDM:

To prevent valve thrombosis and systemic embolism, anticoagulation therapy, such as warfarin, is essential. However, it can also cause serious bleeding making its regular monitoring crucial for patients receiving this type of treatment. The patient was bridged to warfarin with LMWH (enoxaparin) to reach the required INR but then enoxaparin was stopped when aspirin was started to minimize bleeding risk. Target INR indicated for the mechanical valve type that the patient had is 2 to 3 for the first three months and then 1.5 to 2 lifelong. A once-daily 5 mg dose of warfarin was commenced based on the recommendation of Bart's guideline on the initiation of warfarin. Then the dose was modified in a response to patient's INR as recommended in the guideline.

Before warfarin commencement, liver function should be assessed in addition to blood counts. Platelet count less than 50 is a relative contraindication to warfarin.[151] Warfarin monitoring through INR is presented in Table 3.14. Patient's medication on discharge are presented in Table 3.15.

Table 3.13: Patint's INR Monitoring

Date	3/8	4/8	5/8	6/8	7/8	8/8	9/8	10/8	11/8	12/8
INR	1.8	2.6	2.1	2.5	-	3.2	2.8	2.6	2.4	2.0
Dose	5 mg	5 mg	5 mg	6 mg	5 mg	5 mg	5 mg	5 mg	5 mg	6 mg

Table 3.14: Medications on Discharge

Medication and Route	Dose	Frequency	Pharmacy Comments
Warfarin (oral)	as per INR	OD	For mAVR OnX valve (target INR 2-3 for 3 months then 1.5-2.0). Recent dosing / INRs: 6/8 6mg INR 2.5; 7/8 6mg; 8/8 5mg INR 3.2; 9/8 5mg INR 2.8; 10/8 5mg INR 2.6; 11/8 5mg INR 2.4; 12/8 6mg INR 2.0
Ceftriaxone (intravenous)	2 g	OD	*ACUTE* - Course to be complete on 24/8/18 (total 4 weeks post-op) for the treatment of Strep Sanguinis IE. To be managed by Barnet Hospital OPAT service.
Ferrous Sulphate (oral)	200 mg	TID	Anaemia, to review Hb in 3 months to the on-going need.
Enoxaparin (subcutaneous)	120 mg	OD	*ACUTE* Continue until INR >2. 1.9 on discharge.
Aspirin dispersible (oral)	75 mg	OD	Post mAVR OnX valve

3.3 Oncology

Barts Cancer Centre is the specialist regional centre for cancer treatment in north-east London. Barts hospital has highly specialized teams to treat the common cancers (lungs, breast, stomach and bowel, kidney, prostate, ovarian, cervical) as well as some of the rarer forms of cancer such as mesothelioma and melanoma. The hospital is specialized in the use of the most effective and up-to-date treatments combining where necessary surgery with chemotherapy, radiotherapy, hormone treatment, and new drugs for cancer. The oncology wards and day units are based in King George the 5th building at St Bartholomew's hospital (Table 3.16) [160]

Table 3.15: Barts Cancer Centre wards

Ward	Specialty
7A south (BSDU)	Day unit haem-oncology patients
7A north (Paget)	Day unit solid tumor patients
Ward 5A	Medical oncology (solid tumors) – Male
Ward 5B	Medical oncology – Female (breast surgery and RT)
Ward 5C	Haematology oncology ward
Ward 5D	Haematology oncology ward - Transplant Ward

3.3.1 Pharmacist on wards

The pharmacists on the wards start their day by preparing the ward handover. This is where the patients' details and all their essential information are listed to help the pharmacist identify the tasks required for each patient. When on the ward, the pharmacist checks first ward's requests on pharmacy book to ensure that there is no urgent medication that needs to be provided. Next, the pharmacist identifies the new patients for whom a drug history is taken, and medications are ordered. Then the pharmacist does a medication reconciliation to ensure that all the medicines needed for a patient are prescribed on the drug chart as well as to optimize the medications for each case. Pharmacists carry out daily assessments of medication profiles to identify, prevent and manage any drug related problem regarding drug selection, dosage, interactions, administration and side effects by drug chart reviews based on data from clinical notes, laboratory tests, and interviews with patients and/or relatives. As part of the multidisciplinary team, clinical pharmacists participate in the medical round in the mornings. The pharmacist is also active in educating and helping patients and family members understand what to expect during chemotherapy. Whenever necessary, the pharmacist liaises with the health care team to discuss patients' cases and to make medications amendment.

3.3.2 Pharmacist in out-patient Clinic

The primary focus of oncology pharmacists in the clinic is typically the pharmaceutical management of cancer patients.

The pharmacist acts as an integral part in a multidisciplinary team in the outpatient supportive care service, focusing on the management of symptoms resulting from patient's cancer and subsequent cancer treatment.

After being seen by the doctor, clinic patients are then seen by the pharmacist who starts validating the prescription of oral chemotherapy by checking patient's details on the prescription like the name, date of birth, NHS number as well as consultants name and signature, the dates and the diagnosis. For first chemotherapy cycle, the pharmacist firstly needs confirming patient's drug history, then, needs to check lab results including the red and white blood cell count, liver and kidney function in addition to other biomarkers depending on the type of cancer. Afterward, the pharmacist checks whether the regimen prescribed, the doses and the duration fit the patient's diagnosis and clinical situation and makes sure it all matches the prescription on Aria (the software where all chemotherapy prescription are done). Additionally, the pharmacists ensure that supportive therapies are prescribed. The supportive care may include pain management medications, antiemetics, constipation and diarrhoea management medication, medications to treat anaemia, anticoagulation, and anti-infectives. If any changes are to be made, the pharmacist highlights it to the consultant and advises on the most appropriate option for that particular case. Only when the pharmacist is satisfied with the prescription, the chemotherapy order will be written. At that point, the order will be transmitted to the outpatient pharmacy in one of the trust hospitals depending on the patient's preference.

For a subsequent cycle, the pharmacist needs to validate the prescription by checking all patient's details, consultant name and signature, drugs prescribed, the doses, the cycle and its duration, the dates of the previous cycle, blood counts, kidney and liver function among other biomarkers in addition to the supportive medication typically given.

3.3.3 Cytotoxic and immunotherapy preparation

Cytotoxic and immunotherapy preparation for all hospitals within Barts trust typically takes place in the cytotoxic preparation unit on the 7th floor of St. Barts hospital. The worksheets for the preparation of each product are compiled by the oncology pharmacists based on the instructions from the manufacturers. Only accredited personnel, normally qualified technicians, can perform the aseptic preparation of chemotherapy and immunotherapy.

The prescriptions are normally screened by an oncology pharmacist a day ahead of the patients' appointment to receive their chemotherapy. This enables the pharmacist to assess patient's fitness to receive chemotherapy by checking their present states and the most

recent lab results. These clinical analyses must be done within three days before chemotherapy administration. During the screening process, the pharmacist confirms patient demographics, the diagnosis, the cycle, the date of the previous cycle, and the medicines to be administered. Then, assessment is done on their doses according to the patient's body surface area and kidney function as well as the presence of the consultant's name, dates, and signature on the prescription. Moreover, the pharmacist must make sure all the supportive medicines are prescribed with chemotherapy.

After the pharmacist approves the prescription, a technician will generate worksheets and labels for the preparation. A worksheet must include all the primary constituents with their respective quantities calculated using a computer software in addition to all the materials required for the process. Another technician will go through the printed worksheets and confirm all the details. The approved worksheets are sent to the assembly room, where a tray is prepared for each prescription. All constituents and materials necessary for each preparation are gathered in one tray. The approved worksheets are put in a transparent sealable plastic bag in the tray which is sprayed with its content and placed in the hatch to be passed into the process room.

In the process room, a technician receives the tray and confirms its contents. Then the tray is placed in the hatch of the isolator and the worksheet is hung in a visible way so the technician responsible for the preparation can follow it. Cytotoxic preparation is made in a closed system isolator with negative pressure to reduce the risk of occupational contamination and the exposure to cytotoxic materials in the process room. Once in the isolator, the items are sprayed, then the technician begins to prepare the prescription by following the instructions on the worksheet. The second technician will always be around to support the preparing technician and to supply anything needed during the process. When the preparation is ready, it is checked by a highly qualified person accredited for checking.

This includes checking the appearance for the existence of any particles and the colour among other things to ensure the safety of the product. Subsequently, the products are packed in suitable bags depending on their photosensitivity. As soon as the preparation is approved, it is placed in the hatch to be received by the pharmacist in the release area. Here the pharmacist checks again the patient details, clinical situation, lab results and fitness for chemotherapy along with the products prepared and their concordance with the details on the prescription. When the preparation is approved by the pharmacist to be released, it is stored in a special bag with the name of the patient in a locker specific to the ward/day unit where the patient will receive the treatment.

The same process is followed for immunotherapy preparation except for the type of the isolator where positive pressure system is used considering the main concern being product protection enhancement rather than occupational exposure. Finally, it is important to point

out that the staff involved in all aspects of cytotoxic handling must use the most appropriate personal protective equipment.

For some cytotoxic medicines, pre-made products of common doses are kept on designated shelves in the release area. These products have the advantage of fast processing times as the prescriptions will pass from the screening process directly to the release process.

3.3.4 Microbiological control

The requirements for aseptic manipulation of intravenous chemotherapy and immunotherapy formulations mean that strict measures must be applied. These measures include air filtration, restricted access to the preparation areas (only qualified staff and maximum of 5 persons in each area), and minimizing as much as possible objects present in these areas. The cleaning process of the preparation area has several levels. It is performed on a daily, weekly and monthly basis. While in the daily cleaning the isolators and other surfaces used during the day are cleaned, the weekly cleaning is performed more deeply. In the monthly cleaning, everything in the room including the ceiling, the walls, and the ground are washed and disinfected.

To guarantee the effectiveness of the practices followed in the unit, continuous monitoring should be performed. The method of settle plate sampling is used for active air monitoring in the clean areas to ensure the absence of airborne microorganisms that may contaminate or affect cytotoxic preparations. The plates are positioned in various sites in the actual work zone and expected to not have any growth.

For assessing operators' technique and bioburden of products, the finger dap method is used. This is where each operator after each preparation session touches their fingers and thumb onto the agar surface. Normally, the results come in two weeks and the dabs should be bacteria free. Poor operator technique or poor containers spraying practices may lead to bacterial growth in the plates.

3.4 Clinical trials

Bartholomew hospital is a research-active hospital where hundreds of clinical trials are conducted in different areas such as cardiology and oncology. Pharmacy Clinical Trials Department is an established service that offers quality pharmaceutical research in conformity with Good Clinical Practice (GCP) and the EU Clinical Trials Directive. Good clinical practices are legal requirement always when an investigational medicinal products (IMPs) is involved in a clinical trial and it is defined in the EU Directive 2001/20/EC as follows: "Good clinical practice is a set of internationally recognized ethical and scientific quality

requirements which must be observed for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects. Compliance with this good practice provides assurance that the rights, safety, and well-being of trial subjects are protected and that the results of the clinical trials are credible."[161]

The Pharmacy Clinical Trials team combines specialist clinical trial pharmacists, pharmacy technicians, and assistants who have undergone specific training on Good Clinical Practice (ICH/GCP) and, where applicable, Good Manufacturing Practice (GMP) training. This training requirement must be fulfilled prior to undertaking any clinical trials work.

Furthermore, pharmacy clinical trial staff receive regular training to ensure their knowledge is up-to-date in this specialist field. All trials to be conducted at the hospital must be submitted to the Pharmacy Clinical Trials team to carry out a feasibility study. For that, the sponsor needs to provide the essential document set of the study with all the details necessary to conduct the trial. When a Trial is approved by the team, a Pharmacy Site file will be prepared.

For commercial trials, a Clinical Trial Agreement (CTA), which is a contract between the sponsor and the hospital, must be signed off. The objective of a CTA is to clarify the rights and obligations of the contracting parties. A lead technician and a lead pharmacist will be assigned to each trial.

It is the responsibility of the pharmacy clinical trials team to guarantee the safe, proper, and effective implementation and management of clinical trials. Besides, the team must assure that any Investigational Medicinal Product (IMP) employed in a trial is suitable for use and is acquired, handled, stored, and disposed of safely and in accordance with the approved Protocol.

3.4.1 Storage Facilities

IMPs are stored in the pharmacy in safe, temperature-controlled places that entails badge/code access. The room temperature is held between 15 – 25 ºC while the fridge temperature is maintained between 2 – 8 ºC. Sometimes, IMPs needs to be kept outside of the inpatient pharmacy (e.g. at ward/clinics). In such cases, a risk assessment is completed by the pharmacy for each IMP in addition to obtaining a sponsor agreement. IMP stock level is normally under monitoring to guarantee that there are always sufficient amounts for dispensing to trial patients.

An electronic prescribing platform (ARIA) is used for oncology clinical trial prescriptions. The ARIA prescriptions are constructed and verified by the pharmacy team and then validated by

the principal investigator (is the investigator in charge of the running of a specific trial at the site). Paper prescriptions are used for the clinical trials of other specialties.

For IMP dispensing, a trial specific dispensing procedure is followed. This specific procedure is designed and approved by the pharmacy clinical trials team for every single trial. For patient safety reasons, each dispensed IMP need to be labelled with a trial-specific label after adding patient's name, hospital number, and the date of dispensing. Each prescription is dispensed by one person and then checked by an accredited checking senior technician. It is the responsibility of the dispenser and checker to guarantee that the IMP will remain in date for the duration of the prescription.

3.5 Medicines Distribution and Dispensary

Unit dose dispensing is not used at the hospital, instead, one-stop dispensing is employed. One stop dispensing is defined as "the practice of combining inpatient and discharge dispensing into a single supply labelled for discharge".[162] Patients and caregiver are always encouraged to bring Patients' Own Medications (PODs) into the hospital to be used throughout their stay and relabelled when necessary. Using PODs provides a reduction in medication wastage and expenditure. Furthermore, during the process of medication reconciliation at admission patients are asked about their supply at home and the information given are documented on the drug chart so medicines are not re-dispensed at discharge in case the patient has the adequate amount at home. Medicines prescribed during admission are supplied and labelled in a 'ready for discharge' format to facilitate the discharge process and to decrease delays in discharge. Nevertheless, the medicines that are expected to be discontinued (e.g. analgesics) on discharge or to suffer dose amendment (e.g. beta blockers and ACE inhibitors) are provided from the ward stock of previously defined commonly-used-medicines' list in each clinical area. The stock list should include the names and forms of all medicines needed, and the minimum stock level that must be maintained. When a required medicine is not contained within the stock list and when the POD is not available or appropriate for use, it is ordered at the time it is needed. To support the administration process, all medicines used throughout admission including PODs are stored in a bedside locker, which must remain locked when not in use. These lockers help to eliminate the need for pushing a 'medicines trolley' around the ward.

Inpatient dispensary provides medicines to all the wards at St. Bartholomew hospital including surgical, medical, and intensive care unit wards. It is mainly managed by technicians since all the requests sent to the dispensary have been already screened by the clinical pharmacists on the wards to prevent any clinically inappropriate supplies from being dispensed. The team at the dispensary consists of Senior Technicians, Technicians, Student Technicians, and pre-registered pharmacists. The main activities conducted in the dispensary are storing medicines, dispensing, labelling, delivering the medicines to the wards, topping-up medicines

stocks on the wards, and ordering and receiving medicines from the central stores based at The Royal London Hospital (Medicines Distribution Unit). The dispensary deals with three different types of requests: in-patient supply requests for medicines to be used on the wards, to take away medicines (TTA) requests for medications supplied on hospital discharge, and Aria requests which is the electronic prescribing system used for clinical trials and oncology prescriptions. Most of medicines are dispensed via an automated dispensing system (robot). The rest of the medicines and medicinal products are stored in designated areas in the dispensary.

In case of in-patient supply requests and TTAs requests, once the prescriptions have been verified by the pharmacist, an order sheet (appendix XV) is filled with patient's name, hospital number, the drug, form, strength, quantity supplied, expiry date and frequency of administration. The medicines are dispensed by one of the technicians from the in-patient dispensary. In the dispensing process, the dispenser must enter the patient's hospital number and choose correctly the ward where the patient stays into the software. When an original pack needs to be dispensed, it will be supplied by the robot. However, if a split pack or an injection are ordered, for example, these will be dispensed from the shelves in the designated areas. To avoid wasting medicines, there are special shelves for open packs from where split packs are normally dispensed. After selecting the medicines, the labels are produced with patient's name, drug, strength, dosage form, instructions about how often and when to take the drug with cautions when applicable. As dispensing labels are produced, the items requested are delivered by the robot. The dispenser labels and checks the items and then put all the medicines requested with the order sheets together in a tray marked with its destination. A pharmacist or an accredited checking technician will then verify the dispenser's work. After completing the final check, the items are transferred to the relevant wards.

Likewise, ward-based dispensing is commonly practiced at the hospital, where a list of medicines frequently prescribed on the wards are stocked in specified cupboards in dispensing rooms at ward level. Ward-based dispensing is principally useful for urgent requests, when re-labelling PODs, and when processing last-minute discharge medications. Dispensary integrated within the ward provides a faster and efficient discharge prescription dispensing process when all the medicines to be dispensed are included in the stock in the dispensary on the ward. Although patients should be discharged with a minimum of two weeks supplies of medicines, typically, must of the patients are issued 28-day supply. The decision of the quantity of supplied medicines is taken by the pharmacist according to each case.

For oral cytotoxic drug dispensed from the dispensary, the orders are received by the Aria system after clinical validation of the order by a pharmacist. The steps mentioned above are then followed. Cytotoxic drugs are not stored in the robot, instead, there are designated shelves for their storage. The labelling of oral cytotoxic medicines must unambiguously state

the dose and the total number of tablets or capsules to be taken in addition to administration instructions. Labels for medicines of weekly dosing such as methotrexate must contain the phrase 'once a week' and indicate which day of the week the dose should be taken. Considering the risk of chemotherapy drugs being carcinogenic, mutagenic, and teratogenic, a warning sticker with the wording 'cytotoxic, handle with care' should be affixed on all the containers of the drugs. Clinical trial dispensing process is explained in clinical trial section.

All medicines stored in the dispensary are contained within the Trust's medicines formulary.

The temperature in the dispensary is controlled and recorded daily (including weekends) by a pharmacy technician. A refrigeration room is used to store medicines that require refrigeration. Temperatures are recorded at least once daily using a calibrated maximum/minimum thermometer.

The stock of ward-based dispensary is frequently monitored by the pharmacy staff to maintain the minimum level and to do a top-up when necessary. As doing top-up, stoked medicines' expiry dates must be checked.

The outpatient dispensing facilities are managed by Lloyds Pharmacy that deals with Issued prescriptions at out-patient clinics on behalf of the Hospital Trust. Lloyds pharmacy is situated in the hospital main entrance.

3.6 Conclusion

The whole experience was very enriching and enlightening one. During my stay in the hospital I was able to understand and appreciate how fundamental the role of clinical pharmacist is, as part of multidisciplinary team, in the work flow and in patients' care within the hospital. Additionally, this internship made highly appreciate the importance of pharmacy technicians who, by performing the technical part of the work, provide pharmacist with adequate time to carry out their clinical functions in taking care of patients closely.

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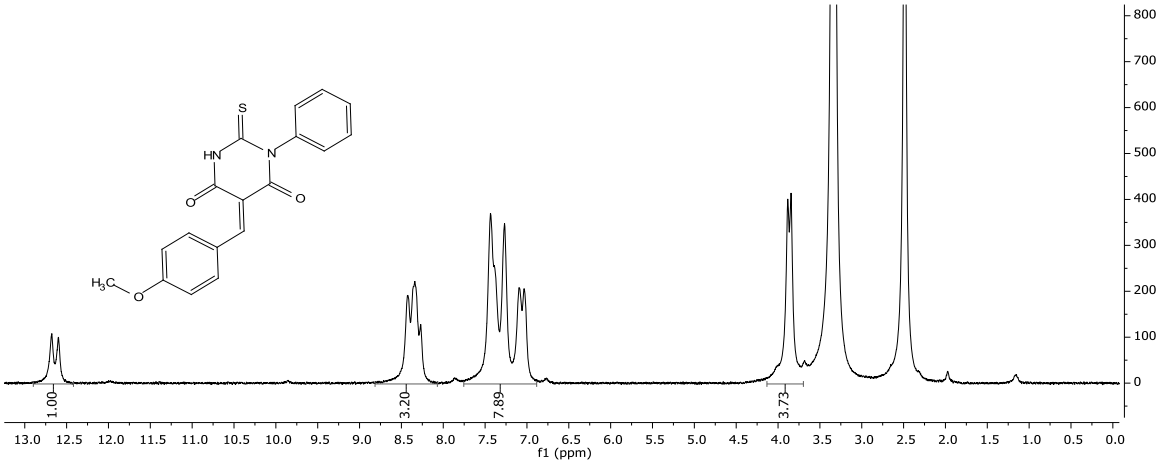
Appendixes

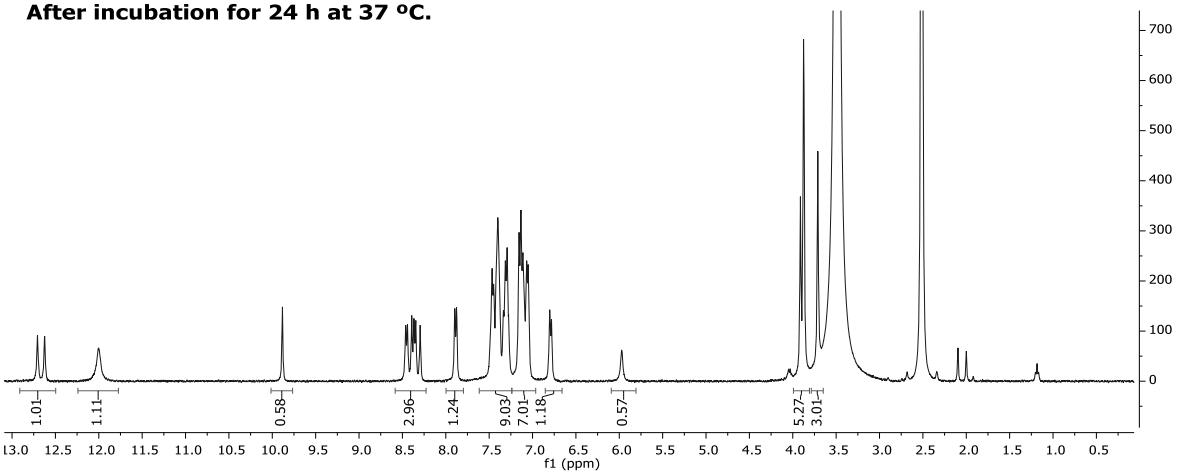
Appendix I: ^{1}H NMR signals of 5-C=CH vinyl from 5-benzilidene(thio)barbiturate **6**, 5-C(O)H from respective benzaldehydes **5**, 5-CCH from 5-benzyl(thio)barbiturates **7**, and 5-CCH2 from 5,5-Dibenzyl(thio)barbiturates **8.**

Entry	5-8	5-CCH from 6	5-C(O)H from 5	5-CCH from 7[a]	5-CCH$_2$ from 8
1	***a***	8.28-8.45 (*E* and *Z)*	9.88	5.98	-
2	***b***	8.46	10.03	6.27	-
3	***c***	8.42	9.96	6.21	-
4	***d***	8.42	9.88	6.20	7.33
5	***e***	8.29	10.03	5.98	-
6	***f***	8.26	9.96	5.92	-
7	***g***	8.27	9.88	5.90	6.79
8	***h***	8.28	-	-	-
9	***i***	8.25	-	-	-
10	***j***	8.37	-	-	3.19

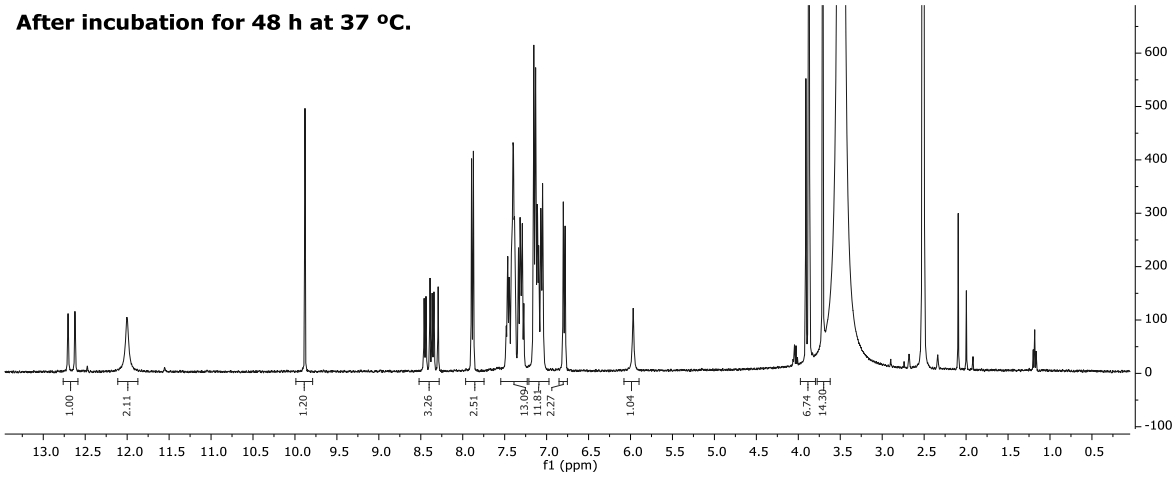
Appendix II: ^{1}H NMR spectrum of 5-(4-methoxybenzyl)-1-phenylpyrimidine-2,4,6(1H,3H,5H)-trione (**6a**) and the resulted spectrum after incubation for 24h at 37 ºC.



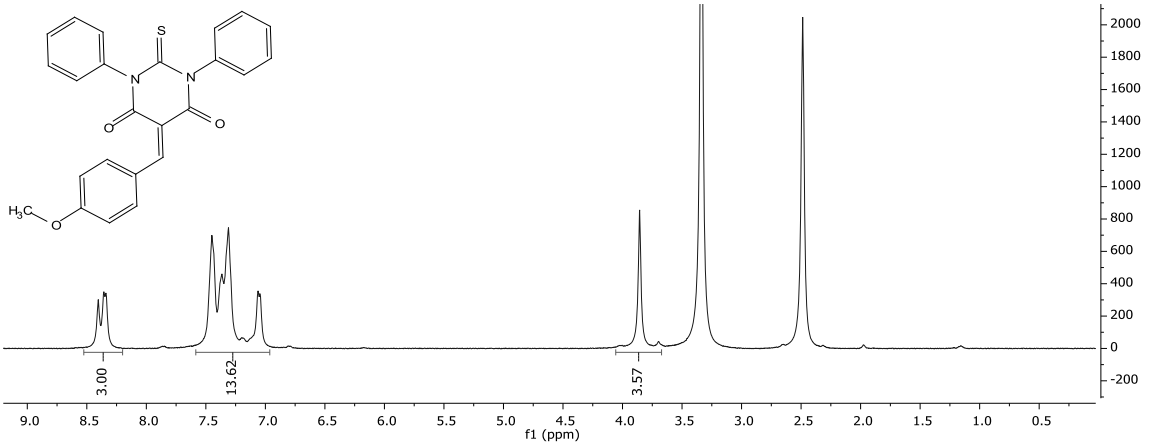
After incubation for 24 h at 37 ºC.

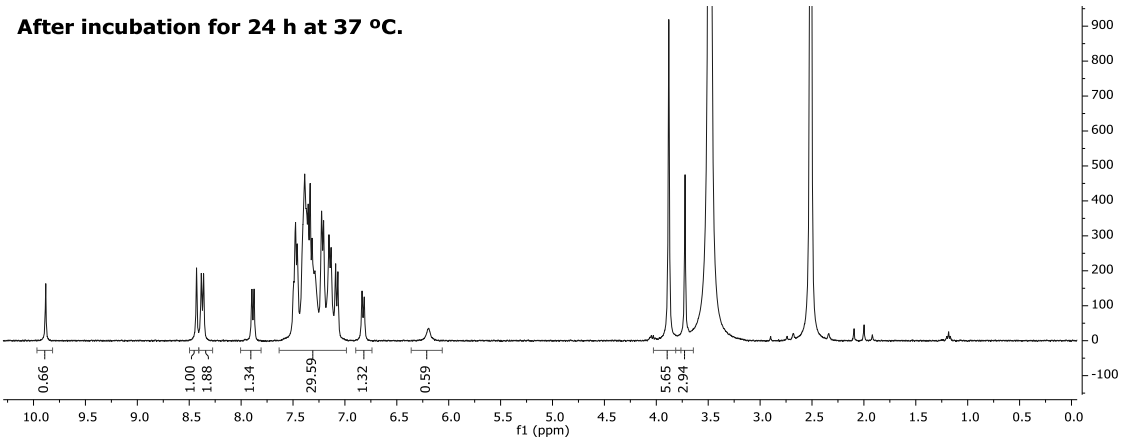


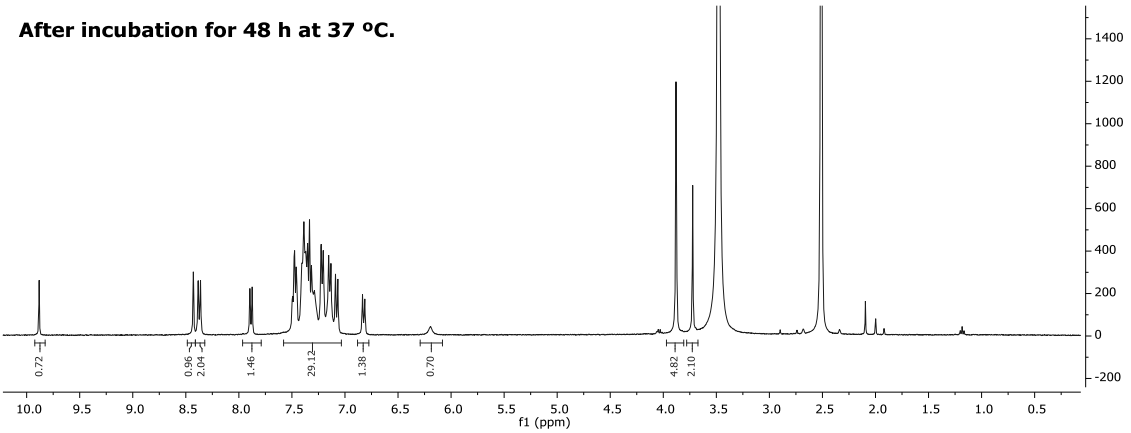
After incubation for 48 h at 37 ºC.



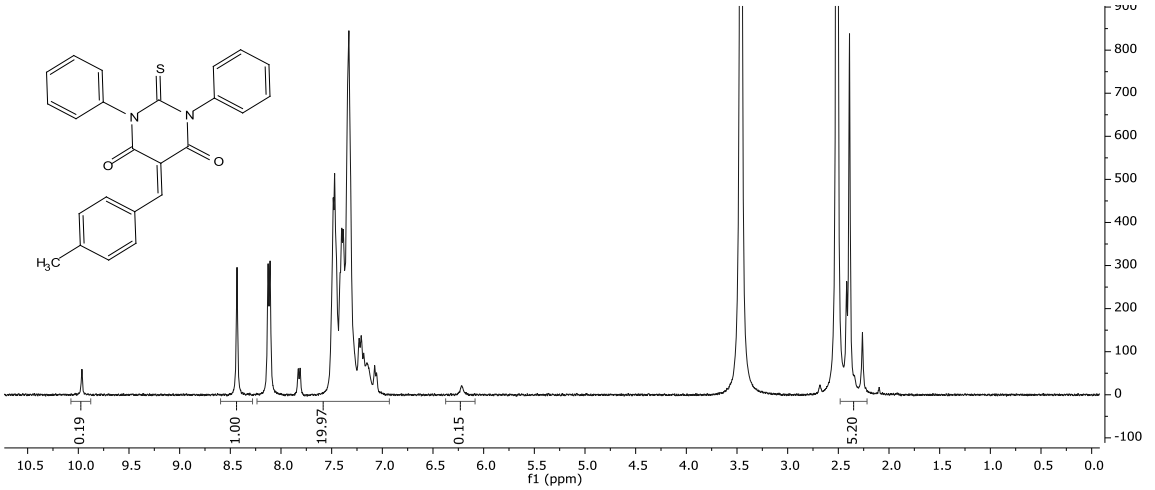
Appendix III: ^{1}H NMR spectrum of 5-(4-methoxybenzylidene)-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (**6b**) and the resulted spectrum after incubation for 24h at 37 ºC.



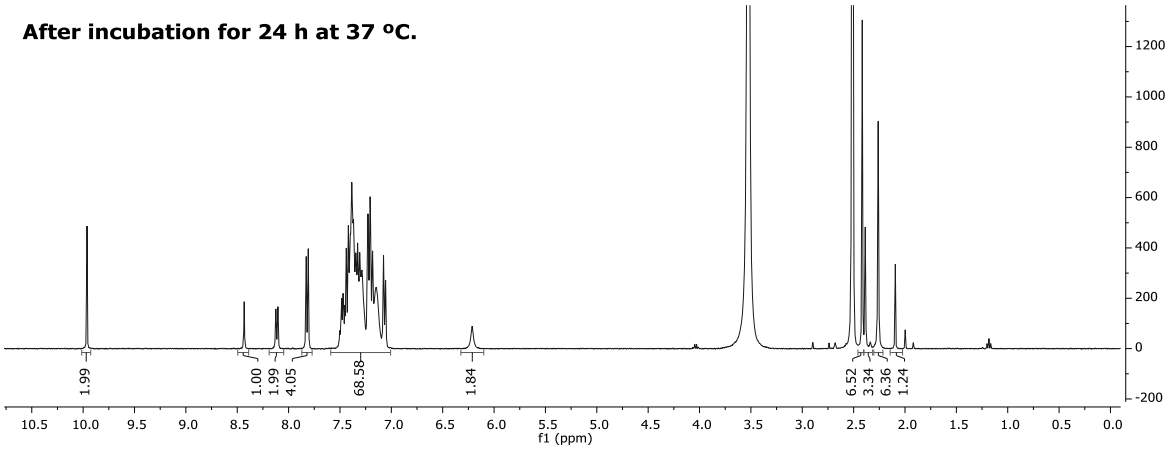




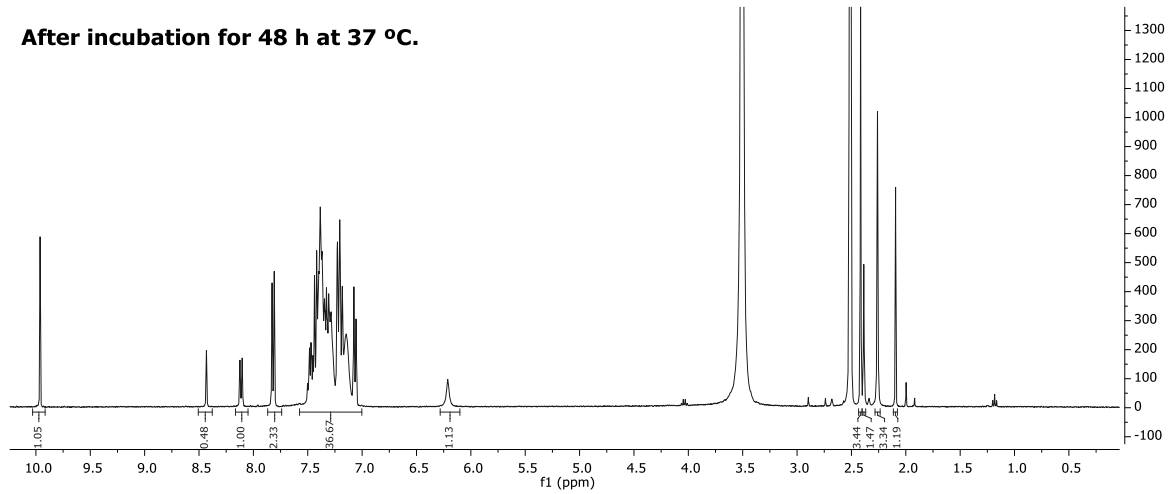
Appendix IV: ^{1}H NMR spectrum of 5-(4-methylbenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione (**6c**) and the resulted spectrum after incubation for 24h at 37 ºC.



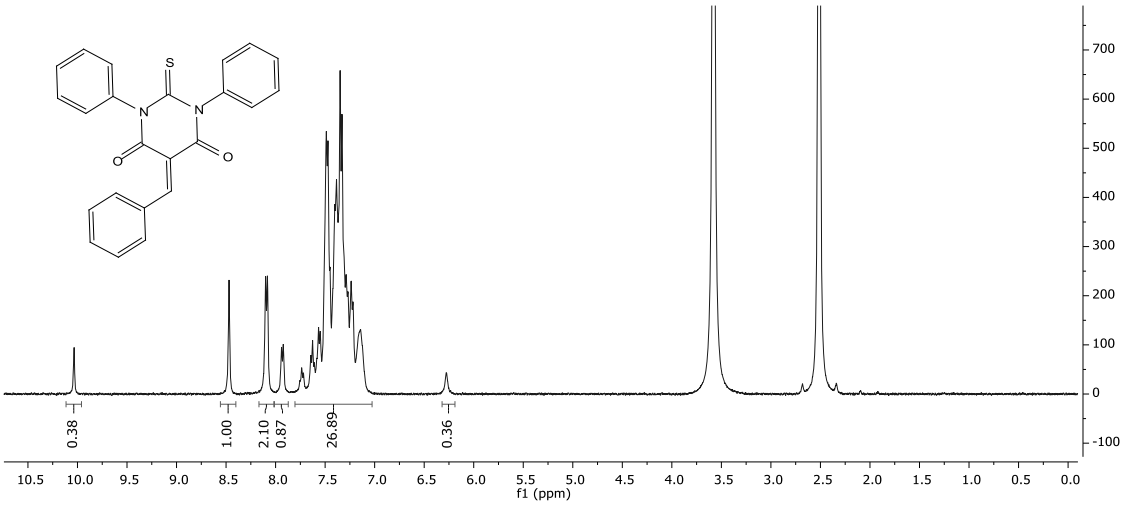
After incubation for 24 h at 37 ºC.

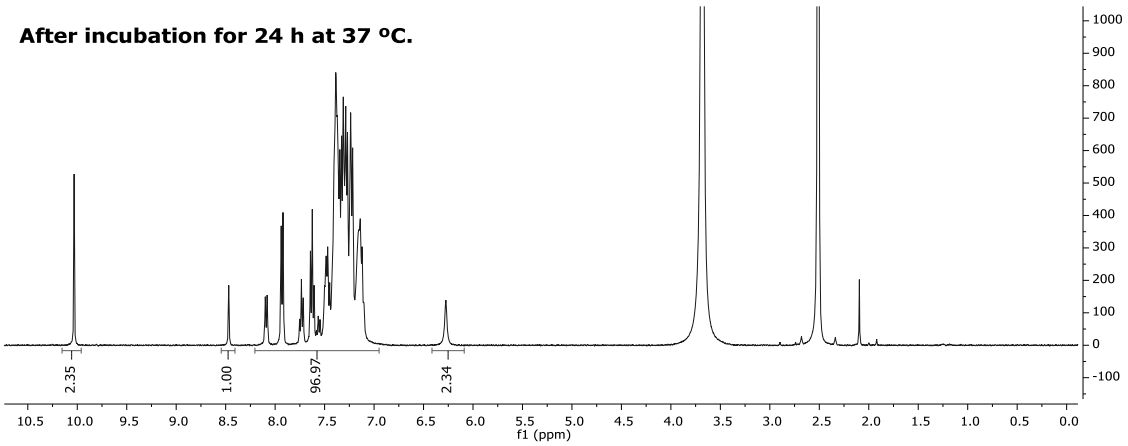


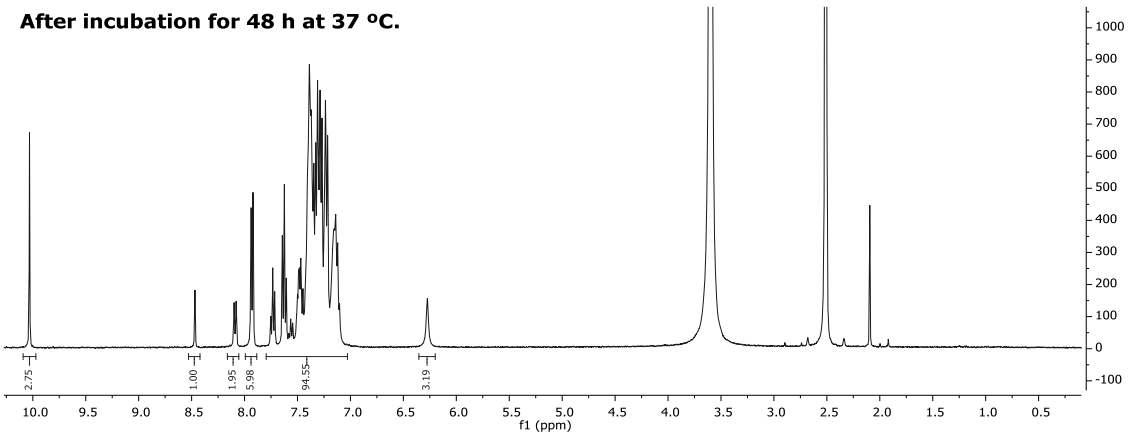
After incubation for 48 h at 37 ºC.



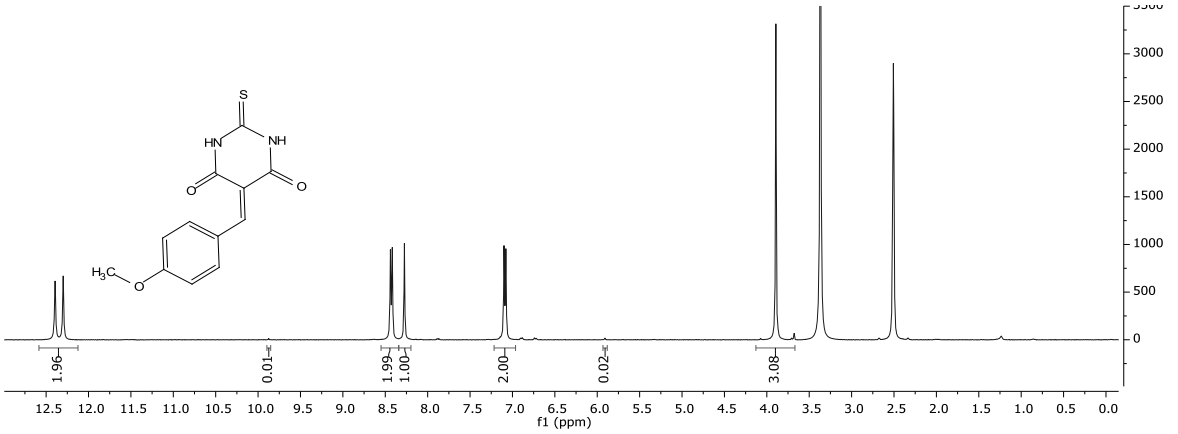
Appendix V: ^{1}H NMR spectrum of 5-benzylidene-1,3-diphenyl-2-thioxodihydropyrimidine-4,6(1H,5H)-dione (**6d**) and the resulted spectrum after incubation for 24h at 37 ºC.



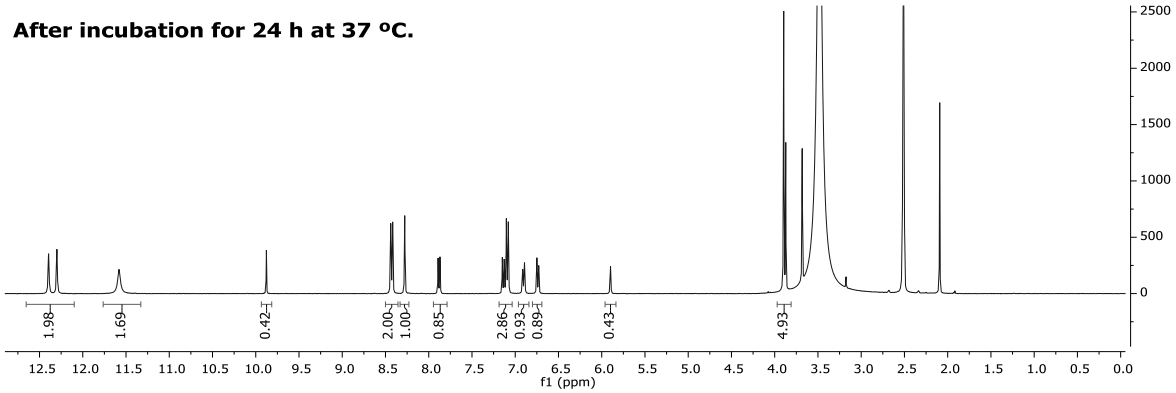




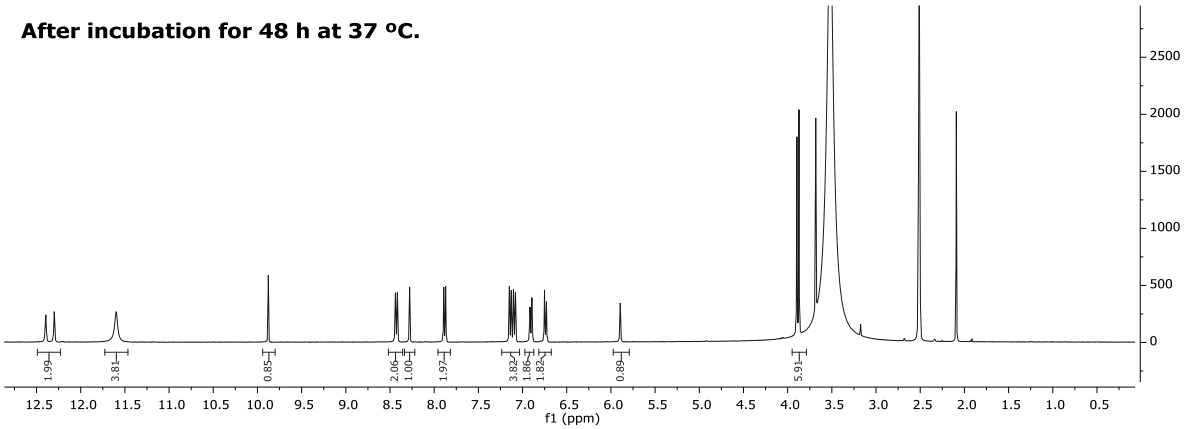
Appendix VI: ^{1}H NMR spectrum of 5-(4-methoxybenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione **(6e)** and the resulted spectrum after incubation for 24h at 37 ºC.



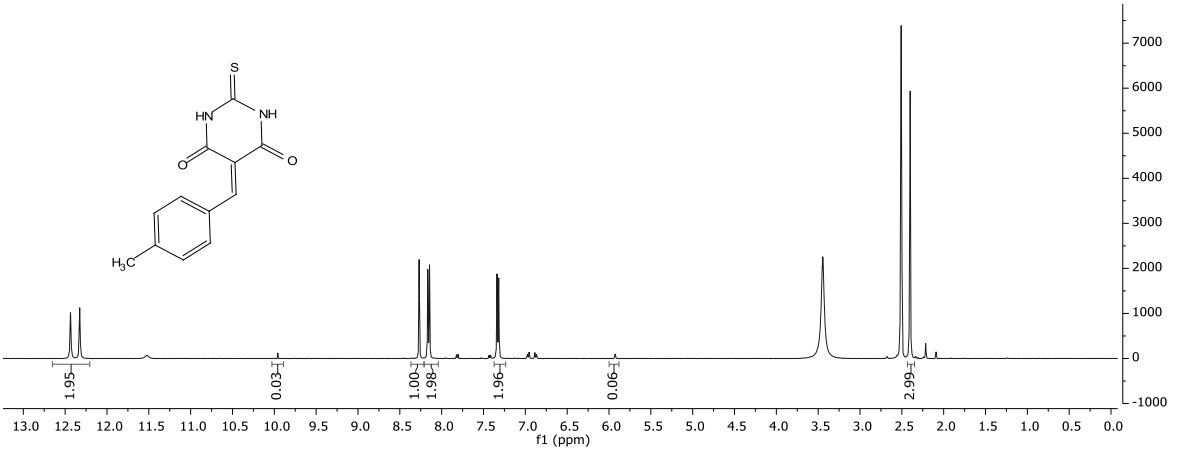
After incubation for 24 h at 37 ºC.



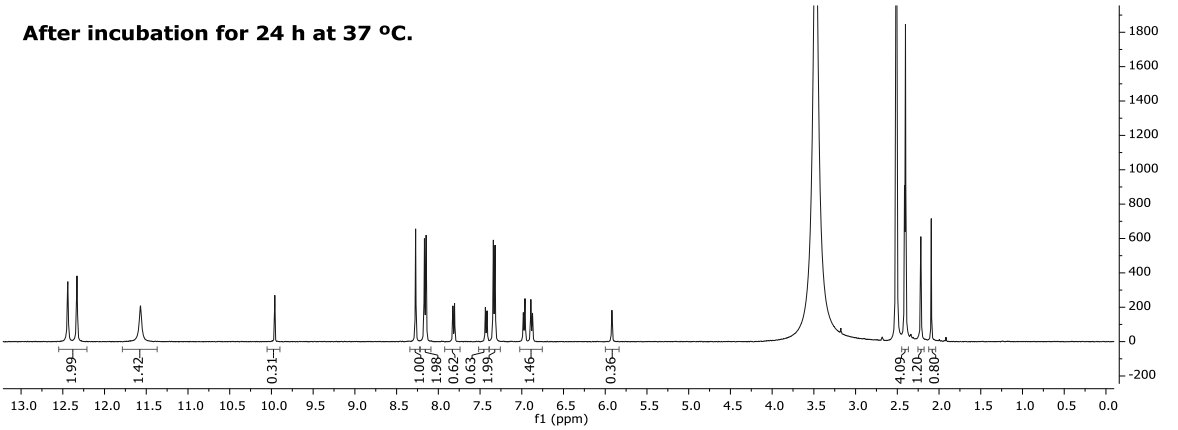
After incubation for 48 h at 37 ºC.



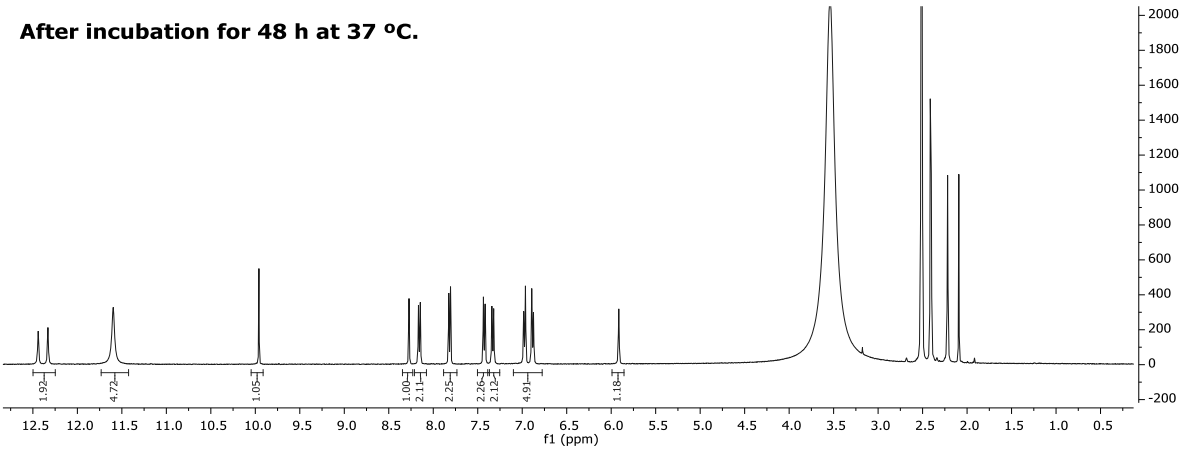
Appendix VII: ^{1}H NMR spectrum of 5-(4-methylbenzylidene)-2-thioxodihydropyrimidine-4,6(1H,5H)-dione **(6f)** and the resulted spectrum after incubation for 24h at 37 ºC.



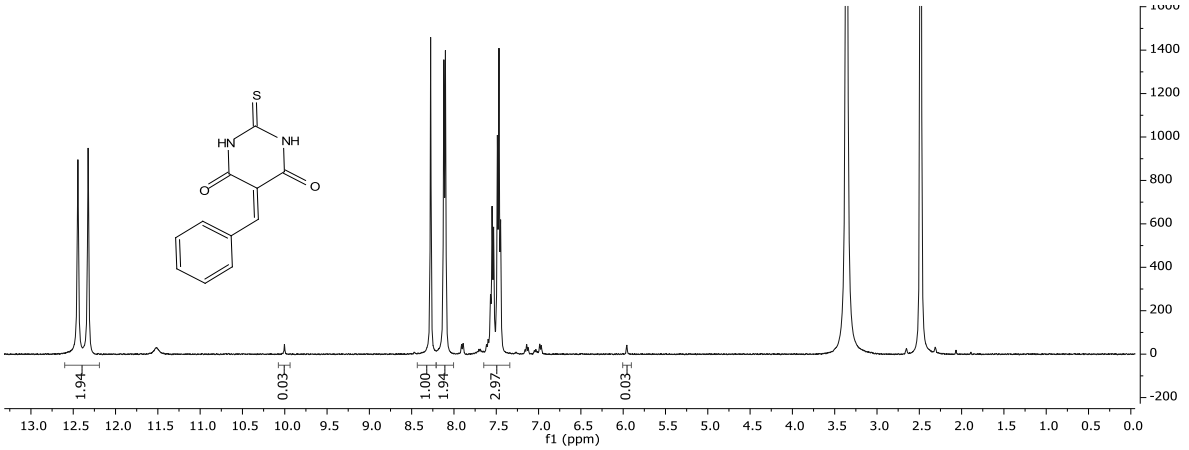
After incubation for 24 h at 37 ºC.



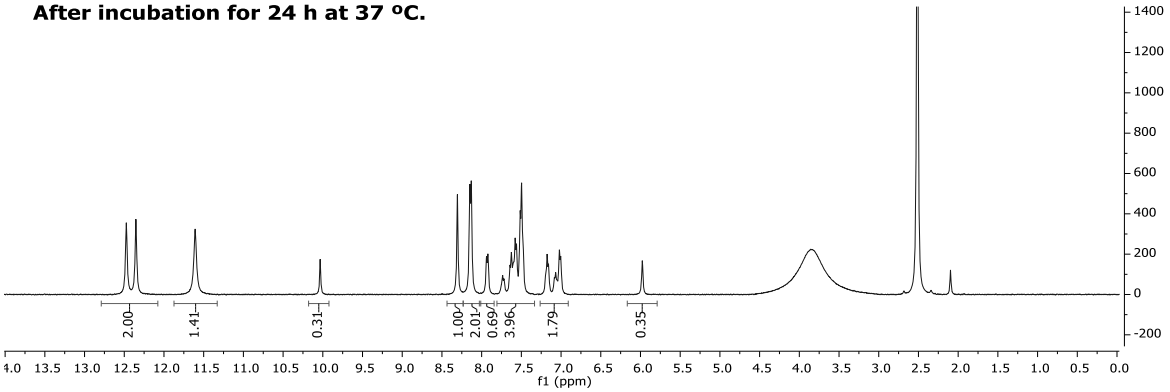
After incubation for 48 h at 37 ºC.



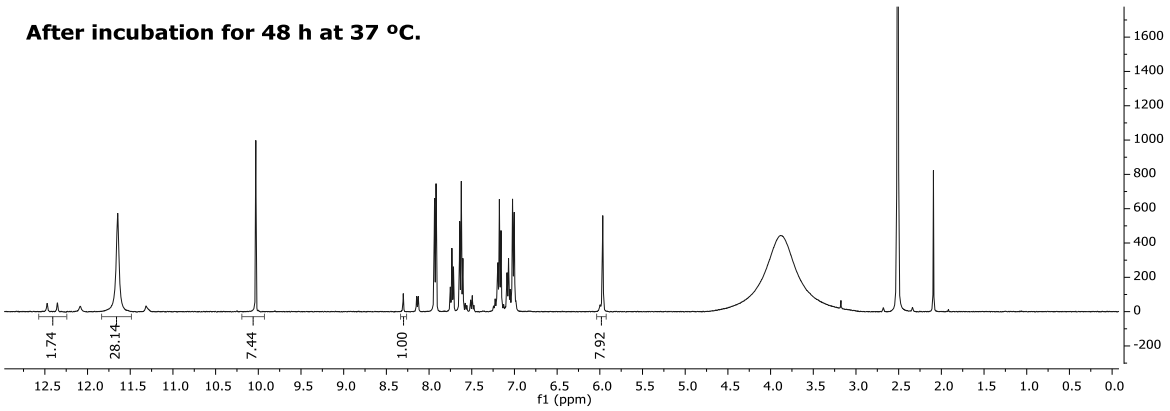
Appendix IIX: ^{1}H NMR spectrum of 5-benzylidene-2-thioxodihydropyrimidine-4,6(1H,5H)-dione **(6g)** and the resulted spectrum after incubation for 24h at 37 ºC.



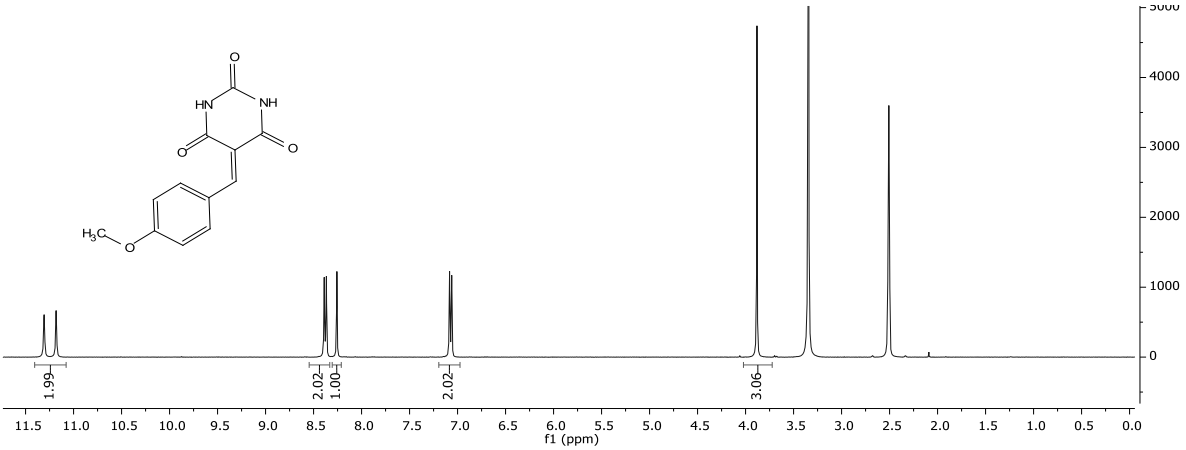
After incubation for 24 h at 37 ºC.



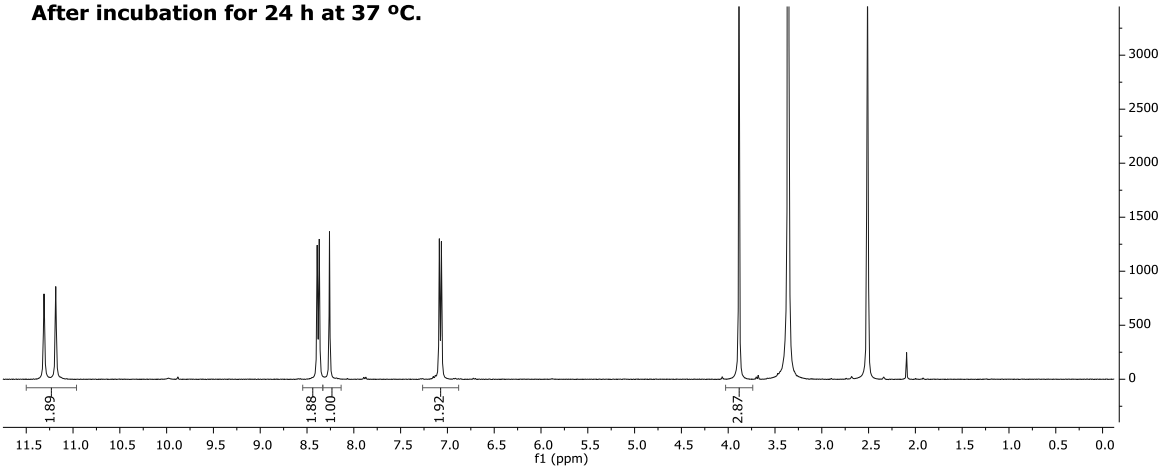
After incubation for 48 h at 37 ºC.



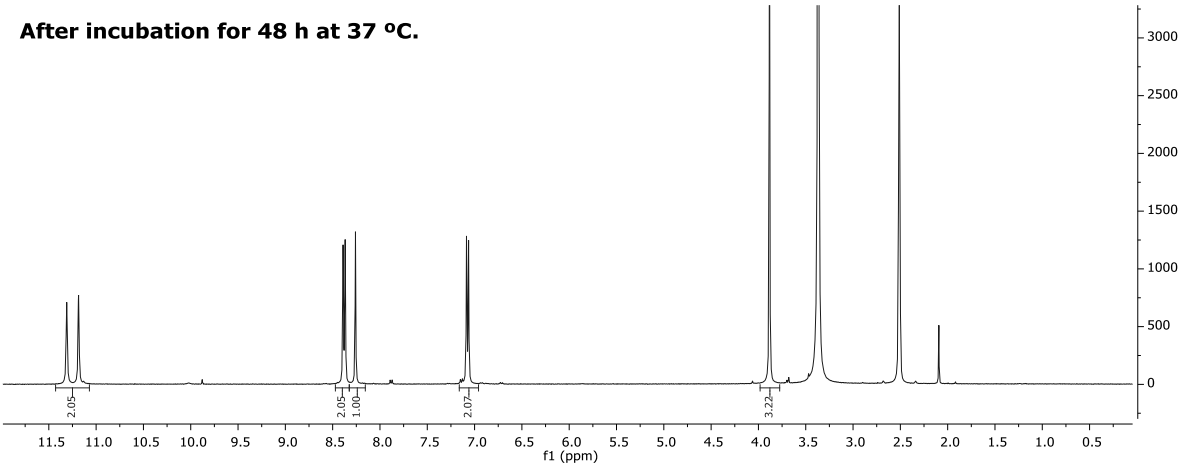
Appendix IX: ^{1}H NMR spectrum of 5-(4-methoxybenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione **(6h)** and the resulted spectrum after incubation for 24h at 37 ºC.



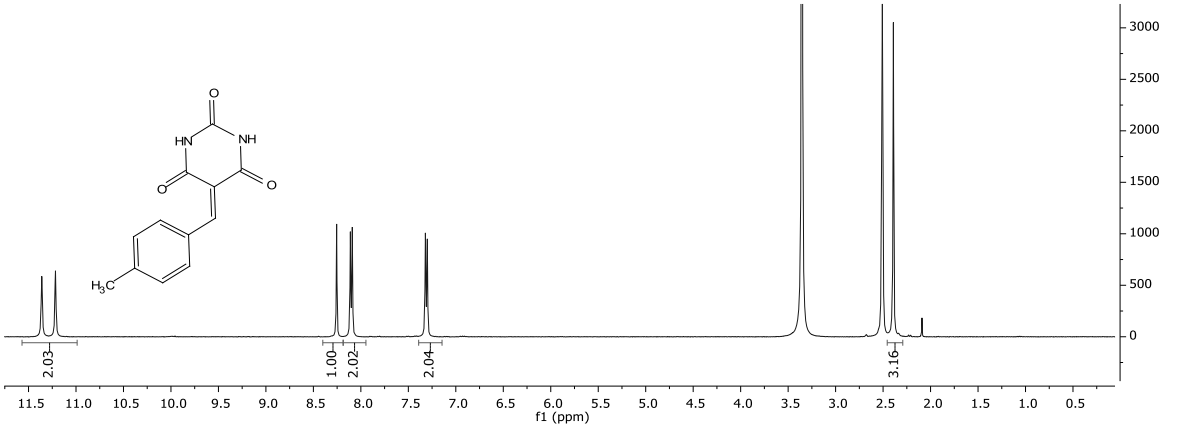
After incubation for 24 h at 37 ºC.

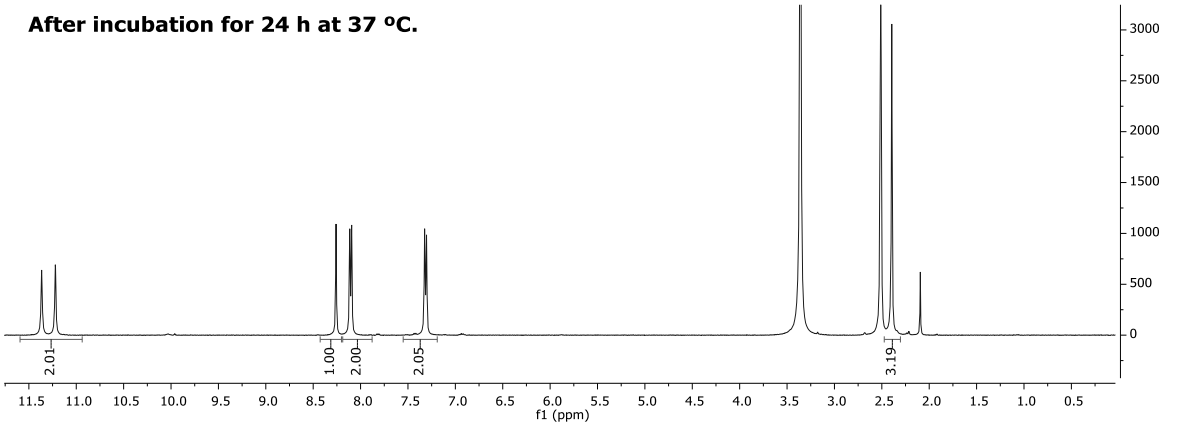


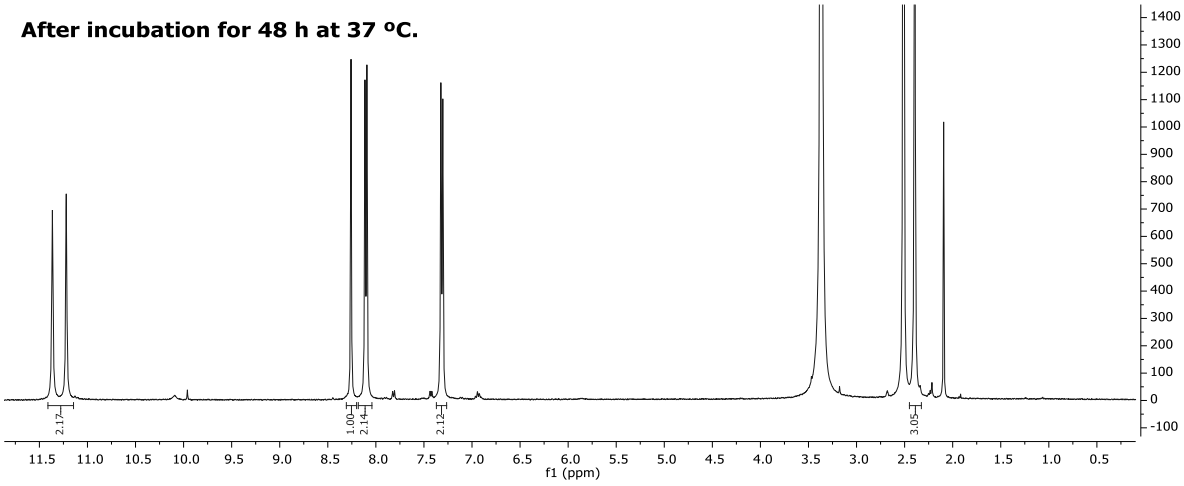
After incubation for 48 h at 37 ºC.



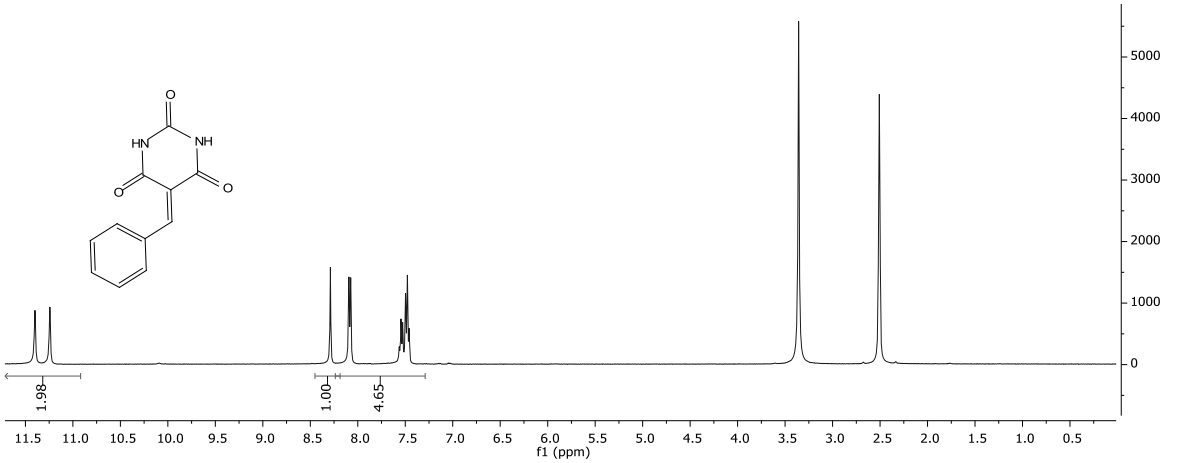
Appendix X: ^{1}H NMR spectrum of 5-(4-methylbenzylidene)pyrimidine-2,4,6(1H,3H,5H)-trione **(6i)** and the resulted spectrum after incubation for 24h at 37 ºC.



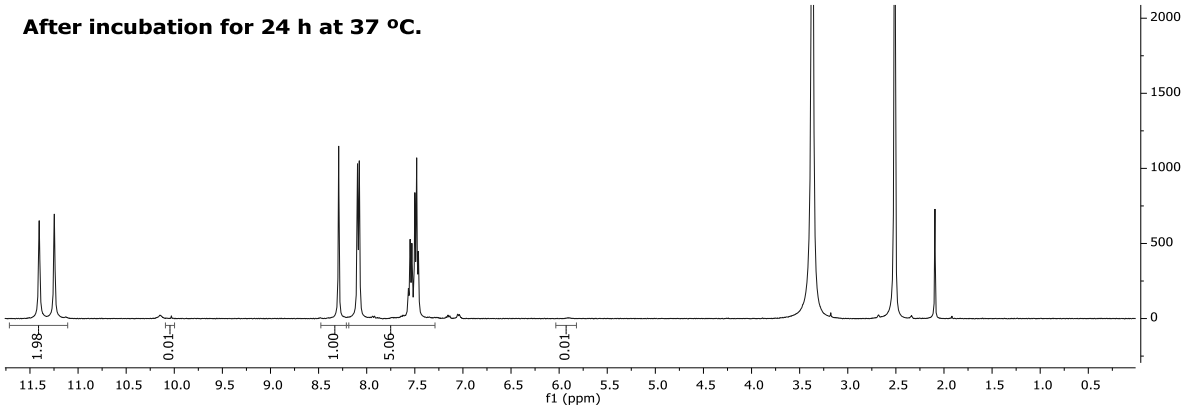




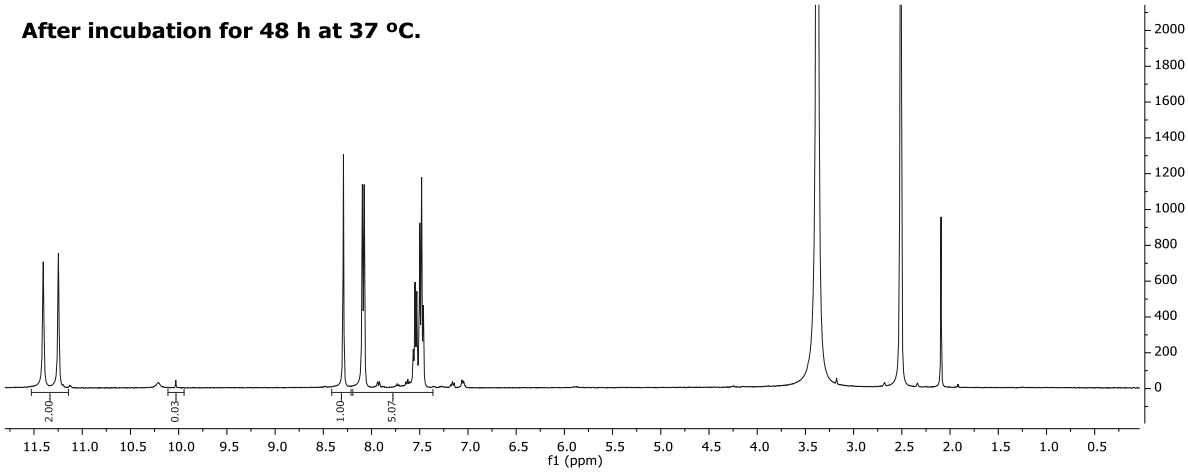
Appendix XII: ^{1}H NMR spectrum of 5-benzylidenepyrimidine-2,4,6(1H,3H,5H)-trione **(6j)** and the resulted spectrum after incubation for 24h at 37 ºC.



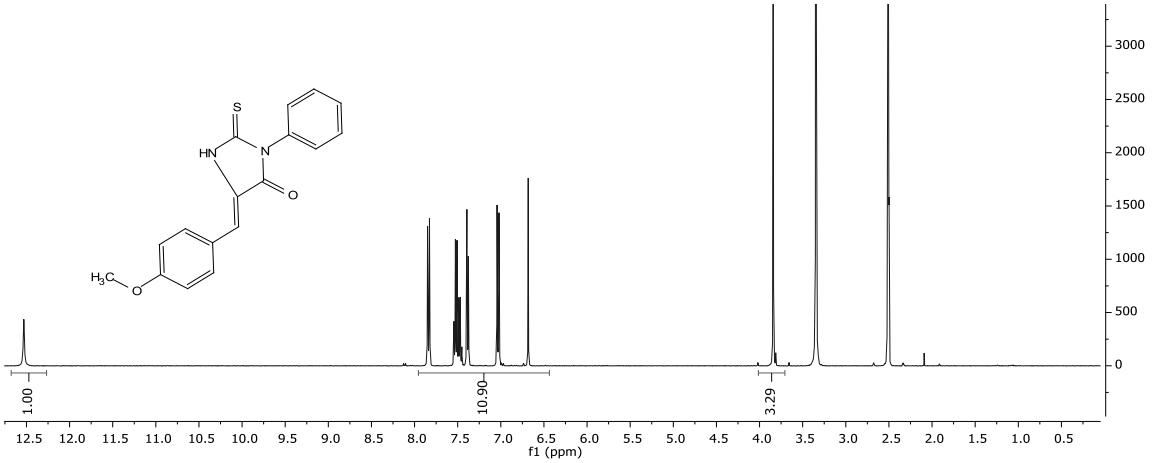
After incubation for 24 h at 37 ºC.



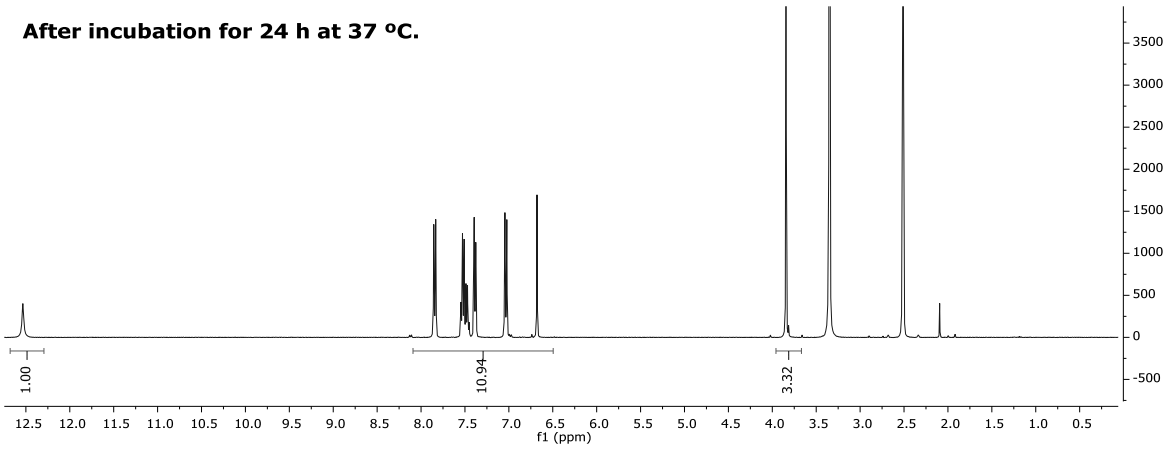
After incubation for 48 h at 37 ºC.



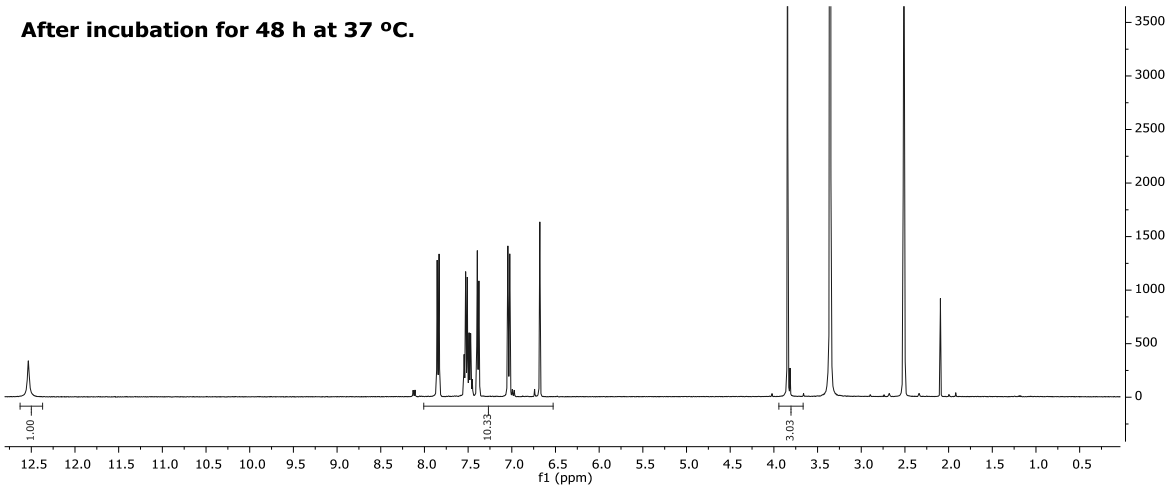
Appendix XIII: ^{1}H NMR spectrum of (5Z)-5-(4-Methoxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one **(2)** and the resulted spectrum after incubation for 24h at 37 ºC.



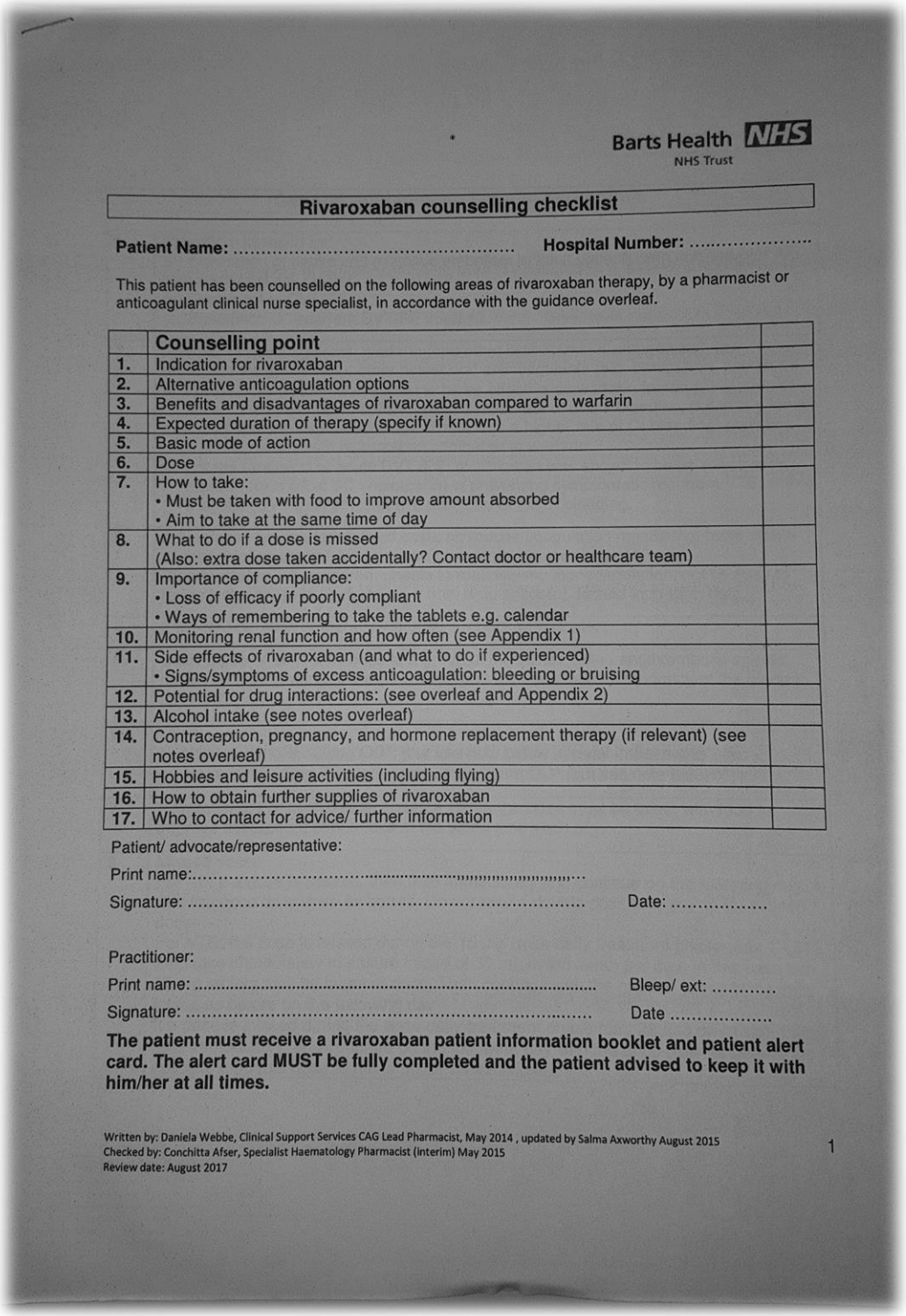
After incubation for 24 h at 37 ºC.



After incubation for 48 h at 37 ºC.



Appendix XIV: Rivaroxaban counselling checklist



Barts Health NHS
NHS Trust

Rivaroxaban counselling checklist

Patient Name: ..... **Hospital Number:** .....

This patient has been counselled on the following areas of rivaroxaban therapy, by a pharmacist or anticoagulant clinical nurse specialist, in accordance with the guidance overleaf.

	Counselling point	
1.	Indication for rivaroxaban	
2.	Alternative anticoagulation options	
3.	Benefits and disadvantages of rivaroxaban compared to warfarin	
4.	Expected duration of therapy (specify if known)	
5.	Basic mode of action	
6.	Dose	
7.	How to take: • Must be taken with food to improve amount absorbed • Aim to take at the same time of day	
8.	What to do if a dose is missed (Also: extra dose taken accidentally? Contact doctor or healthcare team)	
9.	Importance of compliance: • Loss of efficacy if poorly compliant • Ways of remembering to take the tablets e.g. calendar	
10.	Monitoring renal function and how often (see Appendix 1)	
11.	Side effects of rivaroxaban (and what to do if experienced) • Signs/symptoms of excess anticoagulation: bleeding or bruising	
12.	Potential for drug interactions: (see overleaf and Appendix 2)	
13.	Alcohol intake (see notes overleaf)	
14.	Contraception, pregnancy, and hormone replacement therapy (if relevant) (see notes overleaf)	
15.	Hobbies and leisure activities (including flying)	
16.	How to obtain further supplies of rivaroxaban	
17.	Who to contact for advice/ further information	

Patient/ advocate/representative:

Print name:.....

Signature: ..... Date: .....

Practitioner:

Print name: ..... Bleep/ ext: .....

Signature: ..... Date .....

The patient must receive a rivaroxaban patient information booklet and patient alert card. The alert card MUST be fully completed and the patient advised to keep it with him/her at all times.

Written by: Daniela Webbe, Clinical Support Services CAG Lead Pharmacist, May 2014 , updated by Salma Axworthy August 2015
Checked by: Conchitta Afser, Specialist Haematology Pharmacist (interim) May 2015
Review date: August 2017

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Appendix XV: Order sheet for in-patient requisites and TTA.

Barts Health NHS
NHS Trust
Pharmacy Inpatient Ward Sheet
Hospital Name:
Barts/Whipps/NUH/RLH/ME

Date:
Time in:
Circle if urgent Next porter delivery RWR Ext:
Time required
Indication:

Ward:
Nurse:

Log no:
Bag no:

PBR form completed (NUH only) ☐

Patient Name:
MRN:
DOB:
Speciality/Consultant

Medicine name	Form	Strength	Label only	✓ if no directions required	Labelling Directions Include dose & indication if PBR drug	Quantity	Dispensed	Disp. by	Check

Special requirements/notes:
Error code:
To-follows (T/F):
- Highlight T/F item
- T/F form filled in on reverse side
- Critical drugs not to be omitted or delayed

Labelled by:
Time dispensed:
Time checked:

Clinically verified by:
Ext/bleep:

Requested by: (print name)

Important
- **BOLD = compulsory fields**
- One ward sheet per patient/speciality/consultant
- Delays may occur if details are not accurate eg MRN, contact ext/bleep