T.C.
YEDİTEPE UNIVERSITY
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SYNTHESIS AND CYTOTOXICITY STUDIES ON SOME NOVEL 4,4'-DIFLUOROBENZHYDRYLPIPERAZINE DERIVATIVES

MASTER OF SCIENCE THESIS

EMAN ANWER BOBTAINA, B.Pharm.

İSTANBUL – 2017

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Institute	: Yeditepe University Institute of Health Sciences
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This study has been approved as a Master Thesis in regard to content and quality by the Jury.

Chair of the Jury:	Prof. Dr. Hülya AKGÜN Yeditepe University Faculty of Pharmacy Department of Pharmaceutical Chemistry	.....
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APPROVAL

This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated 27.01.2017 and numbered 2017/02-14

Prof. Dr. Bayram YILMAZ
Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

13/01/2017

EMAN BOBTAINA

Dedicated to my mother,

Fatheya Ali

ACKNOWLEDGEMENTS

After an intensive period of time, today is the day: writing this note of thanks is the finishing touch on my thesis. Preparation of this thesis had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

I would like first to thank Libyan High Education Institute and Benghazi University for giving me chance to study abroad and advance my degree. I wish to thank my dedicated advisor, Assist. Prof. Dr. Enise Ece GÜRDAL, who is sincerely giving and always there whenever I need her. For the most part, it is a great honor to be her master student.

I appreciate efforts of Prof. Dr. Mine YARIM YÜKSEL who provided me with the tools that I needed to successfully complete my thesis. I am grateful to Prof. Dr. Hülya AKGÜN, and Prof. Dr. Meriç KÖKSAL AKKOÇ for always being generously supportive with my postgraduate education, and leading me to success.

I am very thankful for the efforts of Prof. Dr. Hakan GÖKER and Assoc. Prof. Dr. Rengül ÇETİN ATALAY, since they conducted our spectral analysis and biological activity measurement respectively.

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Consequently, I want to thank my lovely friend Rosey MOHAMED for her endless support during my education. I appreciate my parents' advices and they never gave up on me. My brother deserves my gratitude as he was always there for me when I needed his device. I also would like to thank my sisters and my little niece Roya.

TABLE of CONTENTS

TABLES

FIGURES and SCHEMES

ABBREVIATIONS

5-FU	5-Flurouracil
5-HT	5-Hydroxytryptamine
AK	Adenylate kinase
ATP	Adenosine-5'- triphosphate
BACE1	Beta-secretase 1
CB_1	Cannabinoid 1 Receptor
CC	Column Chromatography
CML	Chronic Myeloid Leukemia
CPT	Camptothecin
DCM	Dichloromethane
DHFR	Dihydrofolate reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
FSH	Follicle Stimulating Hormones
G_0	Resting Phase
G_1	Gap 1 Phase
G_2	Gap 2 Phase
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GI_{50}	Growth inhibitory concentration

H_1	Histamine 1 Receptors
HCC	Hepatocellular Cancer
HepG-2	Hepatocellular Carcinoma Cell
HeLa	Cervical Cancer Cell
HI	Human Immunodeficiency Virus
HT-29	Colon Carcinoma Cell
HVC	Hepatitis Virus C
IC_{50}	The half maximal inhibitory concentration
IR	Infrared
LDH	Lactate dehydrogenase
LH	Luteinizing Hormones
LHRH	Luteinizing Hormone Releasing Hormone
M	Mitosis Phase
MCF-7	Breast Cancer Cell
MeOH	Methanol
MS	Mass Spectroscopy
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide
NMR	Nuclear Magnetic Resonance
PMS	Phenazine methosulphate
ppm	parts per million

R_f	Retention factor
RNA	Ribonucleic Acid
S	Synthesis Phase
SERM	Selective Estrogen Receptors Modulators
SN	Nucleophilic substitution
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
WHO	World Health Organization
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

ABSTRACT

Bobtaina, E. A. (2017). Synthesis and Cytotoxicity Studies on Some Novel 4,4'-Difluorobenzhydrylpiperazine Derivatives. Yeditepe University Institute of Health Sciences, Department of Pharmaceutical Chemistry, M.Sc. Thesis, Istanbul.

In this study, nine novel compounds, bearing *N*-[2-(4-substitutedpiperazine-1-yl)acetyl]-*N'*-[bis-(4-fluorophenyl)methyl]piperazine structures were synthesized. *In vitro* cytotoxic activities were screened in the comparison with camptothecin (positive control) and 5-fluorouracil (reference).

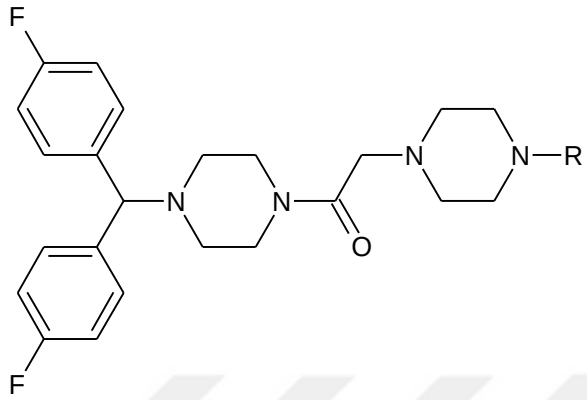
To obtain starting compound, 1-[4-bis(4-fluorophenyl)methyl]piperazine was *N*-acetylated with chloroacetyl chloride. After substitution of chlorine in *N*-(chloroacetyl)-*N'*-[bis(4-fluorophenyl)methyl]piperazine by various piperazine derivatives, the target compounds were gained.

Physical properties of the synthesized compounds were determined and their structures were confirmed by using spectral methods (UV, IR, ^{1}H-NMR, ^{13}C-NMR, Mass spectrometry) and elemental analyses. In addition, their cytotoxic properties were evaluated *in vitro* by NCI-60 Sulforhodamine B (SRB) Assay against human cancer cell lines, Huh7 (hepatocellular), MCF7 (breast) and HCT116 (colorectal).

According to the activity data, most of the compounds are more cytotoxic than 5-fluorouracil against hepatocellular (Huh7) and colorectal (HCT116) cancer cell lines. However, in breast (MCF-7) cancer cell line, all benzhydrylpiperazine derivatives are less cytotoxic than 5-fluorouracil (5-FU). The most active compound for Huh7 cell line is *m*-methoxyphenyl derivative (compound **5**; GI_{50} = 7.04 μM), and the most active compound in colorectal (HCT-116) cancer cell line is *p*-chlorophenyl derivative (compound **1**; GI_{50} = 4.36 μM).

Keywords: Anticancer, benzhydrylpiperazine, cytotoxicity, piperazine, sulforhodamine B

Table. Structures of the synthesized compounds **(1-9).**



Compound	R	M. P. (°C)	Yield (%)
1	*p*-Chlorophenyl	179	30
2	*o*-Hydroxyphenyl	>300(dec.)	35.5
3	*p*-Methylphenyl	194.3	18
4	*m*-Methylphenyl	>300(dec.)	24
5	*m*-Methoxyphenyl	>300(dec.)	40
6	Benzoyl	>300(dec.)	59
7	2,4-Dichlorobenzyl	>300(dec.)	26.2
8	2,6-Dichlorobenzyl	>300(dec.)	22
9	2,4,6-Trimethylbenzyl	120.6	16

ÖZET

Bobtaina, E. A. (2017). Bazı Yeni 4,4'-Diflorobenzhidril Türevleri Üzerine Sentez ve Sitotoksisite Çalışmaları. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Farmasötik Kimya Yüksek Lisans Programı Tezi, İstanbul.

Bu çalışmada, *N*-[2-(4-sübstitütepiperazin-1-il)asetil]-*N'*-[bis-(4-florofenil)metil]-piperazin yapısı taşıyan dokuz yeni bileşik sentezlenmiştir. Bileşiklerin *in vitro* sitotoksik aktivitelerine kamptotesin (pozitif kontrol) ve 5-florourasil (referans) ile karşılaştırmalı olarak bakılmıştır.

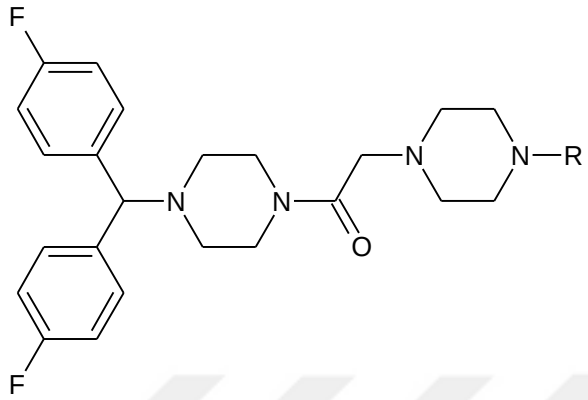
Başlangıç maddesinin eldesi için, 1-[4-Bis(4-florofenil)metil]piperazine kloroasetil klorür ile *N*-asetilasyon yapılmıştır. *N*-(kloroasetil)-*N'*-[bis(4-florofenil)metil]piperazinde bulunan klorun çeşitli piperazin türevleriyle yer değiştirmesi sonucu hedef bileşikler kazanılmıştır.

Sentezlenen bileşiklerin fiziksel özellikleri belirlenmiş, yapıları spektral yöntemler (UV, IR, ^{1}H-NMR, ^{13}C-NMR, kütle spektrometri) ve elementel analizle doğrulanmıştır. Ek olarak, sitotoksik özellikleri *in vitro* NCI-60 Sulforodamin B (SRB) Testi ile Huh7 (hepatoselüler), MCF7 (meme) ve HCT116 (kolorektal) insan kanser hücre hatlarına karşı değerlendirilmiştir.

Aktivite verisine göre, bileşiklerin çoğu hepatoselüler (Huh7) ve kolorektal (HCT116) kanser hücre hatlarına karşı 5-florourasilden daha sitotoksiktir. Fakat, meme (MCF7) kanser hücre hattında tüm benzhidrilpiperazinler 5-florourasilden daha düşük sitotoksisiteye sahiptir. Huh7 hücre hattına karşı en aktif bileşik *m*-metoksifenil türevi (bileşik **5**; GI_{50} = 7.04 μM), kolorektal (HCT116) hücre hattına karşı en aktif bileşik *p*-klorofenil türevi (bileşik **1**; GI_{50} = 4.36 μM) olmuştur.

Anahtar Kelimeler: Antikanser, benzhidrilpiperazin, sitotoksisite, piperazin, sulforodamin B

Tablo. Sentezlenen bileşiklerin (**1-9**) yapıları.



Bileşik	R	E. D. (°C)	Verim (%)
1	*p*-Klorofenil	179	30
2	*o*-Hidroksifenil	>300(dec.)	35.5
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5	*m*-Metoksifenil	>300(dec.)	40
6	Benzoil	>300(dec.)	59
7	2,4-Diklorobenzil	>300(dec.)	26.2
8	2,6-Diklorobenzil	>300(dec.)	22
9	2,4,6-Trimetilbenzil	120.6	16

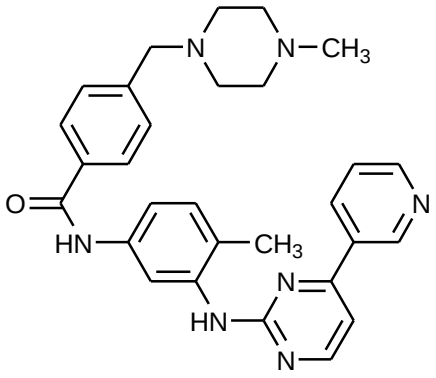
1. INTRODUCTION and PURPOSE

Cancer occurs when alterations in normal cells of the body lead to abnormal growth causing a tumor. Tumor develops and spreads into the normal tissue by blood vessels and lymphatic circulation [1]. World Health Organization (WHO) showed that 4% of all cancers are genetic and the majority of tumors cells are substantially related to lifestyle, diet, and environment, a weak or nonfunctioning immune system, poor health, unhealthy environment and elderly age [2]. There are several types in cancer diffusion, but the worst cases worldwide are lung cancer, for which an estimated 158.080 deaths are expected to occur in 2016. Breast cancer is one of frequently diagnosed cancer and the most common type that leads to death in women, hopefully rate is decreased by 36% from 1989 to 2012 due to early diagnosis and treatment options. Liver cancer death rates have been tripled since 1980. From 2008 to 2012, the rate was increased by 3.5% per year among those above 50 years old but decreased by 3.9% per year among those below 50 years old. Colorectal cancer is the third commonly diagnosed cancer in both men and women, however with early detection and treatment, mortality lessened by 2.8% from 2003 to 2012 [3].

Some reproducing tumor cells and normal cells can take up cytotoxic agents more than resting cells (G_o). Most effected cells belong to hair follicles, bone marrow and digestive tract. Therefore, cancer chemotherapy causes serious problems and side effects like hair loss, immune system suppression together with nausea or diarrhea [4].

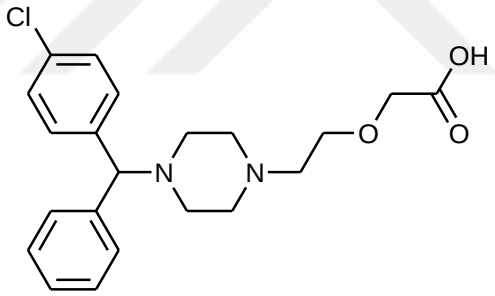
The aim of chemotherapeutic agents is to inhibit the cell cycle process during cell division, because cancer cells can be replicated faster than normal cells. The mechanism of action of these agents is nonspecific against cancer and normal cells. Additionally they are linked with high toxicity. Evaluation of the principles of cancer biology and cellular signaling pathways is aimed to understand the mechanisms of anticancer agents [5].

Piperazine is a common heterocycle for lead compounds. Piperazine based research describes its features in both chemical and pharmacological aspects. Antineoplastic activity of compounds that have piperazine core as an important backbone is studied thoroughly [6-8]. Imatinib, a breakthrough discovery for the treatment of chronic myeloid leukemia (CML), is an inhibitor of BCR-ABL tyrosine kinase [9].



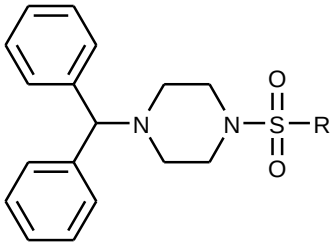
Imatinib

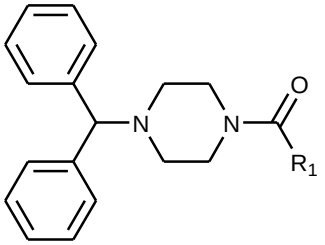
Several benzhydrylpiperazine derivatives are informed to have good antihistaminic activities [10-13], with many drugs in the market, such as cetirizine. Benzhydrylpiperazine derivatives also show many bioactivities such as antimicrobial, anticancer, and cardio protective activity [13].



Cetirizine

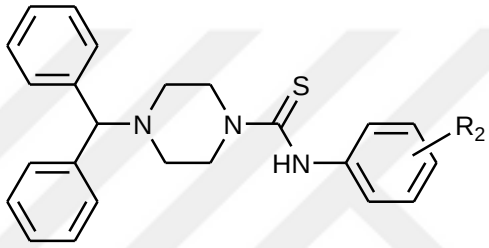
Benzhydrylpiperazine has become crucial motif for improving antiproliferative activity of several compounds [14-19]. Kumar *et al*. showed growth inhibition of several 1-benzhydrylpiperazine derivatives reacted with acid chlorides, sulfonyl chlorides and isothiocyanates. These novel compounds have interesting growth inhibitory effects against four human cancer cell lines breast cancer (MCF-7), liver cancer (HepG-2), colon cancer (HT-29) and cervix cancer (HeLa) cell lines [14].





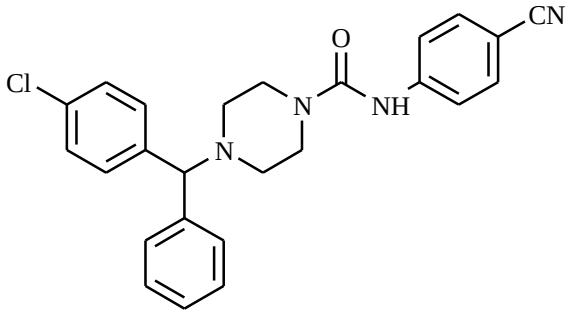
R= isoxazol-5-yl; morpholine-4-yl phenyl; pyrrolidinyl; cyclopropyl

R_1= (4-Cl,2-F)phenyl; Camphoryl

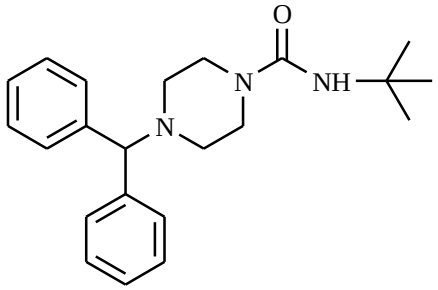


R_2 = 2-OCH_3, 3-OCH_3, 4-OCH_3; 2-Cl, 3-Cl, 4-Cl; 4-F; 2,4-diCl

In addition, Gurdal *et al*., synthesized several benzhydrylpiperazine derivatives that possess potential cytotoxic activity against liver, breast, and colon cancer cell lines. Structures are given below for the most active compounds. Among all derivatives, compounds **1**, **2** and **3** given below were found to be most promising structures [18,19].



1



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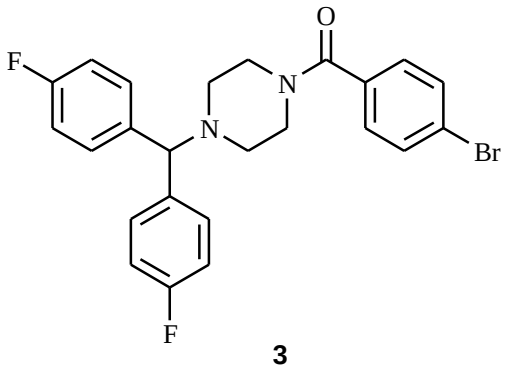
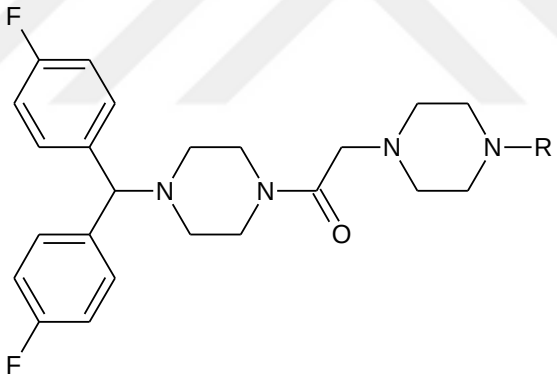


3

This study aims to synthesize and purify novel compounds bearing 4,4'-difluorobenzhydrylpiperazine core as potent cytotoxic agents. We targeted to build structure activity relationships for 4,4'-difluorobenzhydrylpiperazine derivatives based on their cytotoxicity profiles.

Table 1.1. Structures of the synthesized compounds

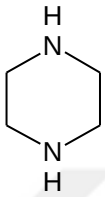


Compound	R	M. P. (°C)	Yield (%)
1	*p*-Chlorophenyl	179	30
2	*o*-Hydroxyphenyl	>300(dec.)	36
3	*p*-Methylphenyl	194.3	18
4	*m*-Methylphenyl	>300(dec.)	24
5	*m*-Methoxyphenyl	>300(dec.)	40
6	Benzoyl	>300(dec.)	59
7	2,4-Dichlorobenzyl	>300(dec.)	26
8	2,6-Dichlorobenzyl	>300(dec.)	22
9	2,4,6-Trimethylbenzyl	120.6	16

2. LITERATURE REVIEW

2.1. Piperazine

Piperazine is a heterocyclic compound containing four carbons and two nitrogens at 1st and 4th position (also called as 1,4-hexahydropyrazine) [20].

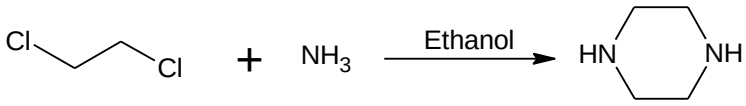


Piperazine

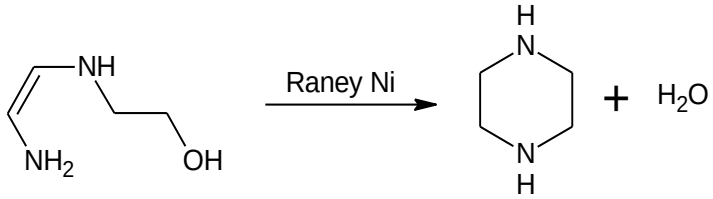
Piperazine is classified as a secondary amine and due to the inductive effect of the second heteroatom, piperazine is a weaker base with pKa 9.8 [21].

2.1.1. Methods of Synthesis

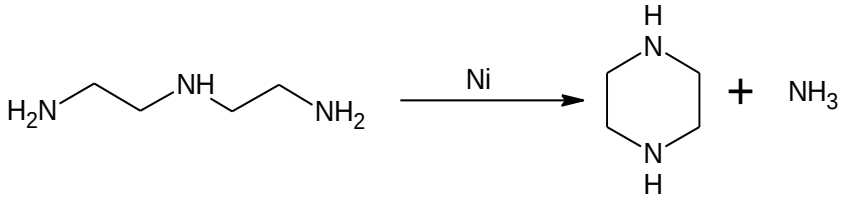
The first synthesis method of Piperazine was reaction of alcoholic ammonia and ethylene chloride [22].



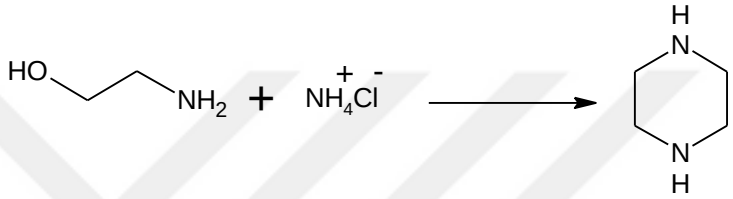
Various catalysts such as Raney nickel is used for synthesis of piperazine by cyclodehydration of *N*-(2-hydroxyethyl)ethenediamine [23].



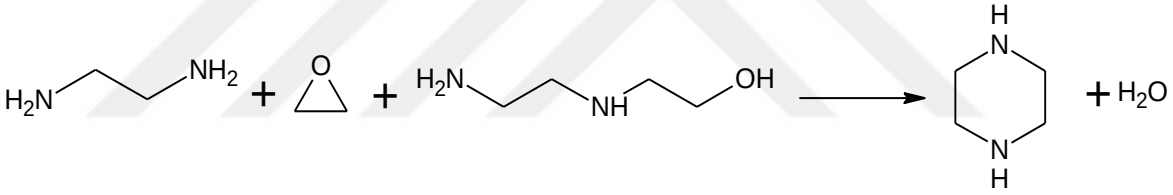
Diethylenetriamine is heated with Raney nickel in autoclave to improve the yields [24]. In a collateral research by Kyrides *et al*. piperazine was gained in good amount when nickel catalyst was reacted in 236 °C with 73 atm in an autoclave [25].



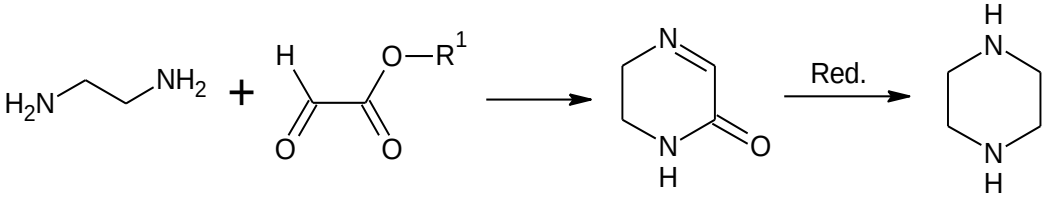
The heating of ethanolamine and ammonium chloride at 250°C leads to formation of piperazine [26].



Piperazine was synthesized in presence of ethylenediamine and oxirane [21].



Piperazine is synthesized from ethylenediamine and glyoxylate esters to produce 3,4-dehydropiperazine-2-one, then reduction with agents such as lithium aluminum hydride ($LiAlH_4$), sodium borohydride ($NaBH_4$), aluminum hydride (AlH_3), potassium borohydride (KBH_4) or diborane (B_2H_6) [27].



2.1.2. Spectral Properties of Piperazine

2.1.2.1. UV Spectroscopy

Piperazine shows absorption in the UV region at 260 nm (A=0.035) and 280 nm (A=0.010) [28].

2.1.2.2. ^{1}H-NMR Spectroscopy

Piperazine CH protons appear at 2.84 ppm in deuterated chloroform ($CDCl_3$) [21].

2.1.2.3. ^{13}C-NMR Spectroscopy

Carbons of piperazine are seen at 47.9 ppm in deuterated chloroform ($CDCl_3$) [21].

2.1.2.4. IR Spectroscopy

N-H stretching vibrations of piperazine give sharp singlet at 3250 cm^{-1}. C-H stretching bands appear at 2950-2700 cm^{-1} [28].

2.1.2.5. Mass Spectroscopy

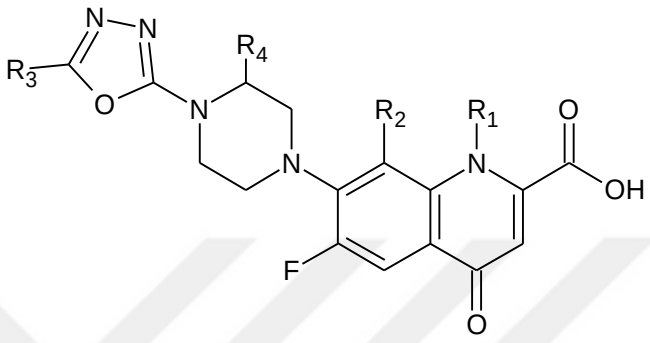
The m/z values of Piperazine. $6H_2O$ in mass spectrum are 86 (M^+), 56, 44 and 30. Base peak corresponds to the fragment $NHCH_2CH_2]^+$ (m/z = 44) [29].

2.1.3. Biological Properties of Piperazine

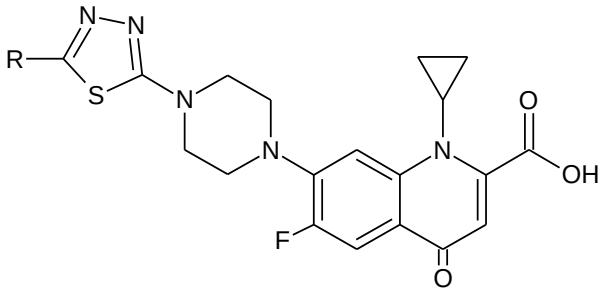
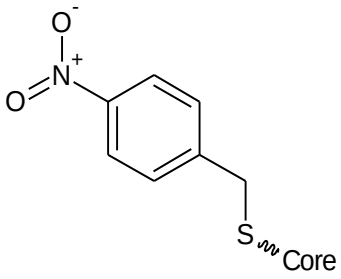
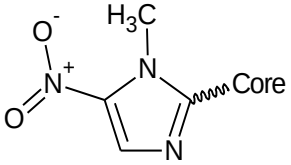
Piperazine is a highly significant heterocycle in drug design studies. Several candidates that are in clinical use having piperazine moiety possess broad biological activities are counted as anticancer (*i.e.* imatinib), analgesic and antiinflamatory (*i.e.* antrafenine, psychoactive (*i.e.* fipexide), antierectile dysfunction (*i.e*. sildenafil, vardenafil), serotonin receptor agonist (*i.e.* quipazine), antianginal *(i.e*. trimetazidine, ranolazine) [13, 30], anxiolytic and antidepressant *(i.e*. amoxapine, buspirone, gepirone [13, 31, 32]), antipsychotic *(i.e.*fluphenazine, perphenazine, clozapine, prochlorperazine, perazine [13, 33, 34]), antihistaminic *(i.e* cinnarizine, cyclizine [13, 35-37]), antibacterial (*i,e*, norfloxacin, ciprofloxacin, levofloxacin) [38-42], antifungal *(i.e*. itraconazole, posaconazole [43]).

2.1.3.1. Antibacterial Activity

Some piperazinyl fluoroquinolone derivatives have been synthesized and found to have good antibacterial activity [13, 44, 45].

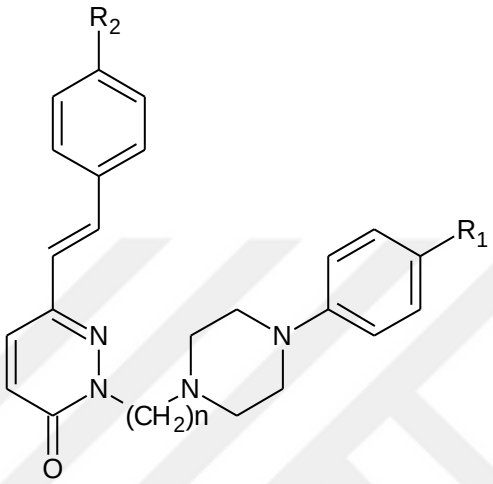


R_1= Cyclopropyl, ethyl; R_2= H, -OCH_3; R_3= Aryl; R_4= -H, -CH_3

Fluoroquinolone core	R
	
	

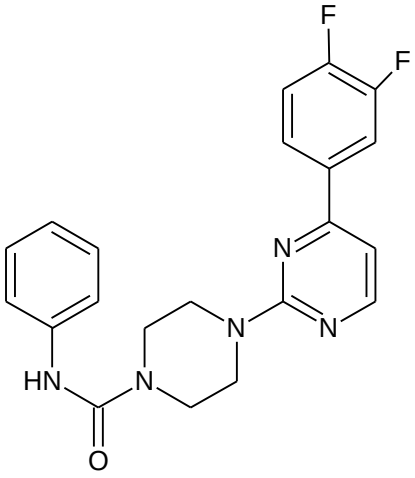
2.1.3.2. Analgesic and Anti-inflammatory Activity

Some alkylpiperazine derivatives have been published for their potent analgesic activity higher than the reference compound indomethacin [13].



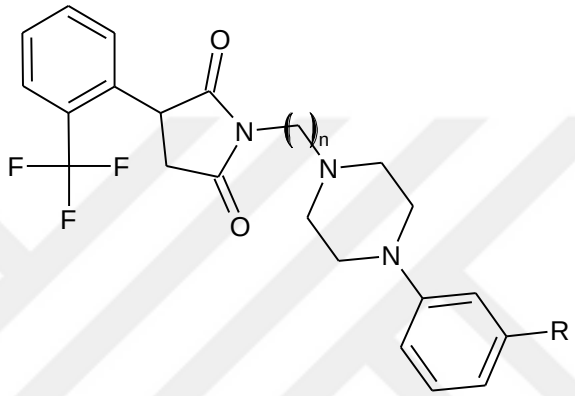
R_1= -Cl, OCH_3; R_2 = -Cl, $-CH_3$; n=1-3

Kono *et al*. established and evaluated a novel serious of piperazine derivatives for their analgesic activity [13].



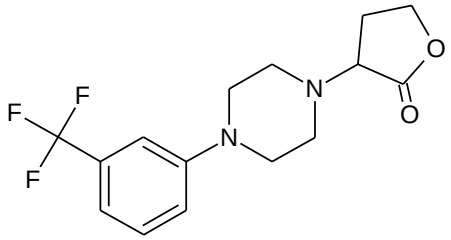
2.1.3.3. Anticonvulsant Activity

Obnisk *et al.* synthesized compounds bearing piperazine moiety and screened for their anticonvulsant activity by using maximum electric shock (MET). Compounds **a** and **b** were mentioned as the highest activity ones with ED_{50} of 20.78 and 132.13 mg/kg respectively [13].



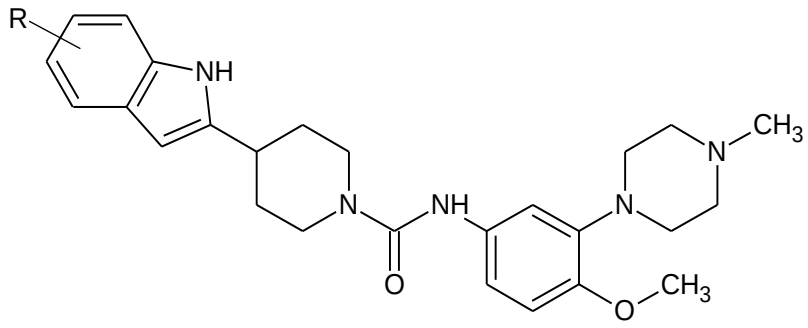
Compound **a**. R= -H, n=1 // Compound **b**. R= $-CF_3$, n=3

Salat *et al.* also mentioned 2-furanone bearing piperazine derivative, as a potent anticonvulsant agent with ED_{50} of 112 mg/kg in the MES model [13].



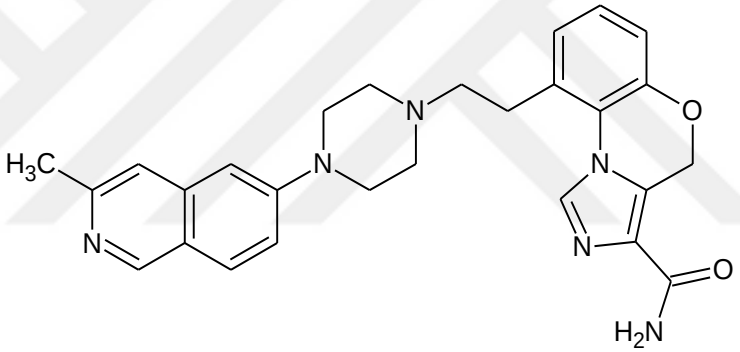
2.1.3.4 Antidepressant Activity

Many antipsychotic agents having piperazine ring are studied by Matzen *et al*. and screened as antidepressant agent. They show significant serotonin reuptake inhibition [13].



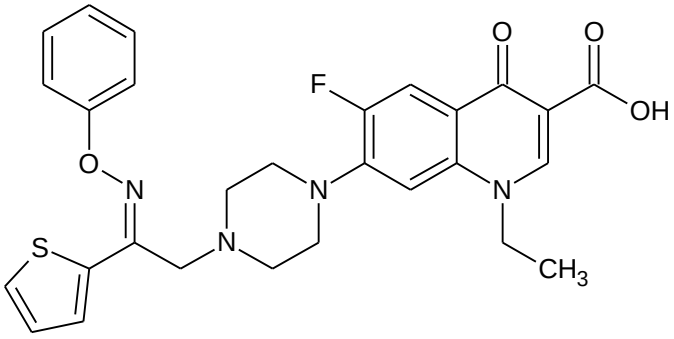
R= 4-F, 5-F, 6-F

New piperazine derivatives bearing 4-xanthone have been published as potent 5-$HT_{1A\B\D}$ receptors antagonist [13].

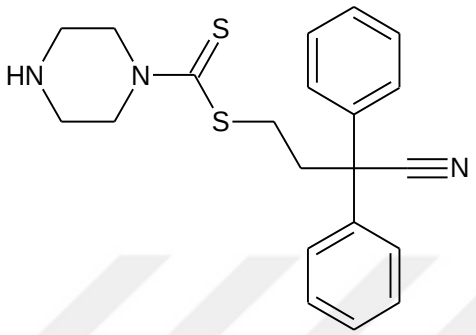


2.1.3.5. Anticancer Activity

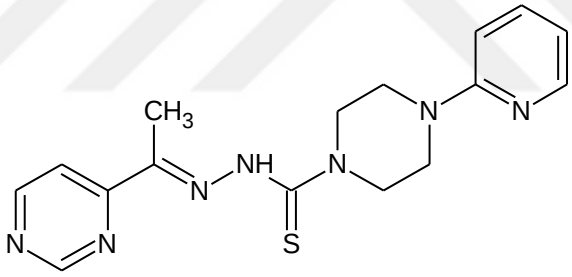
Cytotoxicity potential of a quinolone derivative was determined using human oral epithelial mouth cancer (KB) cell lines to show an IC_{50} value of 4.91 ± 1.94 µg/mL and human squamous cancer (A431) cell lines to have an IC_{50} value of 3.11 ± 0.52 µg/ml [13].



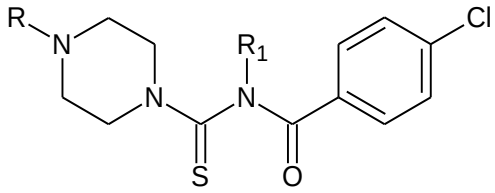
Hou *et al.* reported 4-substituted-piperazine-1-carbodithioic acid esters having anticancer activity with IC_{50} values of 5.3 and 11.5 μM against human promyelocytic leukemia cell lines (HL-60) and hepatoma cell lines (Bel-7402) respectively [13].



A thiosemicarbazone derivative has been reported to have cytotoxic activity against gastric cancer (SGC-7901) cell line with IC_{50} value of 0.032 μM [13].

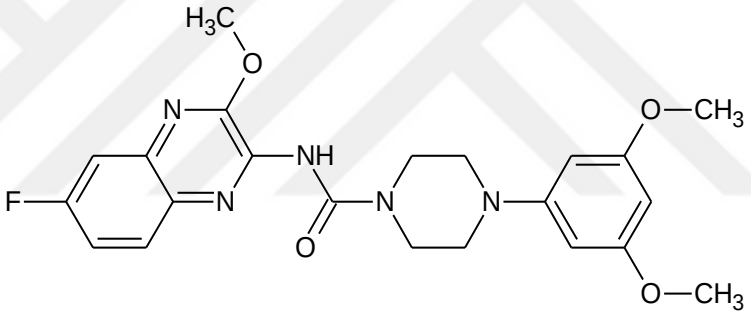


Compounds modified by Ranise *et al.* were found to have cytotoxic activity at low concentrations versus renal cancer cell lines (ACHN) and lung cancer cell lines (NCl-H226) with Gl_{50} of 0.13 μM for both [46].

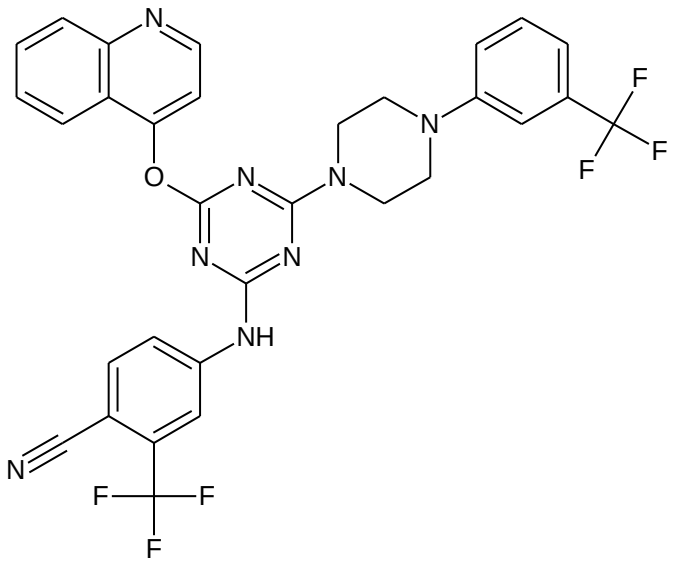


R= CH_3, C_6H_5; R_1= CH_3, C_6H_5

To evaluate cytotoxicity the Sulforhodamine B (SRB) test was used to a quinoxalinyl-piperazine derivative and the compound has potential anticancer activities against breast cancer (MDA-MB-231), renal cancer (Caki, UMRC2) cell lines, pancreas cancer cell line (PANC-1), adenocarcinomic human alveolar basal epithelial cell line (A549), gastric cancer cell line (MKN-45), hepatoma cell line (HepG2), colorectal carcinoma cell lines (HCT-115, HCT-116, HT29), prostate cancer cell line (PC-3), glioma stem cell (U251), cervical carcinoma cells (HeLa), human melanoma (Sk-MEL-28), and ovarian cancer cell lines (OVCAR-3). Lee *et al*. reported that compound inhibited cell cycle in G_2\M phase and induced the apoptosis by down regulation of Bcl-2 protein level also inhibited growth of previously mentioned cancer cell lines. Furthermore, Lee *et al*. reported the compound has potential use for combination therapy with known cancer drugs [47].



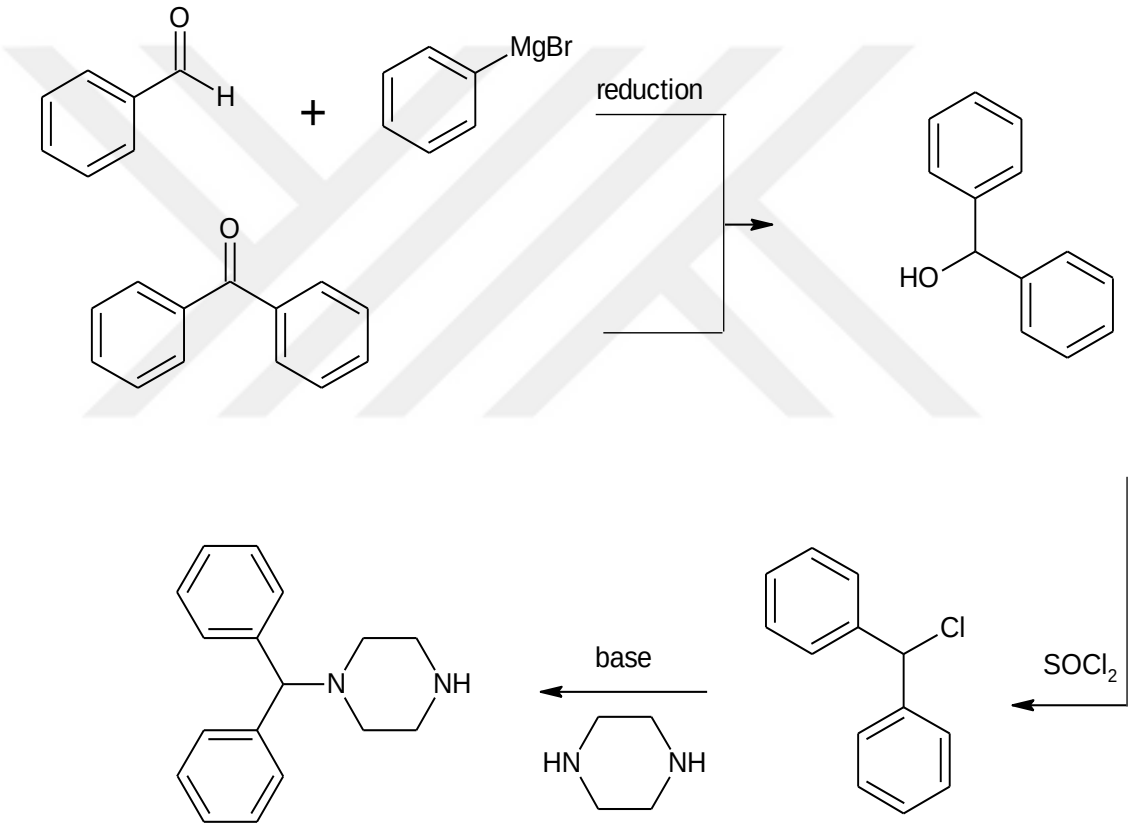
Patel *et al.* prepared a piperazinyltriazine derivative against prostate cancer cell lines (DU-145), with the GI_{50} value of 24.6 µg/mL at concentration level 47.1 µg/mL[48].



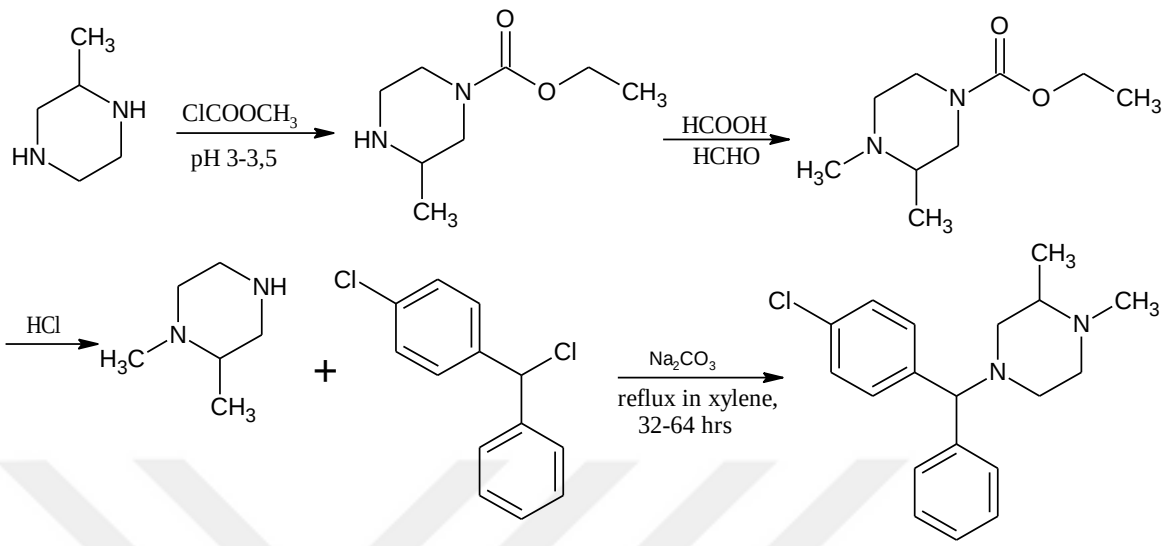
2.2. Benzhydrylpiperazine Derivatives

2.2.1. Methods of Synthesis

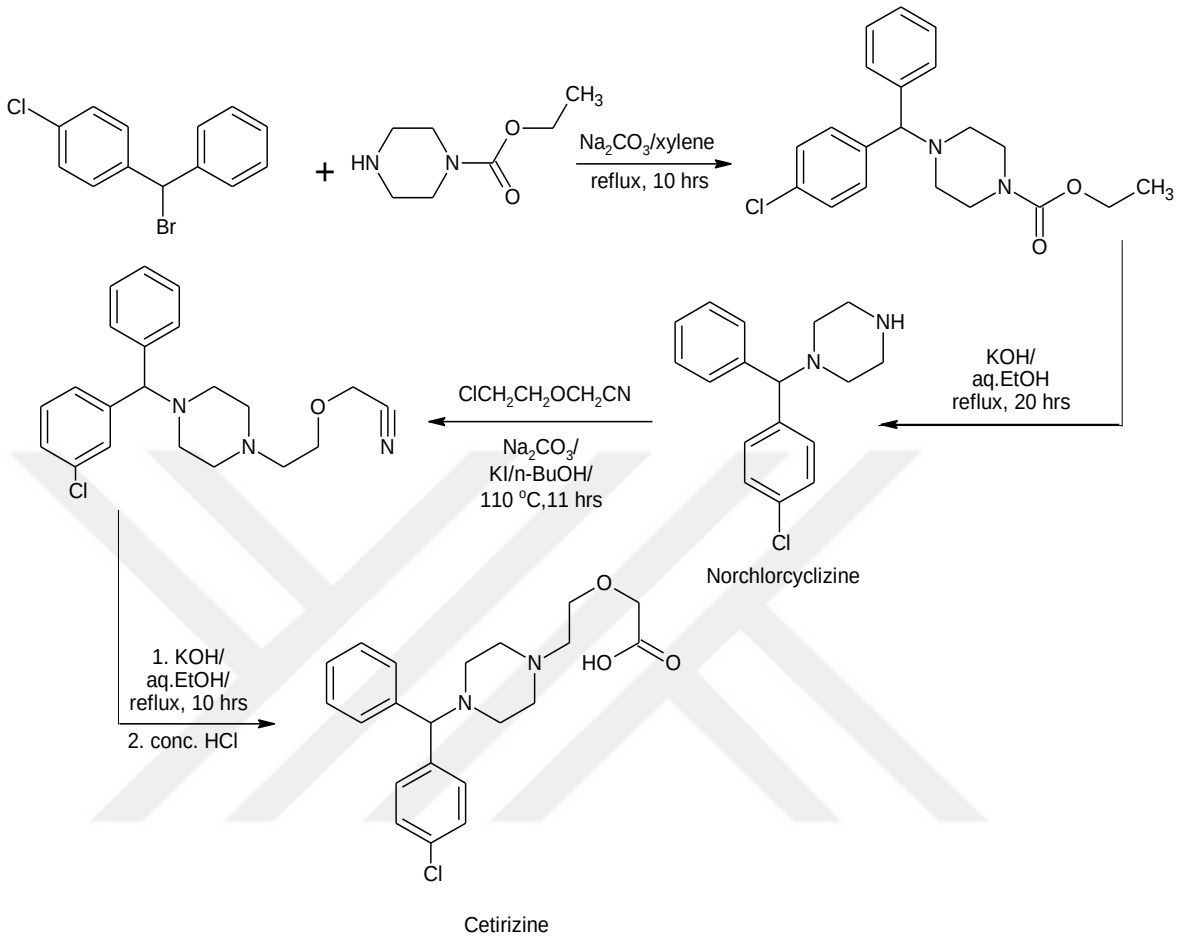
Benzhydrylpiperazine derivatives are commonly synthesized with Grignard reagents from benzaldehydes or through reduction of benzophenones. Afterwards, halogenation of the alcohol is provided with thionyl chloride. Piperazine is then condensed with benzhydrylchlorides in hot and alkali medium [49-51].



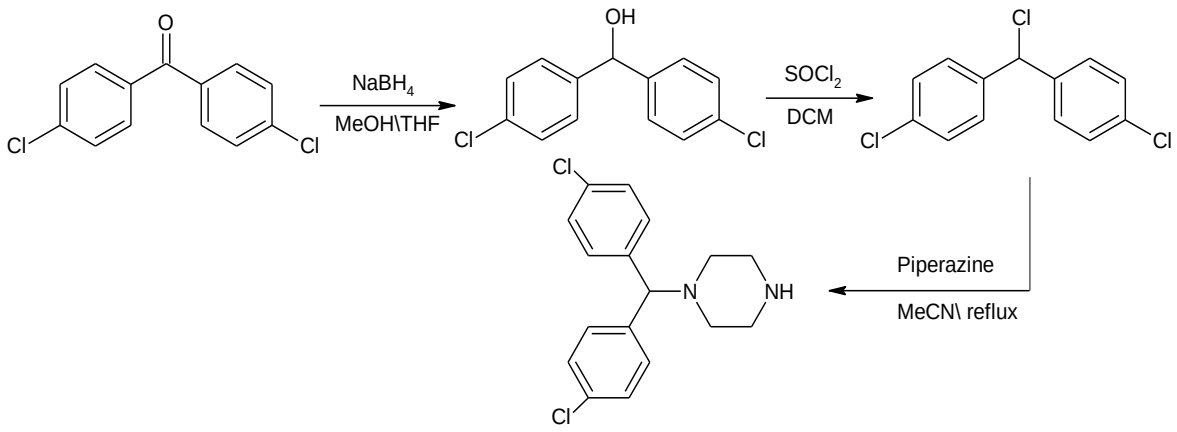
Chlorcyclizine derivatives were prepared by *Eschweiler-Clarke* methylation following carbethoxy protection on piperazine ring [52].



For the synthesis of antihistaminic drug cetirizine alkylation of the ethyl 1-piperazinecarboxylate with 4-chlorobenzhydrylbromide and hydrolysis of piperazine intermediate later yields cetirizine. Reaction of norchlorcyclizine with 2-(2-chloroethoxy)acetonitrile, sodium carbonate, potassium iodide and *n*-butanol at 110ºC for 11 hours produced nitrile bearing derivative which was hydrolyzed to obtain cetirizine [53].



Song and coworkers reported the synthesis of benzhydrole intermediate by reducing benzophenone with sodium borohydrate in valuable yield. Afterwards, halogenation with thionyl chloride to collect benzhydryl chloride and condensation of piperazine in methylnitrite solution were performed to collect 1-benzhydrylpiperazine [54].



2.2.2. Spectral Properties of Benzhydrylpiperazine Derivatives

2.2.2.1. ^{1}H-NMR Spectroscopy

Benzhydrylpiperazine N-H proton gives broad singlet at 2.2 ppm [55]. Piperazine CH protons of 1-[bis(4-fluorophenyl)methyl]piperazine derivatives gives two triplets at 2.19 - 2.29 ppm and 3.11- 3.48 ppm in order. Diphenylmethyl C-H gives a singlet at 4.25 - 4.59 ppm. Phenyl protons are observed as multiplet at 7.15-7.46 ppm [18].

2.2.2.2. ^{13}C-NMR Spectroscopy

Piperazine carbons of 1-benzhydrylpiperazine derivatives are seen at 50 - 54 ppm, diphenylmethyl carbon is observed at 75-85 ppm. Similarly, 1-[bis(4-fluorophenyl)methyl]piperazine derivatives give signal at 52.5 ppm and 54 ppm for piperazine and a peak at 84.5 ppm due to diphenylmethyl carbon [56].

2.2.2.3. IR Spectroscopy

N-H stretching vibrations of 1-[(4-fluorophenyl)(phenyl)methyl]piperazine appear at 3258-3359 cm^{-1}. Furthermore, C=O stretching vibrations of benzhydrylpiperazine carboxamide appear at 1615-1640 cm^{-1}. Other stretching signals are as following; C-H aromatic at 3027-3076 cm^{-1}, C-H aliphatic at 2965-2976 cm^{-1}, C=C at 1540-1626 cm^{-1}, C-N at 1247-1259 cm^{-1} [18].

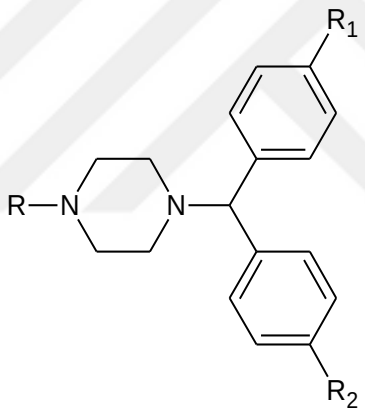
2.2.2.4. Mass Spectroscopy

1-[Bis(fluorophenyl)methyl]piperazine molecular ion has (m/z) (M^+); 290.0 ($(4\text{-F-}C_6H_5)_2CH[N(C_2H_4)_2N]H]^+$; 203.55 (100%, $(4\text{-F-}C_6H_5)_2CH]^+$) [18].

2.2.3. Biological Properties of Benzhydrylpiperazine Derivatives

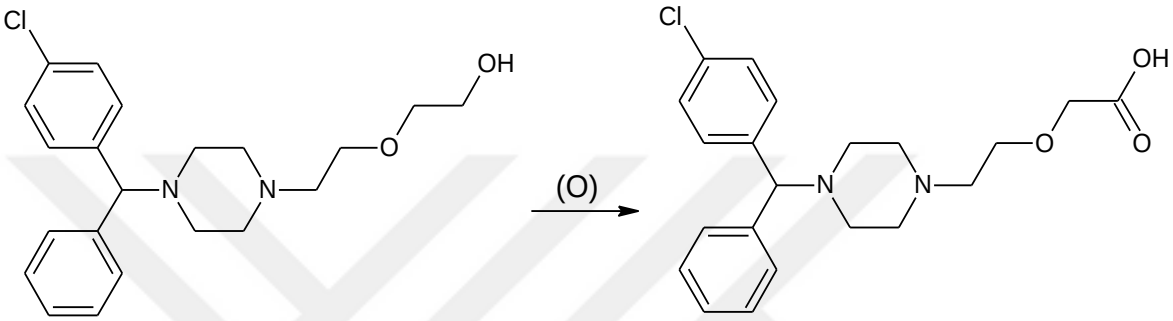
Most of the benzhydrylpiperazines in clinic are in class of first and second generation of antihistaminic drugs. In addition to their antiallergic actions, first generation drugs are also effective against motion sickness. Their sedative side effect is highly overcome by second generation antihistaminics.

Table 2.1. Therapeutically used antihistaminics bearing benzhydrylpiperazine structure



Compound	R_1	R_2	R
Cyclizine	-H	-H	Methyl
Chlorcyclizine	-H	-Cl	Methyl
Meclizine	-H	-Cl	3-Methylbenzyl
Buclizine	-H	-Cl	4-(Tert-butyl)benzyl
Cinnarazine	-H	-H	1-(Phenyl)propen-3-yl
Hydroxyzine	-H	-Cl	2-(2-Hydroxyethoxy)ethyl
Cetirizine	-H	-Cl	2-(Carboxymethoxy)ethyl
Oxatomide	-H	-H	3-(1,3-Dihydro-2H-benzimidazol-2-on-1-yl)propyl
Flunarizine	-F	-F	1-(Phenyl)propen-3-yl

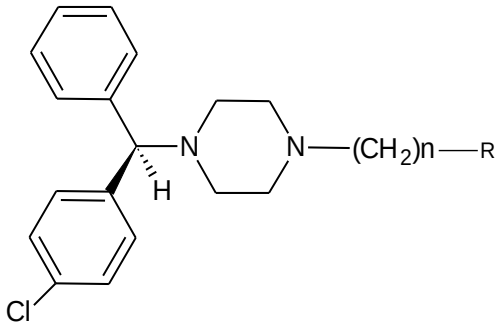
The polarity of second generation antihistaminic drugs make them less able to pass through the blood brain barrier therefore their side effects related to central nervous system are decreased. Bioactive metabolite of hydroxyzine is cetirizine that is polar and amphoteric, *i.e* able to create a zwitterion with a tertiary amine and carboxylic acid functional group. As a result, the drug's sedative effects are decreased [57].



Oxidative biotransformation of hydroxyzine to cetirizine.

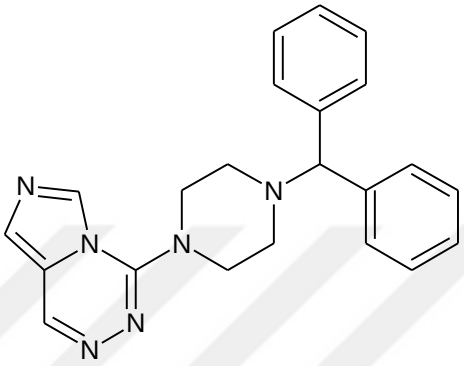
2.2.3.1 Antihistaminic Activity

Piperazine H_1 blocker, with higher affinity to H_1 receptors than the histamine, are usually useful for treatment of allergies. Through these drugs, levocetirizine is third generation antihistaminic drug, regularly recognized for its high efficiency and less side effects. Levocetirizine derivatives are broadly studied as potent histamine receptors antagonist [58].



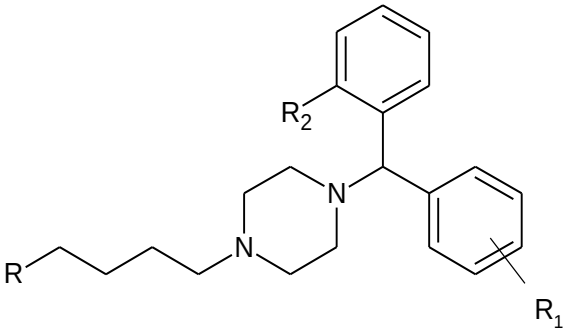
n= 2, 3; R=Sulfonamide

Paul *et al*. published a benzhydrylpiperazine derivative with good antiasthma activity. Those compounds have potent basophil activity when tested for in vitro activity in the mouse passive cutaneous anaphylaxis (PCA) and the guinea pig passive anaphylaxis test [59].



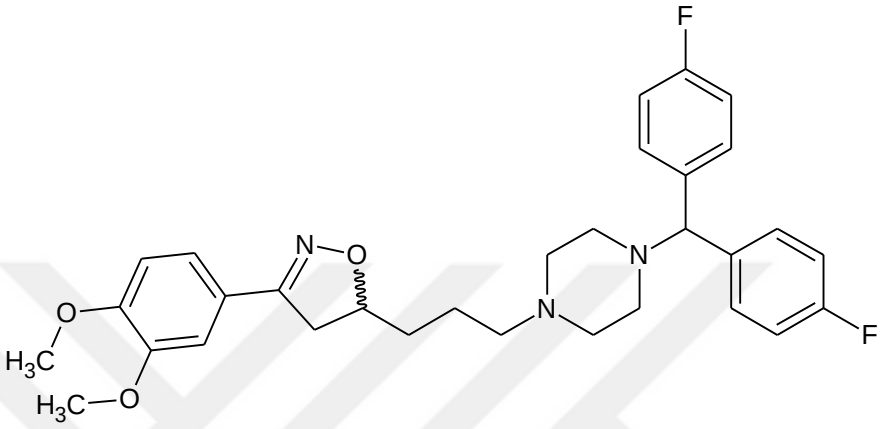
2.2.3.2. Dopaminergic Activity

Sasse *et al*. synthesized a benzhydrylpiperazine derivative with good dopaminergic activity. Benzhydrylpiperazine carrying structures gave remarkable selectivity at D_2 receptors. The present of chlorine substituent allow the structure to have a good hydrophobic interaction with dopamine D_3 receptor [60].



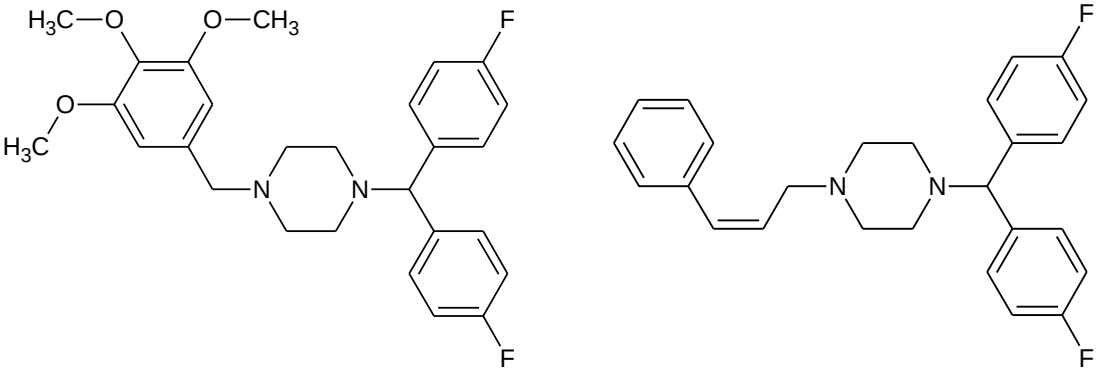
R= naphtalen-2-carboxamide, cinnamide, benzo[b]thiophene-2-carboxamide, cyclohexylcarboxamide; R_1= -H, 4-Cl, 2-OCH_3; R_2= -H, -OCH_3

Jung *et al*. synthesized a chiral piperazylpropylisoxazoline analogue carrying 4-[bis(fluorobenzhydryl)]piperazine moiety. R-configured chiral ligand exhibited higher binding affinity and selectivity for the D_3 receptors over D_4 receptors than S-ligand [61].

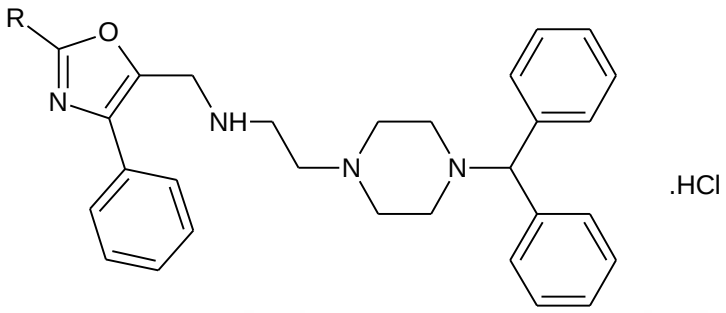


2.2.3.4. Calcium Channel Blocking Activity

Flunarizine and lomerizine were introduced earlier as *N*-type calcium channel blocker in therapy, both carrying 4-bis(fluorophenyl)piperazine moieties as the basic skeleton [62].

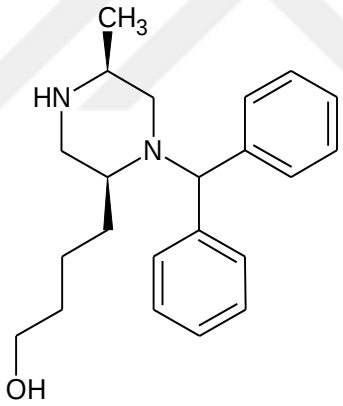


Some benzhydrylpiperazine derivatives were reported to inhibit *T*-type calcium channel [63].



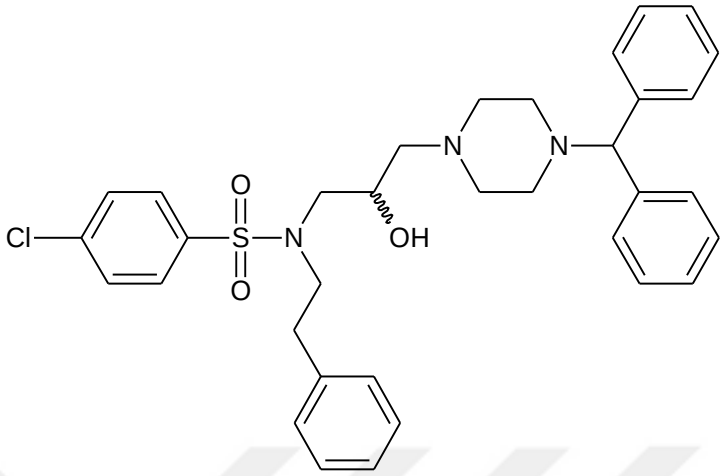
$R_{1=}$ -CH_3, -C_3H_8

Borzenko *et al*. synthesized (1-benzhydryl-5-methylpiperazin-2-yl)butanol to have a potent *N*-type calcium channel blocking activity with IC_{50} of 0.85 μM [64].

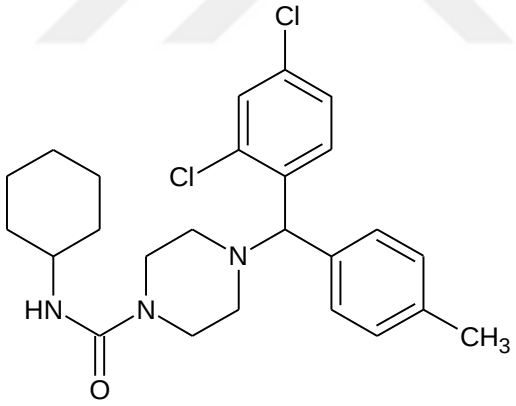


2.2.3.5. Central Nervous System Activity

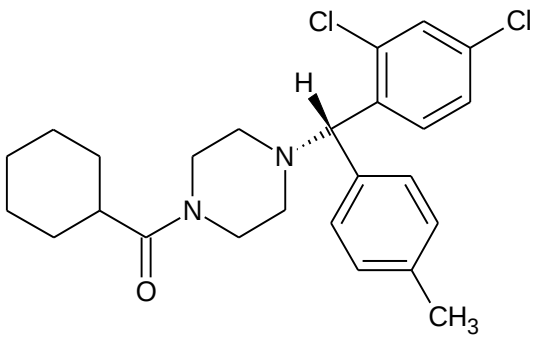
N-[3-(4-benzhydrylpiperazine-1-yl)-2-hydroxypropyl]arylsulfonamide derivatives have been recently prepared and reported as active BACE1 blockers. The most active enantiomers presented IC_{50} values of R-2.3 μM and S-1.9 μM respectively [65].



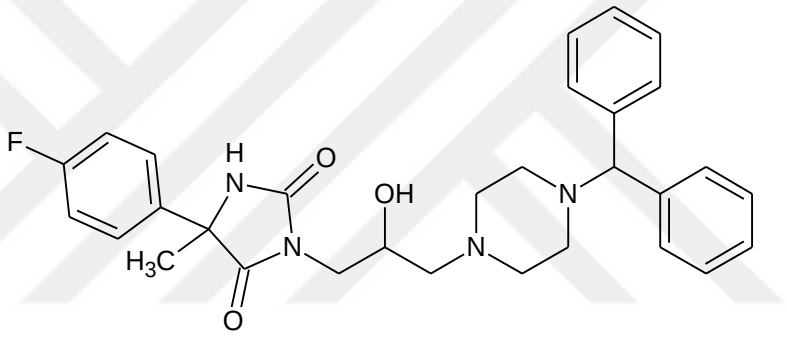
Benzhydrylpiperazine cyclohexylurea derivative was reported as cannabinoid (CB1) receptor antagonist and useful in reducing body weight in diet-induced obese Sprauge-Dawley rats. It showed high potency with K_i = 0.15 nM and selectivity >2000 for hCB1 [66].



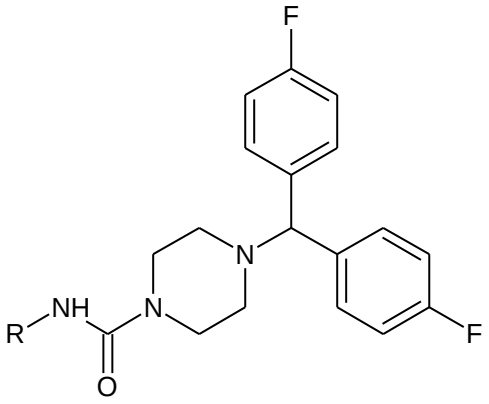
N-cyclohexyl-4-[1-(2,4-dichlorophenyl)-1-(p-toly)methyl]piperazine-1-carboxamide was synthesized in a recent study by Gao *et al.* and the result indicated (R) configured ligand is highly potent and selective as hCB1 inverse agonist with $K_{i\text{-}CB1}$ = 0.15 nM, EC_{50} = 0.87 nM [67].



Following benzhydrylpiperazine derivative was shown to have a potent and full antagonistic action and preferable binding to 5-HT_7R [68].



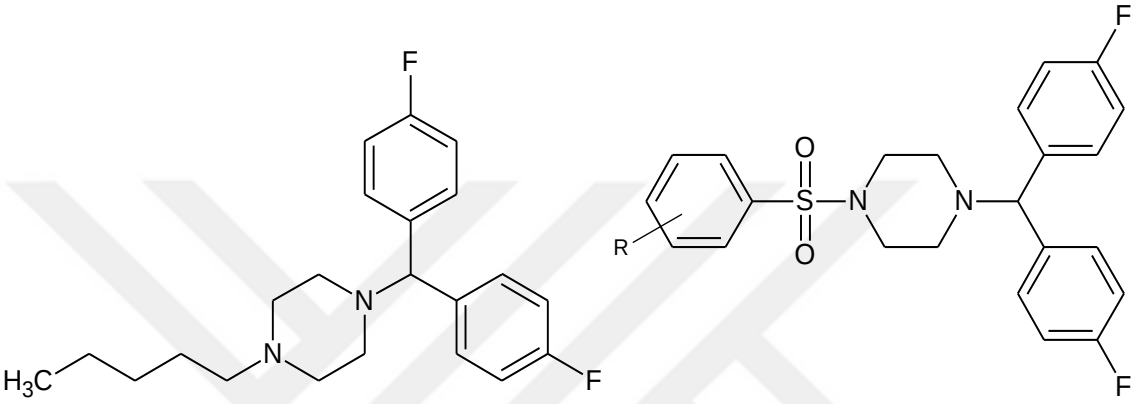
Some benzhydrylpiperazines were found to have antiobesity activity by inhibition of carnitine palmitoyl transferase enzyme [69].



R=2-Phenylethyl, 2-ethylphenyl, 4-heptyloxyphenyl, 4-chlorophenyl, 4-methoxybenzyl

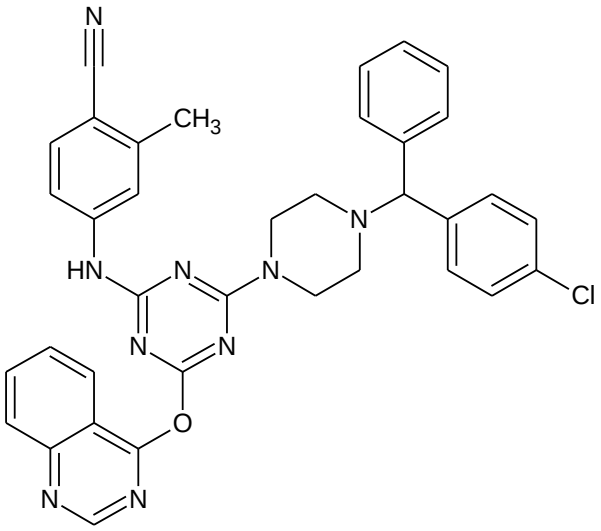
2.2.3.6. Antimicrobial Activity

Chandra *et al*. synthesized benzhydrylpiperazine derivatives, which is effective against different types of bacteria and the range of minimum inhibitory concentration value is 30-169 µg\ml [13].

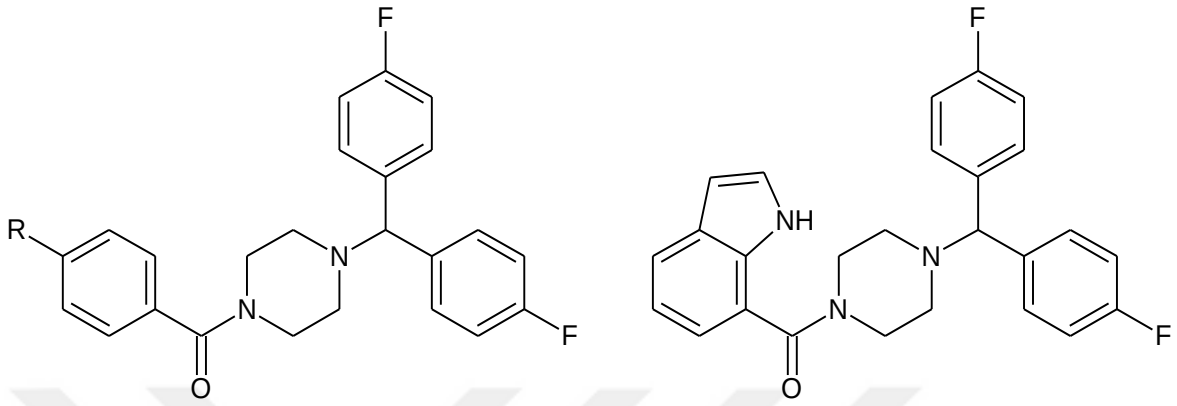


R=4-Cl / 2,6-diCl

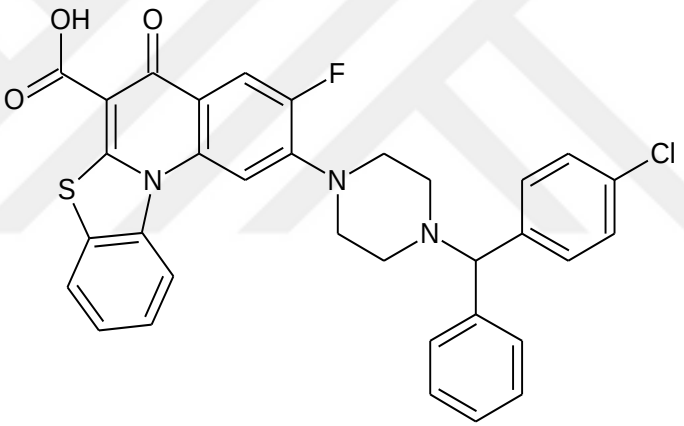
Patel *et al.* developed 2-[(4-cyano-3-methyl)phenylamino)-4-(4-(quinazolinyloxy)-6-benzhydrylpiperazinyl]triazine with highly potent antibacterial activity at MIC of 6.25 µg\ml [13].

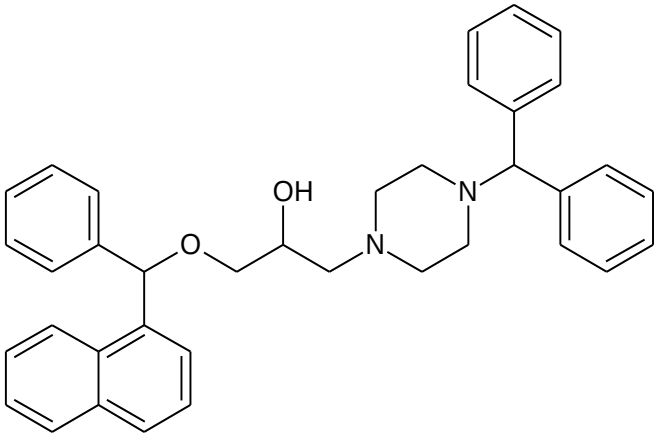


In many studies, benzhydrylpiperazine derivatives showed good antibacterial activities against *Mycobacterium tuberculosis* [70-72].

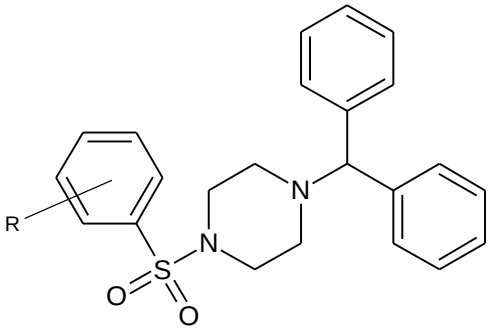


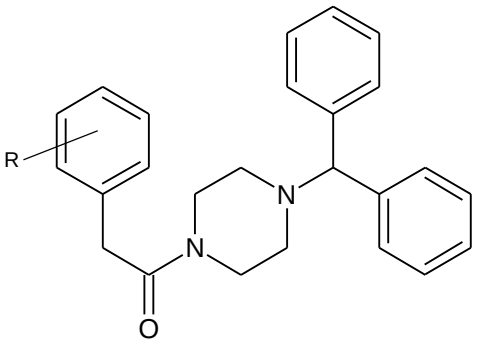
R = -H, -CH_3





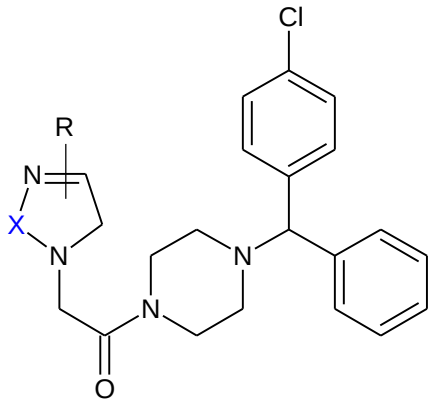
A series of novel 1-benzhydrylpiperazine sulfonamide and benzamide derivatives were synthesized by Kumar *et al*. and reported for their potent antibacterial activities [73].





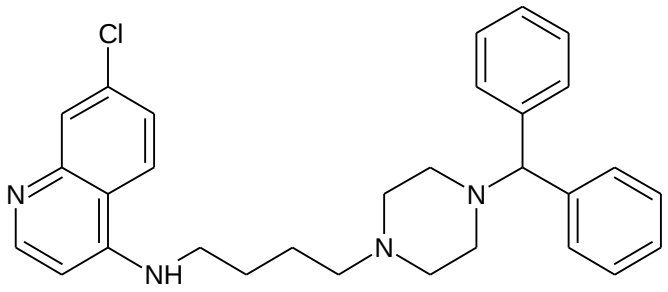
R = 4-Chloro; 2,5-dichloro R=2,4-Dichloro; 2-fluro; 2,6-difluro; 3,5- dinitro

Following benzhydrylpiperazine derivatives were described to have good antibacterial activity against *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Shigella dysenteriae, Escherichia coli, Proteus vulgaris, and Salmonella typhi.* As well as, benzhydrylpiperazine reported for their antifungal activity against *Candida albicans, Sacchorromyces cerevisiae* [74].



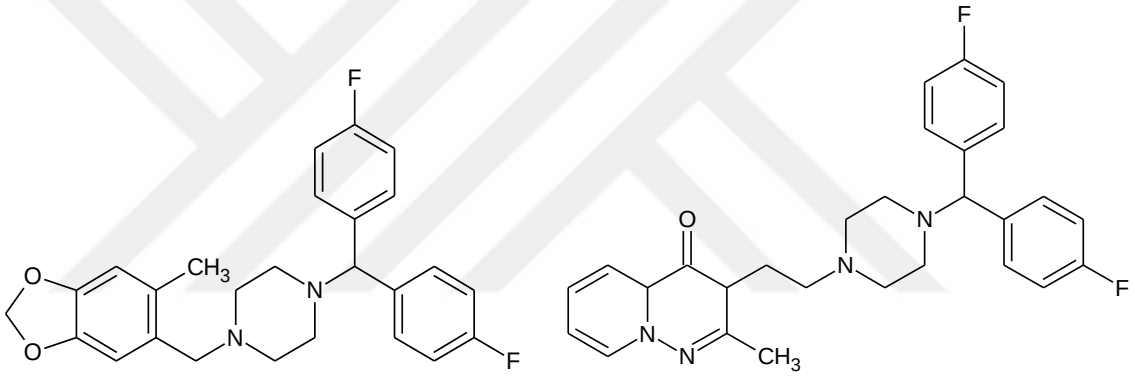
X = -N, -C; R = H, 4-NO_2, 2-CH_3-4-NO_2, 2-C_6H_5, 2-CH_3

Chloroquine derivative benzhydrylpiperazine was informed to have potent antimalarial activity [75].



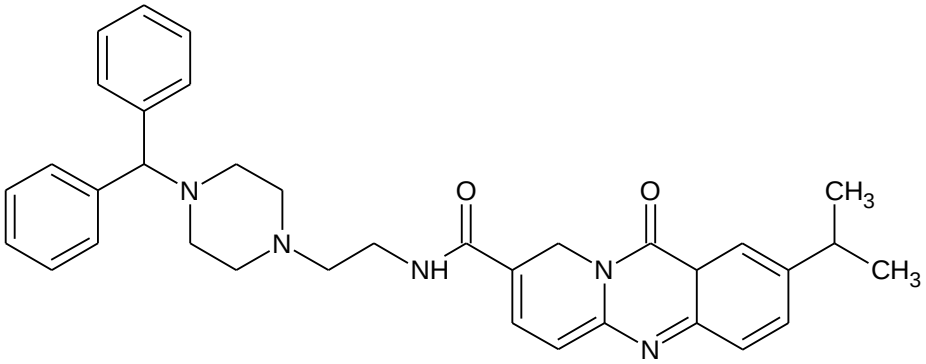
2.2.3.7. Anticholinesterase Activity

Many 1-[bis(4-fluorophenyl)methyl]piperazines were reported as acetylcholinestrase inhibitors [76].



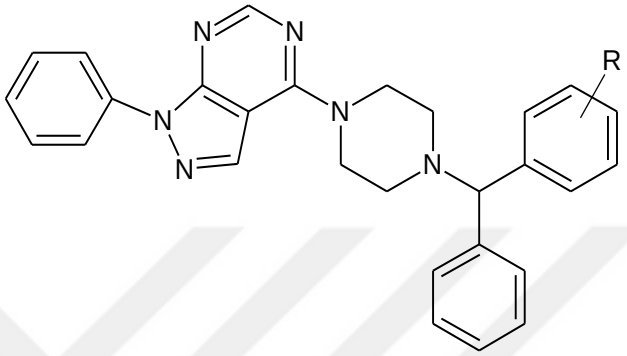
2.2.3.8. Antiallergic Activity

Benzhydrylpiperazines have been reported to relieve anaphylaxis [77].



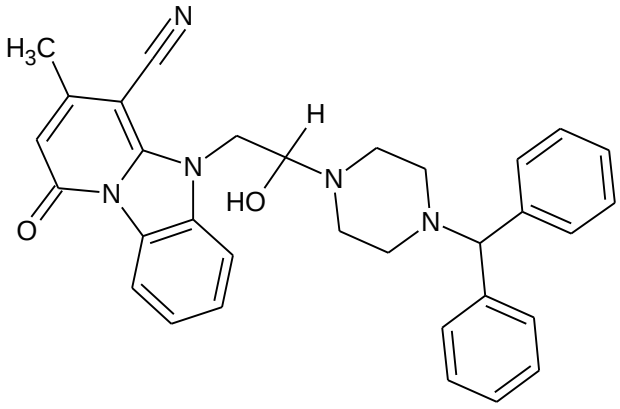
2.2.3.9. Antiviral Activity

Chern *et al.* synthesized pyrazolo[3,4-d]pyrimidines carrying diphenylmethyl-piperazine moiety with *in vitro* antiretroviral activity [78].

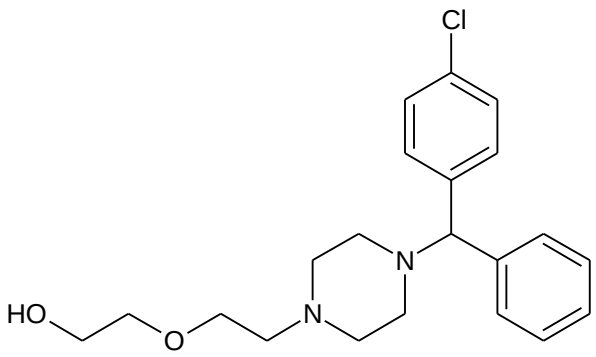


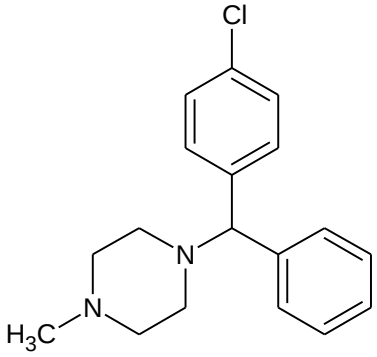
R= -H, 4-Cl, 4-Br, 4-CH_3, 4- CF_3, 4-CN, 3-CN, 2-CH_3

A benzhydrylpiperazine derivative has been published for its activity against human immunodeficiency virus (HIV-1)[79].



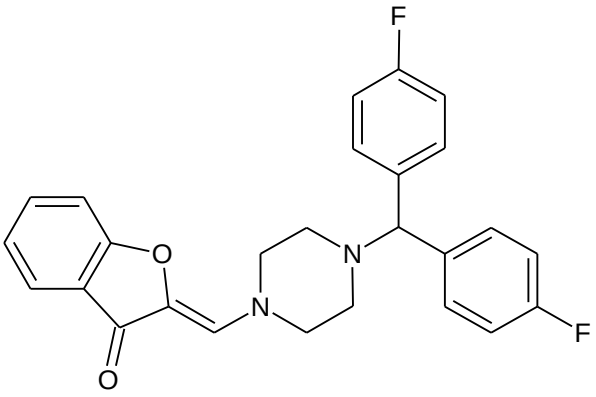
Benzhydrylpiperazines inhibit cholesterol-dependent cellular entry of hepatitis C virus. Hydroxyzine and chlorocyclizine were shown to suppress the infection of HCVcc with estimated IC_{50} values of 19 and 2.3 nM respectively [80].

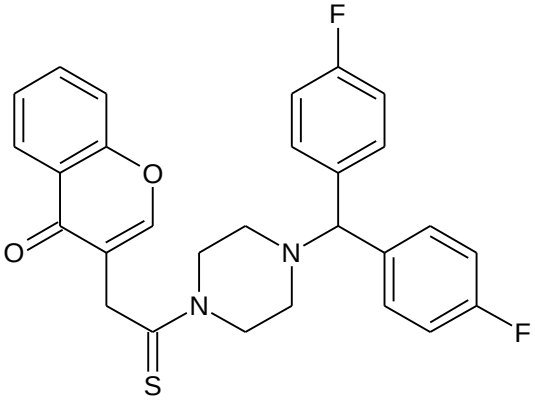




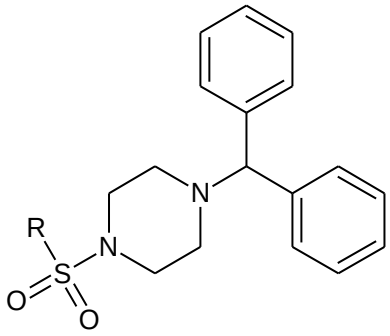
2.2.3.10. Anticancer and Cytotoxicity Activity

In vitro antitumor activities of benzhydrylpiperazine derivatives were evaluated by MTT method on four cancer cell lines, including, HCCLM-7 (liver), Hep-2 (larynx), MDA-MB-435S (mammary adenocarcinoma), and SW-480 (colon). Huang *et al.* synthesized and evaluated benzhydrylpiperazine derivatives which inhibit the cell cycle in resting phase and first growth phase and induce apoptosis on Hep-2 cells [15]. Furthermore, benzhydrylpiperazine derivatives bearing chromone moiety are published as potent antiproliferative effect [16].

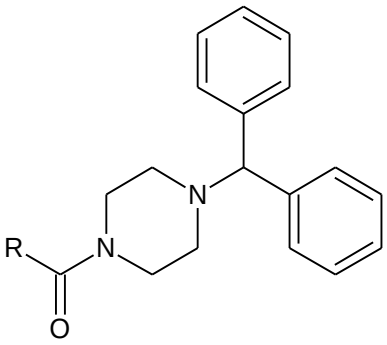




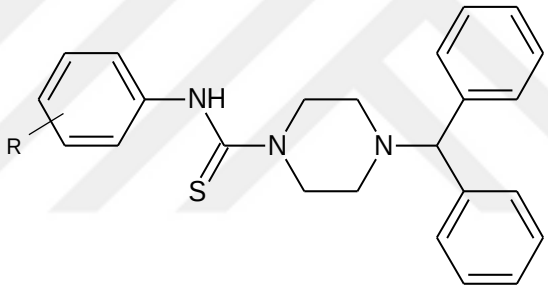
By using MTT assay Kumar *et al.* reported the potential cytotoxic effect of 1-benzhydrylpiperazine derivatives. Compounds were found to have high antitumor activities against MCF-7 (breast cancer cell line), HepG (hepatocellular cancer cell line), HeLa (cervix cancer cell line) and HT-29 (colon cancer cell line). Variation in the functional group at *N*-terminal of piperazine showed good antiproliferative activity versus above cell lines [14].



R= (4-Cl-2-fluoro)phenyl; camphoryl; 2,2,2-trifluroethyl

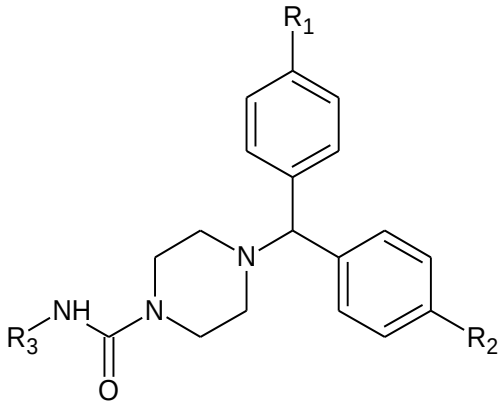


R= isoxazol-5-yl; morpholine-4-yl phenyl; pyrrolidinyl; cyclopropyl



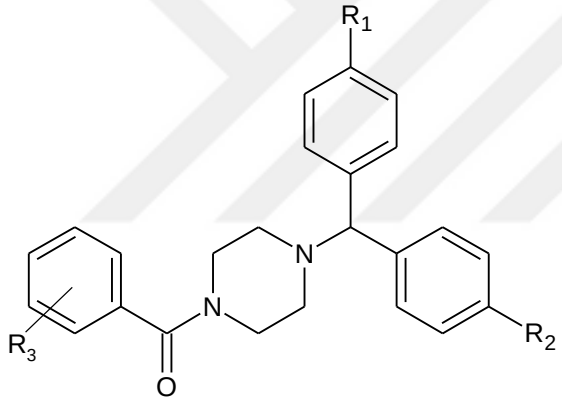
R = 2-OCH_3, 3-OCH_3, 4-OCH_3; 2-Cl, 3-Cl, 4-Cl; 4-F; 2,4-diCl

In another study published by Gurdal *et al.*, benzhydrylpiperazine carboxamide, thiourea, benzamide and sulfonyl derivatives were screened for their cytotoxic activity by sulforhodamine B assay. Cytotoxicity of the synthesized compounds was tested on (Huh7, MCF-7 and HCT-116) cancer cell lines. In general, compounds presented higher cytotoxicity against cancer cell lines than reference drug 5-fluorouracil. Interestingly, 4-chlorobenzhydylpiperazine derivatives were more active than benzhydrylpiperazine and 4,4'-difluorobenzhydrylpiperazine derivatives. Furthermore, thioamide derivatives had much higher cytotoxicity than their carboxamide analogs and benzamide structures were highly active against HCT-116 colon cancer cell line [18,19].



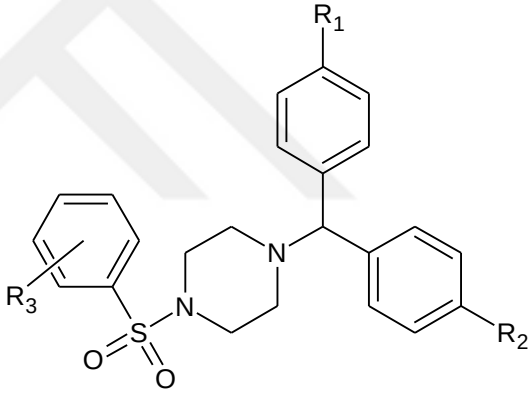
R_1= Cl, F; R_2 =H, F;

R_3 = 4-Bromophenyl; 2,6-dichlorophenyl, 4-cyanophenyl



R_1=-F, -Cl; R_2=-F, -H;

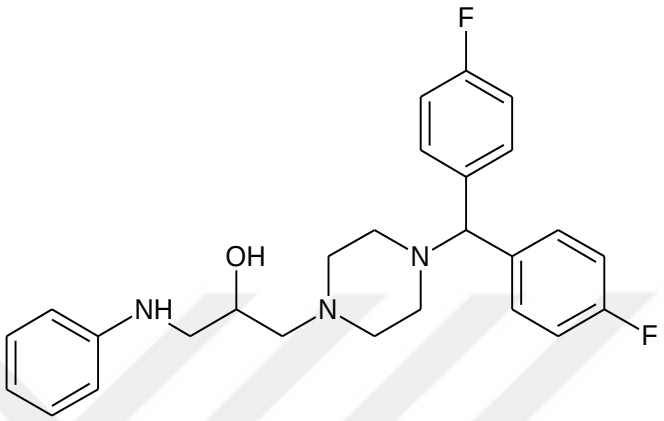
R_3=2-CH_3, 2,4,5-triCl, 4-NO_2



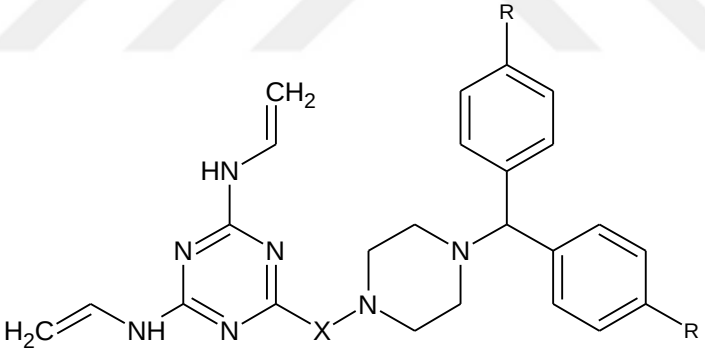
R_1= -F, -Cl; R_2= -F, -H; R_3=3-Br, 4-Br

2.2.3.11. Other Activities

A benzhydrylpiperazine derivative was reported for its high antioxidant activity [81].



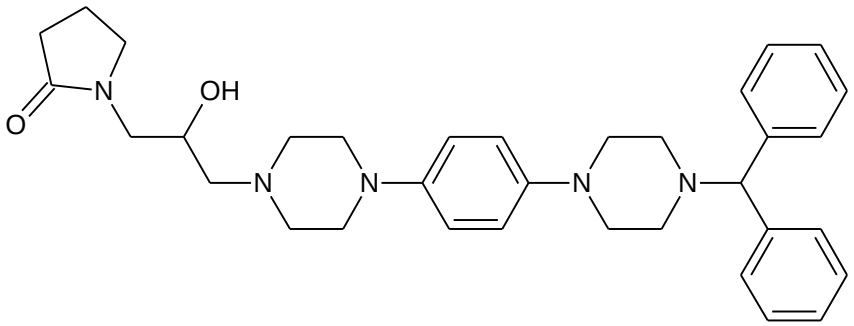
Dhainaut *et al.* synthesized benzhydrylpiperazine derivatives that act as multidrug-resistance modulators [82].



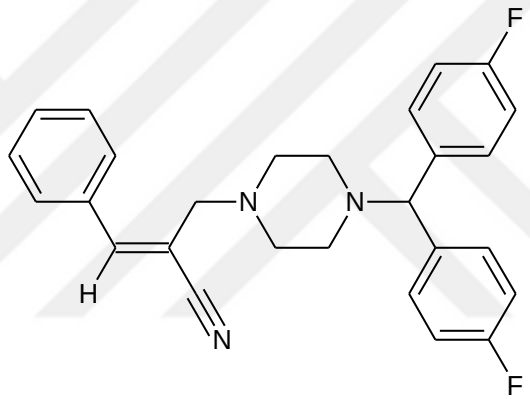
R= -F, -Cl

X= -NH-, no spacer

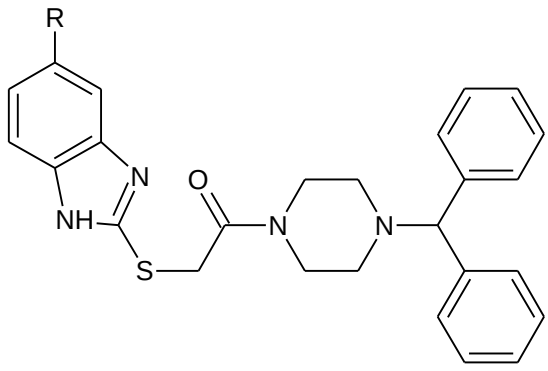
A benzhydrylpiperazine derivative was found as a good α_1 adrenergic receptor antagonist [83].



Santhoshi *et al.* synthesized 1-benzhydrylpiperazine derivative with good ACE inhibitory activity with IC_{50} value 0.874 mM [84].



Following benzhydrylpiperazine derivatives were reported to have anthelmintic activity [85].



R_1= -H, -CH_3

2.3. Biological Activity

2.3.1. Cancer

Cancer is explained by abnormal cell growth mechanisms that govern cell survival, proliferation, and differentiation. The aim of antitumor agents is to decreased the number of tumor cells and avoid their aggregation.

The burden and behavior of specific types of cancer are related to many factors, involving sex, age, race, inherited genetic effect, as well as, lifestyle, tobacco use, physical inactivity, and diet. Moreover, exposure to ionizing radiation and environmental pollutants have been reported as important risk factor for many cancer cases. Additionally, several viruses have been reported as significant factor to cause human tumors. For instance, hepatitis B (HBV) and hepatitis C (HCV) may lead to hepatocellular carcinoma, whereas HIV is associated with Hodgkin's and non-Hodgkin's lymphomas, also human papilloma virus (HPV) is related to cervical cancer.

Cancer is often treated with combination of radiotherapy, surgery, chemotherapy, and targeted therapy. Diagnosis at early stages may prevent further development of disease and increase curing rate with local treatment. In patients suffering from locally advanced disease, chemotherapy is usually combined with radiotherapy to render surgery more efficient. In contrast, chemotherapeutics alone are able to cure less than 10 % of cancerous tumors when the disease is diagnosed at late stages [86].

2.3.1.1. Significant regulations on the Cell and Cancer

Two main principles of cell cycle mechanisms;

- Cell proliferation (DNA replication) and mitosis to manufacture new cells,
- Cell differentiation to produce specialized cells.

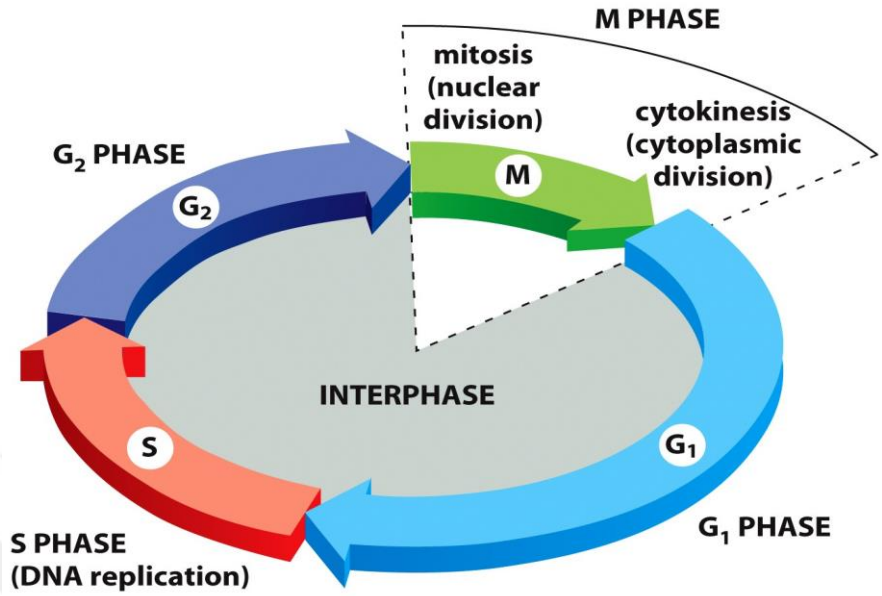
Cell cycle or cell division is an important event that takes place in cell, leading replication of DNA to produce several cells. Furthermore, mechanisms are managed through chemical signals such as growth factors or growth inhibitors. Growth factors serve as signals that force the cell to move through the cell cycle in order to divide. Several cells able to

apply a negative feedback loop to equalize the effect of growth factors, when any defect takes place in cell, generation of growth inhibitor decreased and amount of DNA replication raise until the defective cells are fixed [87].

Growth factors are able to activate cellular proliferation and / or differentiation. In cancer cells, the cell cycle regulation systems has defect. For instance, tumor cells may over produce chemical signals (*i.e.* epidermal growth factors, EGF), under express growth suppressor gene (*i.e.* p53) or over express growth factor receptors. Irregular stimulation of growth factors or reduced expression of suppressor will cause unusual and raising cell replication. As a reason, it is believed that proto-oncogenes that control cellular life are changed into oncogenes. At the end, oncogenes modify normal cellular regulation by stimulating processes that support cellular replication [87].

Some effective cytotoxic drugs are able to inhibit cell proliferation; therefore, they could block activity of the cells that are in process of division. Thus, regular tissues that has rapid replication (*i.e.* bone marrow, hair follicles and intestine epithelium) highly sensitive towards damaging of cytotoxic agents. All cells undergo the same cell cycle division stages which may describe as follows:

1. Presynthetic phase - Gap1 (G_1); increased cellular size and G_1 checkpoint control mechanisms ensure that process is ready for DNA replication.
2. Synthesis phase (S); DNA synthesis occur in this phase.
3. Post synthetic phase - Gap2 phase (G_2); is an interval of protein synthesis and fast cell growth to set the cell for mitosis phase.
4. Mitosis phase (M) - cell growth stops at this stage and the cellular energy is focused on the orderly division into two daughter cells. Metaphase checkpoint ensures that the cell is ready to complete cell division [88].



Scheme 2.1. Cell cycle phases [89]

2.3.1.2. Screened Cancer Cell Types

2.3.1.2.A. Breast Cancer

Breast cancer is most common invasive cancer in females worldwide according to public health and society. Recently breast cancer rate have raised and become difficult to manage.

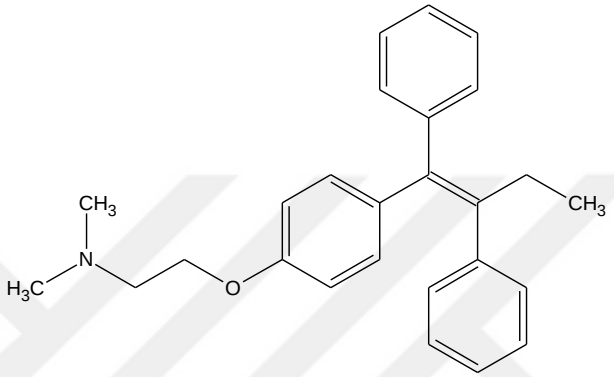
Amongst several danger factors, sex and age are the most significant factors. The breast cancer cases are most commonly found in females, while, male breast cancer is seen rarely. Considering age factors, breast cancer is more common in elder women.

Women in wealthy countries are under risk due to life-style associated choices like unhealthy food, hormonal exogenous drugs and drinking much alcohol. About one-third of breast cancers are related to genetic reasons, caused by mutant genes.

Chemotherapy weakens and destroys cancer cells in the body, by inhibition of hormone synthesis or blockage of hormone receptors. Inhibition of aromatase function cause inhibition of hormone synthesis. Aromatase is an enzyme which is a key regulator

responsible for the synthesis of estrogen by converting androgens into estrogens through aromatization process.

Tamoxifen is the oldest and most prescribed of selective estrogen receptor modulators (SERMs), which have selectivity binding to estrogen receptors on specific cells.



Tamoxifen

There are several chemotherapeutic agents used to treat breast cancer such as doxorubicin, cyclophosphamide, mitoxantrone, methotrexate, vincristine and paclitaxel [90].

2.3.1.2.B. Hepatocellular Carcinoma (HCC)

Hepatocellular carcinoma occurs more often in men than women. Nowadays liver cancer is the third type of cancer that leads to death in worldwide. The epidemic diffusion of hepatitis B and C substantially increased chronic liver disease and posterior development of hepatocellular carcinoma.

Liver cirrhosis is a common reason for HCC. In addition, hepatitis C virus causes increase in epidemic in USA and Europe [91].

Several systemic chemotherapeutics such as doxorubicin, cisplatin, floxuridine, epirubicin, and mitomycin are the most effective drugs for treating localized HCC.

Systemic therapy of metastatic HCC is obtained with chemotherapeutics involving anthracyclines, doxorubicin, 4-epidoxorubicin, and mitoxantrone [92].

2.3.1.2.C. Colorectal Cancer

Colorectal tumor has nearly same diagnosed for men and women; however rectum cancer is widely counts in men.

Risk factors for colorectal cancer include life style, elderly, and gene mutation. Other dangerous factors involve tobacco and alcohol consumption, and exposure to radiation.

For early diagnosis, the most convenient method is colonoscopy. Recently they used Sigmoidoscopy method for screening of patient against cancerous polyps [93].

Combination therapy of 5-fluorouracil and leucovorin (5-FU/LV) or 5-fluorouracil and levamisole (5-FU/LEV) are used for relieving of stage III colon tumor. Patient receives 5-FU/LV plus irinotecan or oxaliplatin as adjuvant treatment [94].

2.3.1.3. Cancer Treatment

Presently, nearly 50% of patients who are early diagnosed with tumor can be cured. In contrast, chemotherapy alone is able to treat less than 10% of all cancer patients when the tumor is discovered at an advanced stage. Specific methods of tumor treatment are used involving surgery, radiotherapy, immune therapy, gene therapy, and cytotoxic agents. Commonly, combined therapy has improved clinical outcomes, such as in surgery in combination with chemotherapy [86].

2.3.1.4. Cancer Chemotherapy

2.3.1.4.A. Drugs Interacting Directly with DNA

Alkylating agents (*e.g*. nitrogen mustard, aziridines, triazenes, nitrosoureas, hydrazines), block DNA synthesis by formation of cross links with DNA strand, some cytotoxic antibiotics (*e.g*. actinomycin D) stabilize DNA by intercalation between cytosine and guanine base pair. In addition, due to formation of intra and interstrand cross links between organic heavy metals (e.g. cispatin and oxaplatin) and nucleic acid, as a result DNA and RNA replication are blocked [86].

2.3.1.4.B. Drugs Interfering with DNA Synthesis

Chemotherapeutic agents are able to interfere with DNA synthesis in S-phase of the cell cycle by inhibiting the responsible enzyme. For instance, antimetabolites such as methotrexate, inhibit action of dihydrofolate reductase which is responsible for the formation of tetrahydrofolate from dihydrofolate [86,95].

2.3.1.4.C. Mitotic Inhibitors

The two notable agents in this class are vincristine and vinblastine. They are mitotic spindle poisons that bind to tubulin, the binding block of the microtubules, this inhibits functions of the spindle during metaphase to destroy mitosis [95].

2.3.1.5. *In vitro* Cytotoxicity Assays

2.3.1.5.A. XTT/PMS Assay

In cell biology assays and histochemical localization studies tetrazolium salts are commonly used as detection reagents. 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxamide sodium salt (XTT) and 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are used as an efficient method to measure cell growth and drug sensitivity in tumor cell lines and apoptosis assays. XTT is colorless or slightly yellow compound that becomes bright orange when reduced, subsequently, reduction of XTT is performed to form formazan product which the intensity can be determined by colorimetric assay. Actually, XTT is more soluble than MTT so it is more preferred. To optimize XTT, the phenazine methosulphate (PMS) is applied as activation reagent induced in the XTT *in vitro* cytotoxic test. However, PMS mediates XTT reduction by catching electrons at the cell surface or in plasma membrane and forms a reactive intermediate that reduces XTT to formazan product. Furthermore, mitochondrial function, levels of NADH, and glucose are important factors for controlling the result of these assay [96, 97].

2.3.1.5.B. Neutral Red Assay

Neutral red assay is widely used for *in vitro* cytotoxicity screening. Neutral red (3-amino-7-diethylamino-2-methylphenazinehydrochloride) is used as indicator of

cytotoxicity in various cell lines, as it is taken up by living cells and dye is collected in the lysosomes [98].

2.3.1.5.C. Alamar Blue Assay

Alamar Blue indicates cellular health by using the reducing ability of viable cells to quantitatively measure the proliferation of various cell lines. Alamar blue assay uses the mitochondrial reductase to transform resazurin blue to fluorescent resorufin, which can be easily measured by colorimetric or fluorometric reading. [99].

2.3.1.5.D. ATP Cell Viability Assay

Cell viability assay is a homogenous method to determine the number of living cells in culture based on quantification of the present ATP. Luminometer is used for measuring the light generated using the luciferase-luciferin reagent. A typical apoptotic level exhibits a significant decrease in ATP level. Therefore, loss of ATP level in cell indicates cell death. Bioluminescent detection of the ATP levels accounts for a rapid screening of apoptosis and cell proliferation. The assay is applied by two steps, as the first adding ADP as substrate for adenylate kinase and ATP is formed. In the second step luciferase is utilized to catalyze the formation of light from ATP and luciferin and the intensity of light can be measured by using a luminometer or a β counter [99].

2.3.1.5.E. [^{3}H]-Thymidine Incorporation Assay

A radioactive nucleoside [^{3}H]-thymidine is incorporated into a new strand of DNA during mitotic cell division in thymidine incorporating assay. A scintillation beta-counter measures the radioactivity in DNA recovered from the cells [99].

2.3.1.5.F. Enzyme Release Based Cytotoxicity Assay

Lactate dehydrogenase release from lysed tumor cells is evaluated for screening cytotoxicity [100].

2.3.1.5.G. Sulforhodamine B (SRB) assay

SRB assay is one of the major techniques used for *in vitro* cytotoxicity testing, which was developed by Skehan *et al*. in 1990 [101].

This assay is used for determination of cell viability, dependent on protein mass. Sulforhodamine is an anionic aminoxanthene dye that forms an electrostatic complex with basic amino acid remnant under moderately acidic media, and dissociate under basic media. Determination of total protein content by using spectrophotometric method follows for measurement of cytotoxic activity.

SRB dye is taken up by living cells, in presence of trichloroacetic acid (TCA), cells are lysed and the liberated dye will form more intense color of greater absorbance.

The SRB assay provides a better linearity with cell number, high sensitivity, and the stable end point that does not need time-sensitive measurement and causes lower cost [101,102].

3. MATERIALS AND METHODS

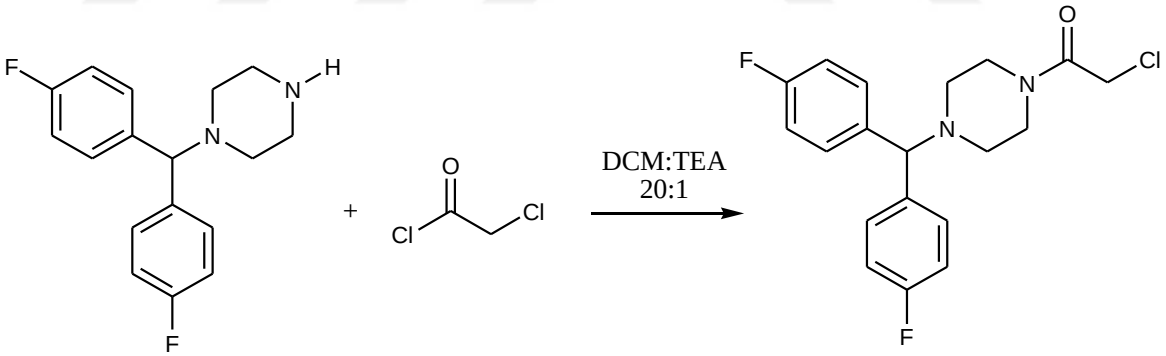
3.1. Chemistry

3.1.1. Materials

In this work, 1-[bis(4-fluorophenyl)methyl]piperazine was purchased from Chemicals International Turkey. Potassium carbonate, anhydrous sodium sulphate, ammonium chloride, hydrochloric acid, and benzene were purchased from Riedel de Häen. Triethylamine was purchased from J.T. Baker. Other piperazine derivatives, sulforhodamine B, absolute ethanol, methanol, ethyl acetate, *n*-hexane and dichloromethane were purchased from Sigma-Aldrich.

3.1.2. Methods of Synthesis

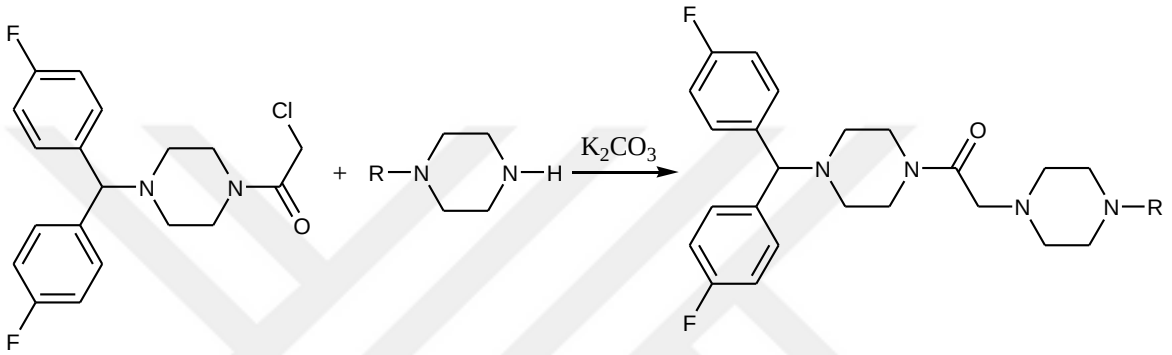
3.1.2.1. Synthesis Method of Starting Compound *N*-(2-chloroacetyl)-*N*'-[bis(4-fluorophenyl)methyl]piperazine [103]



0.01 Moles (2.84 g) of 1-[bis(4-fluorophenyl)methyl]piperazine was dissolved in 35 ml of dry dichloromethane. Triethylamine (2 ml) was added to the solution in room temperature. 0.01 Moles of chloroacetyl chloride (4.2 ml) was dissolved separately in 5 ml of dry dichloromethane and the resulting solution was added dropwise into the piperazine mixture over 15 minutes. Reaction continued mixing for two days at room temperature. Reaction was followed by thin layer chromatography in *n*-hexane:ethyl acetate (50:50) mobile phase and silica gel stationary phase. After the reaction was over, extraction with ammonium chloride solution (10%) and distilled water was applied to the reaction medium.

Followed by drying on sodium sulphate anhydrous dichloromethane was evaporated to get the crude product. Column chromatography with *n*-hexane:ethyl acetate (5:5) mobile phase and silica gel stationary phase gave pure product.

3.1.2.2. Synthesis Method of Target Compounds *N*-[2-(4-substitutedpiperazin-1-yl)acetyl)-*N*'-[bis(4-fluorophenyl)methyl]piperazine [104]



0.0017 Moles (0.6484 g) of *N*-(2-chloroacetyl)-*N*'-[bis(4-fluorophenyl)methyl]-piperazine was dissolved in dry acetone (20 ml) and 0.0017 moles (0.4167 g) of potassium carbonate was added to the solution. After ten minutes of mixing, piperazine derivative (0.0017 moles) in 10 ml of acetone was added drop by drop into the mixture. Reaction continued mixing for two days at room temperature. Reaction was followed by thin layer chromatography in *n*-hexane:ethyl acetate (10:90) mobile phase and silica gel stationary phase. After the reaction was over, potassium carbonate was removed by filtration. Acetone was evaporated under vacuum. Solid crude products were crystallized by absolute ethanol.

3.1.3. Analytical Methods

3.1.3.1. Melting Point Determination

Melting points (°C) of compounds were determined by using a Mettler Toledo FP62 capillary melting point apparatus and are uncorrected.

3.1.3.2. Controls by Thin layer Chromatography

Materials:

Plates : TLC aluminum sheets 20x20 cm silica gel 60 F_{254} (Merck).

Solvent system: Three different solvent systems were prepared to be used in chromatographic controls of compounds.

S-1: Ethyl acetate: *n*-Hexane (90:10)

S-2: Ethyl acetate: *n*-Hexane (80:20)

S-3: Ethyl acetate: *n*-Hexane (50:50)

Method:

Dragging condition: Solvent systems were poured to chambers and kept for 1 hour for adequate saturation.

Reactions were monitored with TLC after dissolving the synthesized compounds and starting materials with suitable solvents and application of them with Pasteur pipettes onto silica gel plates. The plates were dragged for 10 cm at room temperature. R_f values of compounds were calculated.

Stain determination: Stains of synthesized compounds and their starting materials were determined by light (254/365 nm) and *Dragendorff reagent* was sprayed over the plate in order to visualize orange colored spots of piperazine residues.

Preparation of Dragendorff reagent [105]:

Solution I: 2 g Bismuth subnitrate is dissolved in 25 ml acetic acid and 100 ml distilled water.

Solution II: 40 g Potassium iodide is dissolved in 100 ml distilled water.

Spray solution: 10 ml of solution I, 10 ml of solution II and 20 ml acetic acid are mixed and diluted with 100 ml of distilled water.

3.1.3.3. Purification by Column Chromatography

Materials:

Stationary phase: Silica gel - 60 mesh.

Mobile phase: Ethyl acetate: *n*-hexane (50:50)

Method:

Column was filled in accordance with wet method. Elution was controlled with TLC using silica gel plates and ethyl acetate: *n*-hexane (50:50), (80:20)

3.1.4. Spectrometric Analysis

3.1.4.1. Infrared Spectroscopy

Infrared spectra were recorded on a Perkin-Elmer spectrum one series FT-IR apparatus (version 5.0.1), using potassium bromide pellets, frequencies were expressed in cm^{-1}.

3.1.4.2. ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were recorded with a Varian Mercury-400 FT-NMR spectrometer (Varian Inc., Palo Alto, CA, USA), using tetramethylsilane (TMS) as the internal reference, with chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6) as solvent. Chemical shifts were reported in parts per million (ppm).

3.1.4.3. ^{13}C-NMR Spectroscopy

^{13}C-NMR spectra were recorded with aVarian Mercury-400 FT-NMR spectrometer (Varian Inc., Palo Alto, CA, USA), using tetramethylsilane (TMS) as the internal reference, with chloroform ($CDCl_3$) as solvent. Chemical shifts were reported in parts per million (ppm).

3.1.4.4. Mass Spectrometry

Mass spectra were recorded with Waters 2695 Alliance Micromass ZQ LC\MS instrument (Water Corp., Milford, MA, USA).

3.1.4.5. Elemental Analysis

Elemental analysis data were collected by LECO 932 CHNS (LECO-932, St. Joseph, MI, USA) instrument.

3.2. Anticancer Activity

3.2.1. Cytotoxicity Analysis of the Compounds

Cytotoxic activities of the synthesized compounds were investigated on liver (Huh7), breast (MCF-7) and colon (HCT-116) cancer cell lines, by means of sulforhodamine B (SRB) assays in triplicate. Serial dilutions from 100 µM to 2.5 µM were used, 5-fluorouracil (5-FU) was the reference compound and camptothecin (CPT) was the positive control for the cytotoxic effect.

3.2.1.1. Cell Culture

Human cancer cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. Each cell line was maintained in an incubator at 37 °C supplied with 5% CO_2 and 95% air.

3.2.1.2. NCI-60 Sulforhodamine B (SRB) Assay [104]

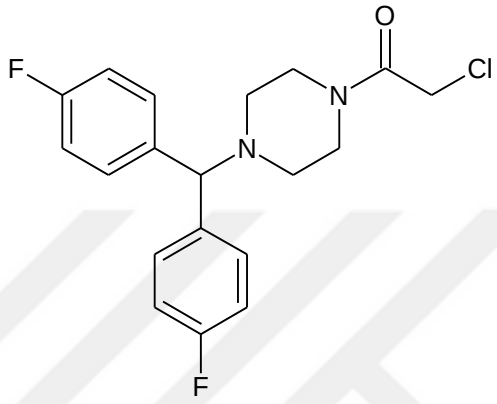
Cancer cells (range of 2000 cell/well to 5000 cell/well) were inoculated into 96-well plates in 200 µl of media and incubated in 37 °C incubators containing 5% CO_2 and 95% air. After a 24h incubation period, one plate for each cell line was fixed with 100 µl 10% ice-cold trichloroacetic acid (TCA). This plate represents the behavior of the cells just prior to drug treatment and is accepted as the time-zero plate. The compounds to be tested were dissolved in DMSO to a final concentration of 40 mM and stored at +4 °C. While treating the cells with the compounds, the corresponding volume of the compound was applied to the cell to achieve the desired drug concentration and diluted through serial dilution. After drug treatment, the cells were incubated in 37 °C incubators containing 5% CO_2 and 95% air for 72 hours. Following the termination of the incubation period after drug treatment, the cells were fixed with 100 µl 10% ice-cold TCA and incubated in the dark at +4 °C for 1 hour. Then the TCA was washed away with ddH_2O five times and the plates were left to air dry. For the final step, the plates were stained with 100 µl of 0.4% sulforhodamine B (SRB) solution in 1% acetic acid solution. Following staining, the plates were incubated in dark for 10 min at room temperature. The unbound dye was washed away using 1% acetic acid and

the plates were left to air dry. To measure the absorbance results, the bound stain was then dissolved using 200 µl of 10 mM Tris-Base. The OD values were obtained at 515 nm.

4. RESULTS

4.1. Chemical Data

N-(chloroacetyl)-*N'*-[bis(4-fluorophenyl)methyl]piperazine [103]



1-[Bis(4-fluorophenyl)methyl]piperazine (0.01 moles, 2.84 g) and chloroacetyl chloride (0.01 moles, 4.2 ml) were reacted according to general method at 3.1.2.1. The yield is 35.13%.

The form of compound is yellow liquid. It is soluble in methanol and hot ethanol; chloroform and acetone at room temperature. R_f values at S-1, S-2, S-3 solvent systems are 0.73, 0.61, 0.85 respectively.

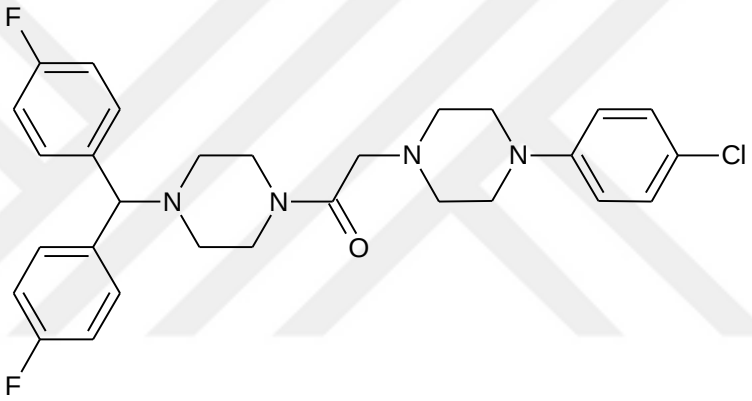
FT-IR (KBr, cm^{-1}); 3028 (C-H, aromatic), 2971 (C-H, aliphatic), 1736 (C=O, amide), 1600 (C=C, aromatic), 1291 (C-N).

^{1}H-NMR ($CDCl_3$, ppm); 2.37 (t, 2H, piperazine H_2, J=4.8 Hz); 2.41 (t, 2H, piperazine H_6, J= 4.4 Hz); 3.52 (t, 2H, piperazine H_3, J= 4.8 Hz); 3.62 (t, 2H, piperazine H_5, J= 5.2 Hz); 4.03 (s, 2H, -$NCOCH_2$-); 4.24 (s, 1H, $(Ar)_2CH$-); 6.98 (t, 4H, phenyl H_3,H_5,$H_{3'}$,$H_{5'}$, J= 8.4 Hz); 7.34 (dd, 4H, phenyl H_2,H_6,$H_{2'}$,$H_{6'}$, J_1= 5.2 Hz, J_2= 8.4 Hz).

Elemental analysis of $C_{19}H_{19}ClF_2N_2O$ (MW: 364.82 g/mol);

	% C	% H	% N
Calculated	62.55	5.25	7.68
Found	61.62	5.54	7.25

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[4-(*p*-chlorophenyl)-1-piperazinyl]acetyl}-piperazine (Compound 1)**



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then 1-(4-chlorophenyl)piperazine (0.0017 moles, 0.3612 g) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2. The yield is 0.271 g (30%).

The form of compound is white, clustered crystals, and the compound has a melting point of 179 °C. It is soluble in methanol and hot ethanol; chloroform and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.43, 0.42, 0.20 respectively.

UV (MeOH, λ_{max}, nm); 225 (log ε: 4.76), 269 (log ε: 4.67).

FT-IR (KBr, cm^{-1}); 3011 (C-H, aromatic), 2970 (C-H, aliphatic), 1640 (C=O, amide), 1601 (C=C, aromatic), 1241 (C-N).

^{1}H-NMR ($CDCl_3$,ppm); 2.35 (t, 4H, piperazine $H_{2,}H_{6,}$ J=7.6 Hz); 2.63 (t, 4H, benzhydrylpiperazine $H_{2',}H_{6'}$, J=4.4 Hz); 3.13 (t, 4H, piperazine $H_{3',}H_{5'}$, J=4.8 Hz); 3.21 (s, 2H, -N$\underline{CH_2}$CO-); 3.62 (t, 4H, benzhydrylpiperazine $H_{3,}$ $H_{5,}$ J=4.8 Hz); 4.22 (s, 1H, $(Ar)_2CH$-); 6.82 (dd, 2H, *p*-chlorophenyl H_3,$H_{3',}$ J_1=2.4 Hz, J_2=7 Hz); 6.98 (m, 4H, bis(*p*-fluoro)benzhydryl H_2,H_6,$H_{2'}$,$H_{6'}$); 7.20 (dd, 4H, *p*-chlorophenyl H_2,$H_{2',}$ J_1=7.2 Hz, J_2=2.4 Hz); 7.35 (dd, 4H, bis(*p*-fluoro)benzhydryl H_3,H_5,$H_{3'}$,$H_{5',}$ J_1=5.2 Hz, J_2=8.8 Hz).

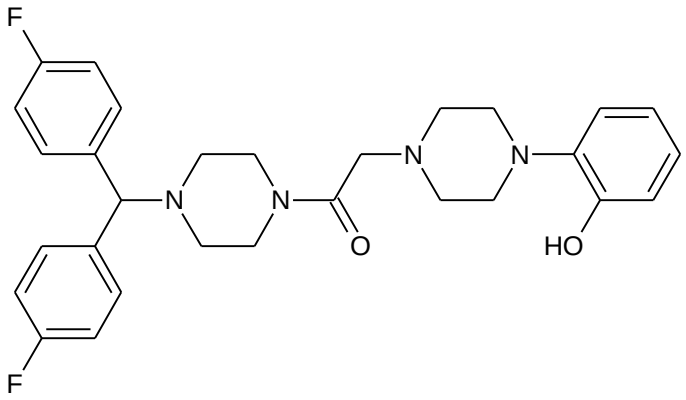
^{13}C-NMR ($CDCl_3$, ppm); 49 ($C_{14,16}$); 51.64 ($C_{15,17}$); 52.11 ($C_{20,22}$); 52.91 ($C_{21,23}$); 61.23 (C_{19}); 74.29 (C_7); 117.19 ($C_{25,29}$); 124.59 ($C_{26,28}$); 128.92 ($C_{2,6,9,13}$); 129.15 ($C_{3,5,10,12}$); 137.6 (C_{24}); 149.78 ($C_{1,8}$); 160.67 (C_{27}); 163.12 ($C_{4,11}$); 167.67 (C_{18}).

LC-MS (m/z); 525.6 (M^+); 527.9 (M^{+2}); 323.3 (4-Cl-C_6H_5-N(CH_2CH_2)$_2$N-CH_2CO-N(CH_2CH_2)$_2$N$\rceil^+$); 203.0 (100%, (4-F-C_6H_5)$_2$CH$\rceil^+$).

Elemental analysis of $C_{29}H_{31}ClF_2N_4O$ (MW: 525.03 g/mol);

	% C	% H	% N
Calculated	66.39	5.95	10.67
Found	66.19	6.12	10.46

_N_-[bis(4-fluorophenyl)methyl]-_N'_-{[4-(_o_-hydroxyphenyl)-1-piperazinyl]-acetyl}piperazine (Compound 2)



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then1-(2-hydroxyphenyl)piperazine (0.0017 moles, 0.3307 g) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2. The yield is the yield is 0.3274 g (35.5%).

The form of compound is white, opaque, powdered crystals and the compound has a melting point above 300 °C. It is soluble in methanol and hot ethanol; DMSO and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.44, 0.39, 0.18 respectively.

UV (MeOH, λ_{max}, nm); 204 (log ε: 4.97), 274 (log ε: 4.09).

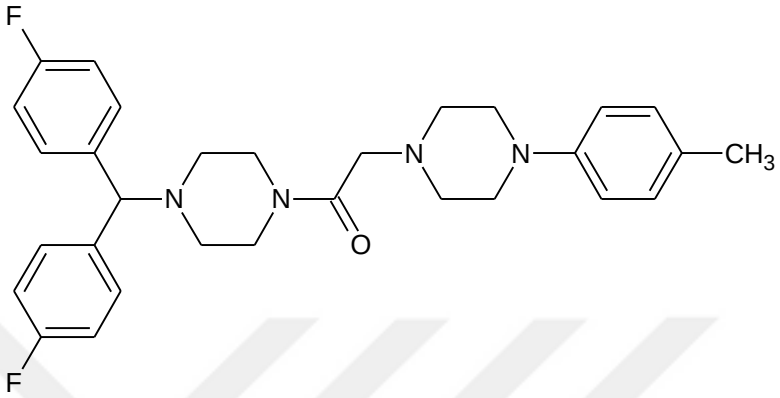
FT-IR (KBr, cm^{-1}); 3375-2993 (OH), 3069 (C-H, aromatic), 2955 (C-H, aliphatic), 1636 (C=O), 1602 (C=C), 1232 (C-N).

^{1}H-NMR ($CDCl_3$, ppm); 2.39 (bs, 4H, piperazine H_2, H_6); 2.83 (bs, 4H, benzhydrylpiperazine $H_{2'}$, $H_{6'}$); 2.95 (bs, 4H, piperazine $H_{3'}$, $H_{5'}$); 3.36 (bs, 2H, -N$\underline{CH_2}$CO-); 3.62 (bs, 4H, benzhydrylpiperazine H_3, H_5); 4.26 (s, 1H, $(Ar)_2CH$-); 6.85 (m, 1H, *o*-hydroxyphenyl H_4); 6.93 (dd, 2H, hydroxyphenyl H_6,H_5, J_1=3.5 Hz, J_2=6.9 Hz); 7.05 (m, 4H, bis(*p*-fluoro)benzhydryl H_2, H_6, $H_{2'}$, $H_{6'}$); 7.14 (dd, 1H, *o*-hydroxyphenyl H_3, J_1=3.0 Hz, J_2=7.1 Hz); 7.35 (bs, 4H, bis(*p*-fluoro)benzhydryl H_3, H_5, $H_{3'}$, $H_{5'}$).

Elemental analysis of $C_{29}H_{32}F_2N_4O_2$ (MW: 506.58 g/mol);

	% C	% H	% N
Calculated	68.76	6.37	11.06
Found	68.04	6.37	10.69

***N*-[bis(4-fluorophenyl)methyl]-*N'*- {[*p*-methylphenyl)-1-piperazinyl]acetyl}-piperazine (Compound 3)**



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then 1-(4-methylphenyl)piperazine (0.0017 moles, 0.3057 g) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2. The yield is 0.158 g (18%)

The form of compound is white, powdered crystals and the compound has a melting point 194.3 °C. It is soluble in methanol, hot ethanol, chloroform and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.48, 0.40, 0.20 respectively.

UV (MeOH, λ_{max}, nm); 204 (log ε: 4.94), 225 (log ε: 4.91), 265 (log ε: 4.81) .

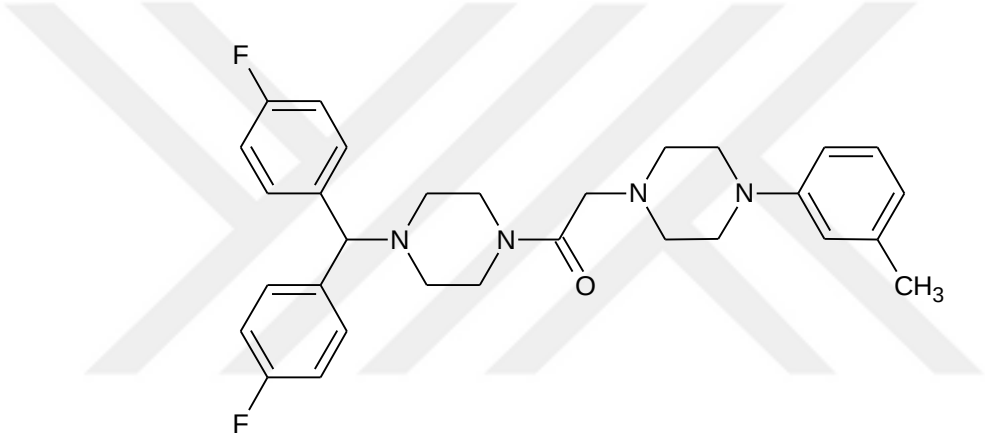
FT-IR (KBr, cm^{-1}); 3008 (C-H, aromatic), 2969 (C-H, aliphatic), 1641 (C=O), 1601 (C=C), 1211 (C-N).

^{1}H-NMR ($CDCl_3$, ppm); 2.1 (s, 3H, CH_3); 2.35 (bs, 4H, piperazine H_2, H_6); 2.82 (bs, 4H, benzhydrylpiperazine $H_{2'}$, $H_{6'}$); 3.20 (bs, 4H, piperazine $H_{3'}$, $H_{5'}$); 3.34 (bs, 2H, -N$\underline{CH_2}$CO-); 3.61 (bs, 4H, benzhydrylpiperazine H_3, H_5), 4.23 (s, 1H, $(Ar)_2CH$-); 6.83 (d, 2H, *p*-methylphenyl H_3, H_5, *J*= 6.8 Hz); 6.98 (t, 4H, bis(*p*-fluoro)benzhydryl H_2, H_6, $H_{2'}$, $H_{6'}$, *J*=6.9 Hz); 7.07 (d, 2H, *p*-methylphenyl H_2, H_6, *J*=7.0 Hz); 7.4 (bs, 4H, bis(*p*-fluoro)benzhydryl H_3, H_5, $H_{3'}$, $H_{5'}$).

Elemental analysis of $C_{30}H_{34}F_2N_4O$ (MW: 504.61 g/mol);

	% C	% H	% N
Calculated	71.41	6.79	11.10
Found	70.97	6.89	11.01

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[4-(*m*-methylphenyl)-1-piperazinyl}acetyl-piperazine (Compound 4)**



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then 1-(*m*-methylphenyl)piperazine (0.0017 moles, 0.331 g) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2. The yield is 0.211 g (24%).

The form of compound is white, powdered crystals, and the compound has a melting point above 300 °C. It is soluble in methanol and hot ethanol, chloroform and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.46, 0.47, 0.23 respectively.

UV (MeOH, λ_{max}, nm); 231 (log ε: 5.14), 206 (log ε: 5.09), 269 (log ε: 5.07).

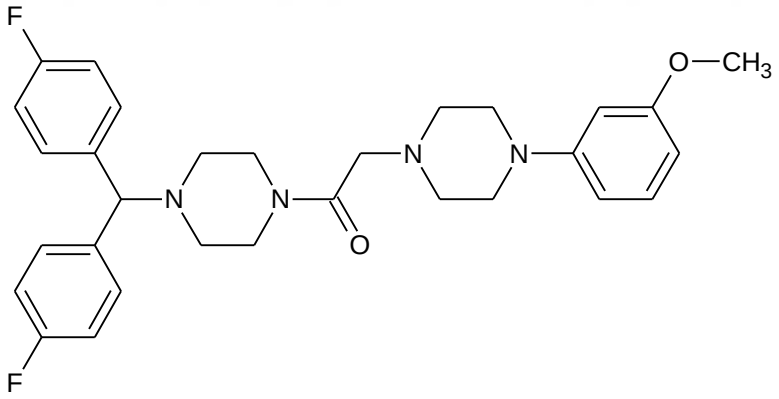
FT-IR (KBr, cm^{-1}); 3045 (C-H, aromatic), 2966 (C-H, aliphatic), 1638 (C=O), 1602 (C=C, aromatic), 1215 (C-N).

^{1}H-NMR ($CDCl_3$, ppm); 2.31 (s, 3H, CH_3); 2.36 (bs, 4H, piperazine $H_{2,}$ H_6); 2.82 (bs, 4H, benzhydrylpiperazine $H_{2'}$, $H_{6'}$); 3.24 (bs, 4H, piperazine $H_{3',}$ $H_{5'}$); 3.35 (bs, 2H, -N$\underline{CH_2}$CO-); 3.61 (bs, 4H, benzhydrylpiperazine H_3, H_5), 4.23 (s, 1H, $(Ar)_2CH$-); 6.72 (t, 3H, *m*-methylphenyl H_4, $H_{5,}$ $H_{6,}$ *J= 8.4 Hz*); 6.98 (t, 4H, bis(*p*-fluoro)benzhydryl $H_{2,}$ $H_{6,}$ $H_{2',}$ $H_{6',}$ *J*=8.8 Hz); 7.15 (t, 1H, *m*-methylphenyl $H_{2,}$ *J*=8.0 Hz); 7.34 (bs, 4H, bis(*p*-fluoro)benzhydryl $H_{3,}$ $H_{5,}$ $H_{3',}$ $H_{5'}$).

Elemental analysis of $C_{30}H_{34}F_2N_4O$ (MW: 504.61 g/mol);

	% C	% H	% N
Calculated	71.41	6.78	11.10
Found	71.50	6.89	11.18

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[4-(*m*-methoxyphenyl)-1-piperazinyl]-acetyl}piperazine (Compound 5)**



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then 1-(3-methoxyphenyl)piperazine (0.0017 moles, 0.254 ml) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2.The yield is 0.1549 g (40%).

The form of compound is yellow, powdered crystals and the compound has melting point above 300 °C. It is soluble in methanol and hot ethanol, $CHCl_3$ and acetone at room

temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.38, 0.39, 0.20 respectively.

UV (MeOH, λ_{max}, nm);208 (log ε: 5.12).

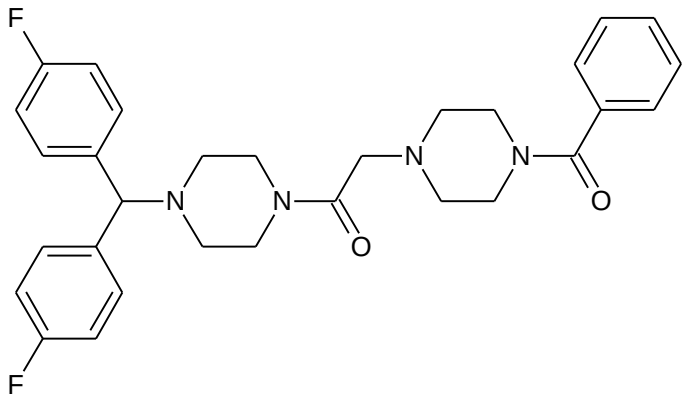
FT-IR (KBr, cm^{-1}), 3069 (C-H, aromatic), 2932 (C-H, aliphatic), 1640 (C=O), 1603 (C=C, aromatic), 1213 (C-O).

^{1}H-NMR ($CDCl_3$, ppm); 2.34 (m, 4H, piperazine H_2, H_6); 2.62 (t, 4H, benzhydrylpiperazine $H_{2'}$, $H_{6'}$, *J*=4.6 Hz); 3.16 (t, 4H, piperazine $H_{3'}$ $H_{5'}$, *J*=4.8 Hz); 3.2 (s, 2H, -NCH_2CO-); 3.62 (q, 4H, benzhydrylpiperazine H_3, H_5, *J*=4.8 Hz*);* 3.79 (s, 3H, -OCH_3); 4.21 (s, 1H, $(Ar)_2CH$); 6.44 (m, 2H, *m*-methoxyphenyl, H_4,H_6); 6.51 (dd, 1H, *m*-methoxyphenyl H_5, J_1=6.4 Hz, J_2=3.2 Hz); 6.97 (t, 4H, bis(*p*-fluoro)benzhydryl H_2, H_6, $H_{2'}$, $H_{6'}$, *J*=7.0 Hz); 7.18 (t, 1H, *m*-methoxyphenyl H_2, *J*=8.0 Hz); 7.34 (dd, 4H, bis(*p*-fluoro)benzhydryl H_3, H_5, $H_{3'}$, $H_{5'}$, J_1=3.2 Hz, J_2=6.4 Hz*).*

Elemental analysis of $C_{30}H_{34}F_2N_4O_2$ (MW: 520.6 g/mol);

	% C	% H	% N
Calculated	68.21	6.58	10.76
Found	68.25	6.98	10.83

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[4-benzoyl-1-piperazinyl]acetyl}piperazine (Compound 6)**



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then 1-benzoylpiperazine (0.0017 moles, 0.2745 g) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2. The yield is 0.530 g (59%).

The form of compound is white, opaque, powdered crystals and the compound has a melting point above 300 °C. It is soluble in methanol and hot ethanol, chloroform and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.06, 0.26, 0.06 respectively.

UV (MeOH, λ_{max}, nm); 203 (log ε: 4.32), 225 (log ε: 4.28)

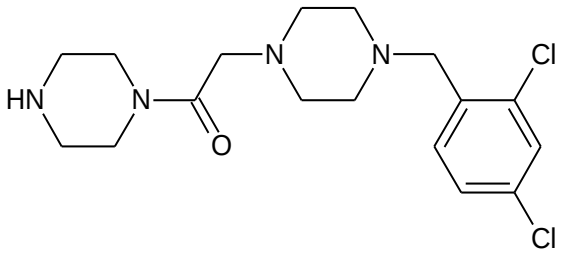
FT-IR (KBr, cm^{-1}), 3059 (C-H, aromatic), 2977 (C-H, aliphatic), 1624 (C=O, overlap), 1600 (C=C), 1265 (C-N).

^{1}H-NMR (DMSO, ppm); 2.21 (t, 4H, piperazine H_2, H_6, *J=4 Hz*); 2.26 (m, 4H, benzhydrylpiperazine $H_{2'}$, $H_{6'}$); 3.13 (s, 2H, -NCH_2CO-); 3.42 (t, 4H, piperazine $H_{3'}$, $H_{5'}$, *J*=4.6 Hz); 3.52 (t, 4H, benzhydrylpiperazine H_3,H_5, *J*=4.8 Hz); 4.39 (s, 1H, $(Ar)_2CH$-); 7.11 (t, 4H, bis(*p*-fluoro)benzhydryl H_2, H_6, $H_{2'}$, $H_{6'}$, *J=7.0 Hz*); 7.33 (m, 2H, benzoyl H_2, H_6), 7.32 (m, 3H, benzoyl H_3, H_4, H_5); 7.38 (m, 4H, bis-(*p*-fluoro)benzhydryl H_3, H_5, $H_{3'}$, $H_{5'}$).

Elemental analysis of $C_{30}H_{32}F_2N_4O_2 .H_2O$ (MW: 536.61g/mol);

	% C	% H	% N
Calculated	67.15	6.39	10.44
Found	67.36	6.43	10.43

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[4-(2,4-dichlorobenzyl)piperazinyl]acetyl}-piperazine (Compound 7)**



N-[bis(4-fluorophenyl)methyl]-*N'*-(2-chloroacetyl)piperazine (0.0017 moles, 0.6484 g) and potassium carbonate (0.0017 moles, 0.4167 g) in dry acetone (20 ml), were mixed for ten minutes then 1-(2,4-dichlorobenzyl)piperazine (0.0017 moles, 0.3423 ml) in 10 ml acetone were added according to the general synthesis method at 3.1.2.2. The yield is 0.2638 g (26%).

This compound forms yellow, flat crystals and compound has melting point above 300 °C. It is soluble in methanol and hot ethanol; chloroform and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.02, 0.19, 0.13 respectively.

UV (MeOH, λ_{max}, nm); 233 (log ε: 4.93).

FT-IR (KBr, cm^{-1}); 3070 (C-H, aromatic), 2966 (C-H, aliphatic), 1631(C=O amide), 1604 (C=C, aromatic), 1245 (C-N).

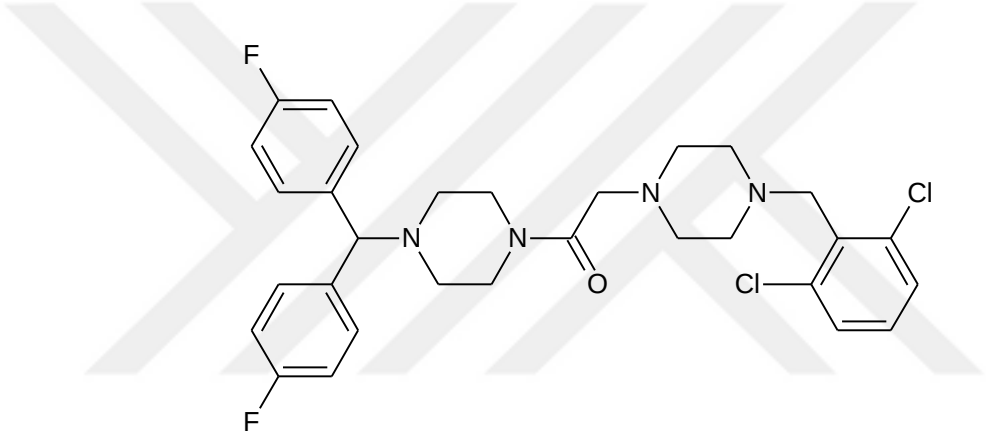
^{1}H-NMR ($CDCl_3$, ppm); 2.30 (m, 4H, piperazine H_2, H_6); 2.65 (bs, 8H, benzhydrylpiperazine); 3.25 (s, 2H, -NCH_2CO-); 3.60 (m, 4H, piperazine H_3,H_5); 3.70 (s, 2H, -N$\underline{CH_2}$Ph); 4.25 (s, 1H, $(Ar)_2CH$-); 7.0 (t, 4H, bis(p-fluoro)benzhydryl H_2, H_6, $H_{2'}$, $H_{6'}$ *J=6.8 Hz*); 7.2 (s, 1H, 2,4-dichlorophenyl H_3); 7.34 (d, 1H, 2,4-dichlorophenyl H_6, *J=7.34 Hz*); 7.38 (d, 4H, bis-*p*-fluoro)benzhydryl H_3, H_5, $H_{3'}$, $H_{5'}$, *J=7.37 Hz*); 7,56 (bs, 1H, 2,4-dichlorophenyl H_5).

LC-MS (m/z); 573.6 (M^+); 575.9 (M^{+2}); 371.5 (2,4-diCl-$(C_6H_5)CH_2$-$N(CH_2CH_2)_2N$-CH_2CO-$N(CH_2CH_2)_2N$Ⅎ$^+$); 203.1 (100%, (4-F-$C_6H_5)_2CH$Ⅎ$^+$).

Elemental analysis of $C_{30}H_{32}Cl_2F_2N_4O$ (MW: 573.50 g/mol);

	% C	% H	% N
Calculated	62.83	5.62	9.77
Found	61.98	5.67	9.80

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[(2,6-dicholorobenzyl)-1-piperazinyl]-acetyl}piperazine (Compound 8)**



N-(2-chloroacetyl)-*N'*-bis (4-flurophenyl) methyl piperazine (0.0017 Moles, 0.6484 g), (0.0017 Moles 0.4167 g) of potassium carbonate in dry acetone (20 ml), mixing for ten minutes then 1-(2,6-dichlorobenzyl)piperazine (0.0017 moles, 0.4167 g) in 10 ml acetone were reacted according to the general synthesis method at 3.1.2.2. The yield is 0.200 g (22%).

The form of compound is white, white powdered crystals and the compound has a melting point of above 300 °C. It is soluble in methanol and hot ethanol, chloroform and acetone at room temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.53, 0.51, 0.30 respectively.

UV (MeOH, λ_{max}, nm); 211 (log ε: 5.16), 225 (log ε: 5.15).

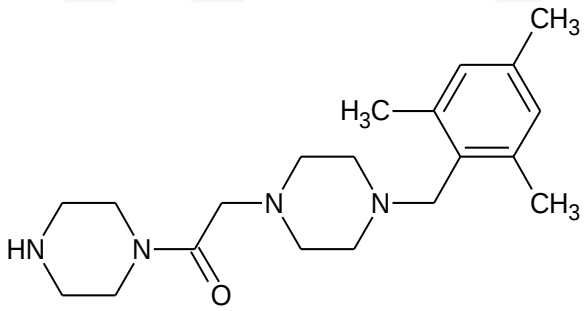
FT-IR(KBr, cm^{-1}); 2962 (C-H,aromatic), 2931 (C-H,aliphatic), 1638 (C=O), 1601 (C=C), 1245 (C-N).

^{1}H-NMR ($CDCl_3$, ppm); 2.32 (m, 4H, piperazine H_2, H_6); 2.62 (bs, 4H, benzhydrylpiperazine $H_{2'}$, $H_{6'}$); 2.70 (bs, 4H, piperazine $H_{3'}$, $H_{5'}$); 3.3 (s, 2H, -N$\underline{CH_2}$Ph); 3.60 (t, 4H, benzhydrylpiperazine H_3, H_5 *J*=3,5 Hz); 3.80 (s, 2H, NCH_2O); 4.25 (s, 1H, $(Ar)_2CH$); 6.98 (m, bis-*p*-fluoro)benzhydryl, H_2, H_6, $H_{2'}$,$H_{6'}$); 7.14 (t, 1H, 2,6-dichlorophenyl H_4 , *J*=8.4 Hz); 7.3 (d, 4H bis(p-fluoro)benzhydryl H_3, H_5, $H_{3'}$, $H_{5'}$, *J*=7.37 Hz); 7.4 (dd, 2H, 2,6-dichlorophenyl H_3, H_5, J_1= 5.2 Hz, J_2=8.4 Hz).

Elemental analysis of $C_{30}H_{32}Cl_2F_2N_4O$ (MW: 573.50 g/mol);

	% C	% H	% N
Calculated	62.83	5.62	9.77
Found	62.89	5.78	9.74

***N*-[bis(4-fluorophenyl)methyl]-*N'*-{[4-(2,4,6-trimethylbenzyl)-1-piperazinyl]-acetyl}piperazine (Compound 9)**



N-(2-chloroacetyl)-*N'*-[bis(4-fluorophenyl)methyl]piperazine (0.0017 Moles, 0.6484 g), (0.0017Moles 0.4167 g) of potassium carbonate in dry acetone (20 ml), mixing for ten minutes then 1-(2,4,6-trimethylbenzyl)piperazine (0.0017 moles, 0.4051g) in 10 ml acetone were reacted according to the general synthesis method at 3.1.2.2. The yield is 0.156 g (16%).

The form of compound is white, powdered crystals and the compound has melting point of 120.6 °C. It is soluble in methanol and hot ethanol; $CHCl_3$ and acetone at room

temperature. R_f values in TLC at S-1, S-2, S-3 solvent systems are 0.46, 0.52, 0.33 respectively.

UV (MeOH, λ_{max}, nm); 204 (log ε: 5.0) 236 (log ε: 5.11).

FT-IR (KBr, cm^{-1}); 2986 (C-H, aromatic), 2962 (C-H, aliphatic), 1632 (C=O), 1600 (C=C, aromatic), 1247 (C-N).

^{1}H-NMR ($CDCl_3$, ppm); 2.35 (s, 2H, -N$\underline{CH_2}$Ph); 2.38 (s, 9H, $(CH_3)_3$); 2.55 (bs, 8H, piperazine H_2, H_6, benzhydrylpiperazine $H_{2'}$, $H_{6'}$); 3.18 (s, 2H, -NCH_2CO); 3.60 (t, 8H, piperazine H_3, H_5, benzhydrylpiperazine $H_{3'}$, $H_{5'}$ *J*=5.6 Hz); 4.20 (s, 1H, $(Ar)_2CH$); 6.84 (s, 1H, 2,4,6-trimethylphenyl); 7.0 (m, 4H, bis(fluoro)benzhydryl) H_2, H_6, $H_{2'}$, $H_{6'}$); 7.3 (m,4H, bis(fluoro)benzhydryl) H_3, H_5, $H_{3'}$, $H_{5'}$).

LC-MS (m/z); 547.8 (M^+); 549.1 (M^{+2}); 345.4 (2,4,6-$(CH_3)_3$-C_6H_5-CH_2-$N(CH_2CH_2)_2N$-CH_2CO-$N(CH_2CH_2)_2N$ไ$^+$); 203.1 (100%, (4-F-$C_6H_5)_2CH$ไ$^+$).

Elemental analysis of $C_{33}H_{40}F_2N_4O$ .H_2O (MW: 564.71 g/mol);

	% C	% H	% N
Calculated	70.19	7.50	9.92
Found	71.15	7.52	10.03

4.2. Pharmacological Studies

Cytotoxic activity results of synthesized molecules are given at Table 4.1.

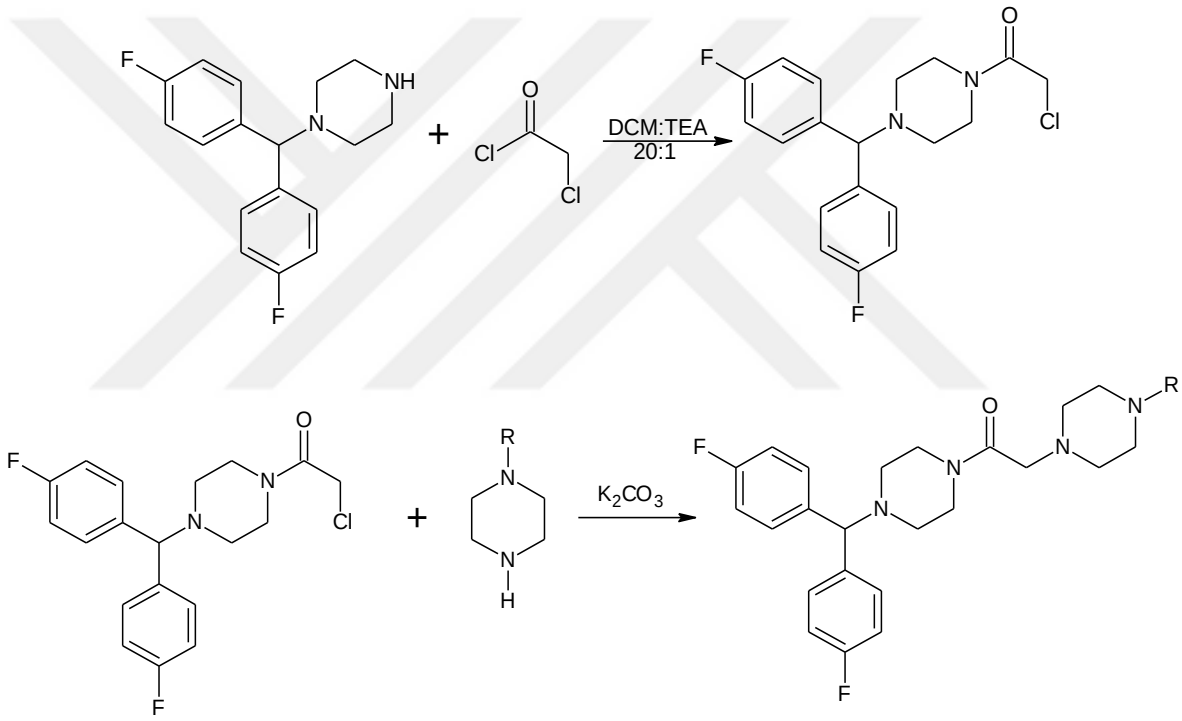
Table 4.1. GI_{50} values of compounds screened in liver (Huh7), breast (MCF-7) and colorectal (HCT-116) cancer cell lines by means of Sulforhodamine B assay.

Compound	R	Cancer Cell Line (GI_{50}, µM)*		
		Huh7	**MCF-7**	**HCT-116**
1	*p*-Chlorophenyl	10.09	17.81	4.36
2	*o*-Hydroxyphenyl	9.57	20.74	10.37
3	*p*-Methylphenyl	15.75	103.69	14.13
4	*m*-Methylphenyl	9.60	15.56	9.48
5	*m*-Methoxyphenyl	7.04	16.19	10.07
6	Benzoyl	93.87	618.97	22.96
7	2,4-Dichlorobenzyl	8.87	14.00	8.66
8	2,6-Dichlorobenzyl	9.67	11.05	7.03
9	2,4,6-Trimethylbenzyl	11.40	18.27	6.50
5-Fluorouracil		18.67	3.51	30.66

* All tests were performed as triplicate ($0 < R^2 < 1$).

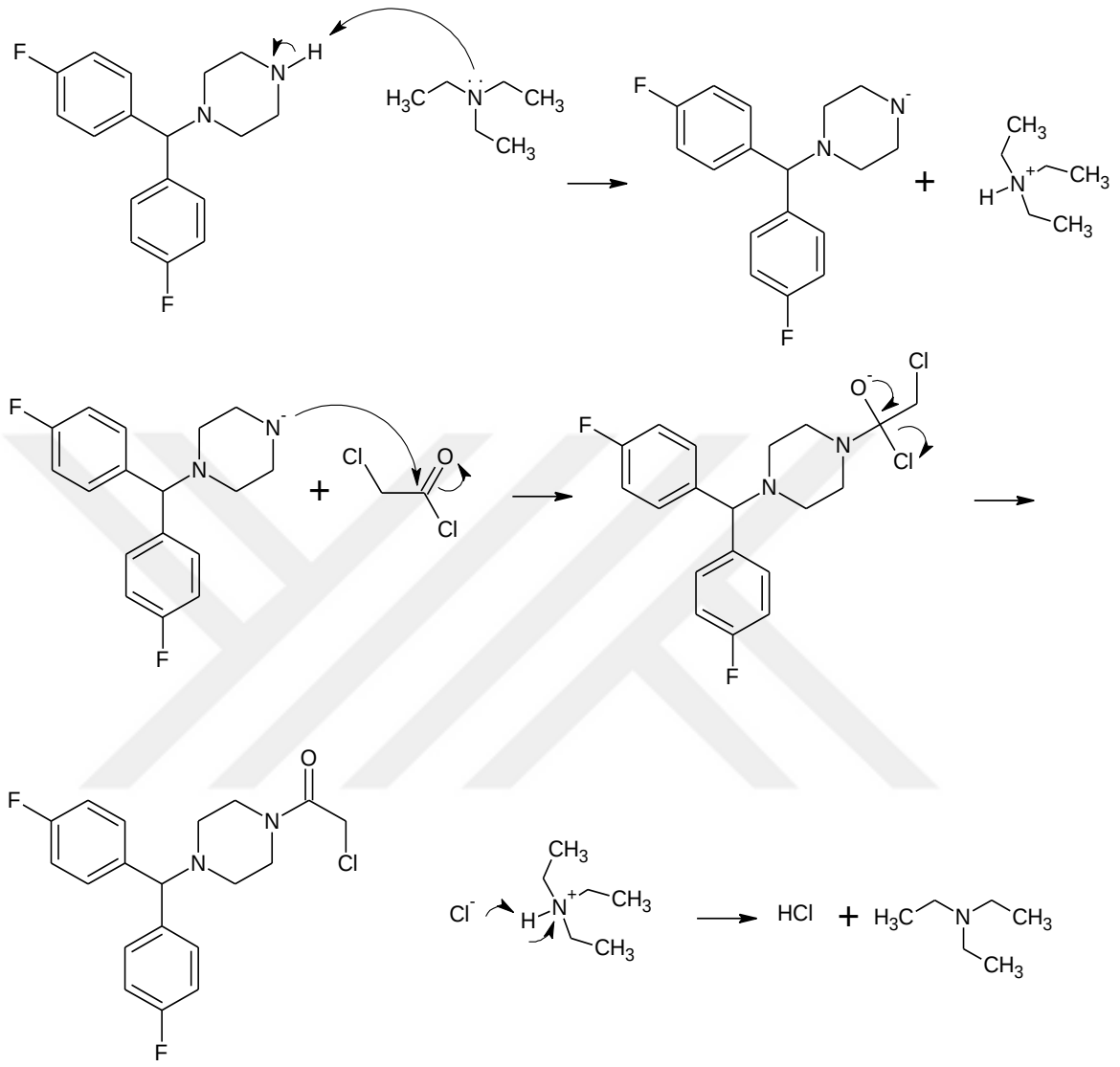
5. DISCUSSION AND CONCLUSION

In this study, 9 novel compounds having 1-[bis(4-fluorophenyl)methyl]piperazine derivatives were prepared and evaluated for their *in vitro* cytotoxic activity on breast (MCF-7), hepatocellular (Huh7) and colorectal (HCT-116) carcinoma cell lines. UV, IR, ^{1}H-NMR, ^{13}C-NMR, and mass spectra assisted the confirmation of the structures. In addition, purity of the compounds was determined by elemental analysis. The target compounds mentioned in this study were prepared according to the synthetic pathway depicted in scheme 5.1.



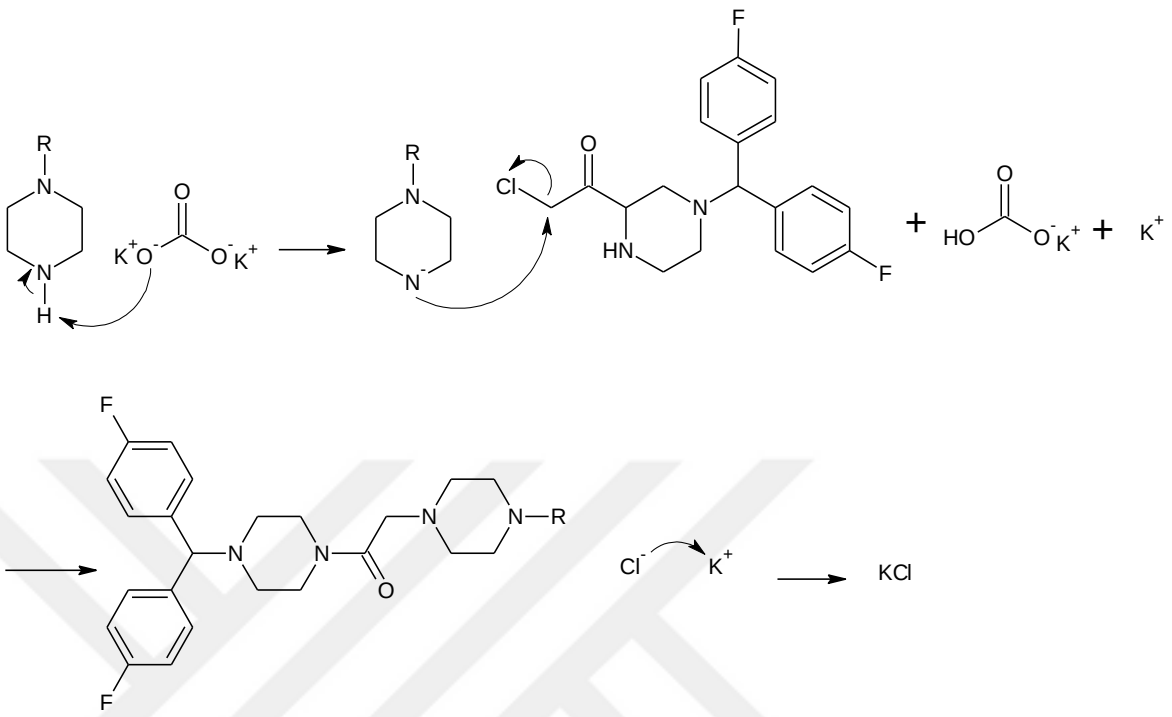
Scheme 5.1. General synthesis pathway of compounds.

N-(bis-4-fluorophenyl)methyl)piperazine was reacted with chloroacetyl chloride in alkaline medium to obtain *N*-(chloroacetyl)-*N*'-[bis(4-fluorophenyl)methyl]piperazine. Nucleophilic substitution reaction afforded oily product purified by column chromatography. *n*-Hexane:Ethyl acetate (1:1) mobile phase and silica gel stationary phase yielded pure starting compound.



Scheme 5.2. Proposed reaction mechanism of *N*-acetylation

N-(chloroacetyl)-*N*'-bis(4-fluorophenyl)methyl)piperazine was reacted with piperazine derivatives in alkaline medium in order to prepare *N*-[2-(4-substitutedpiperazin-1-yl)acetyl]-*N*'-[bis(4-fluorophenyl)methyl]piperazines. *N*-alkylation reaction afforded target compounds in yields of 20-60%.



Scheme 5.3. Proposed reaction mechanism of *N*-alkylation

Following the synthesis of compounds, elucidation of their structures with spectral analysis was carried out. Spectral data are found to be in accordance with expected structures. Compound **1** is chosen as the representative structure for characterization of compounds in the series.

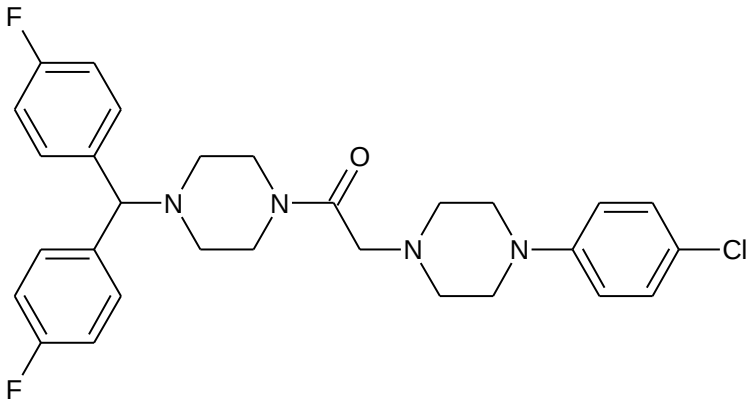


Figure 5.1. Structure of compound **1**

In UV spectra of compound **1** there are two significant bands at 225 nm (log ε: 4.76) and 269 nm (log ε: 4.67) which represent π → π* transition of aromatic rings and n → π* transition of amide carbonyl group.

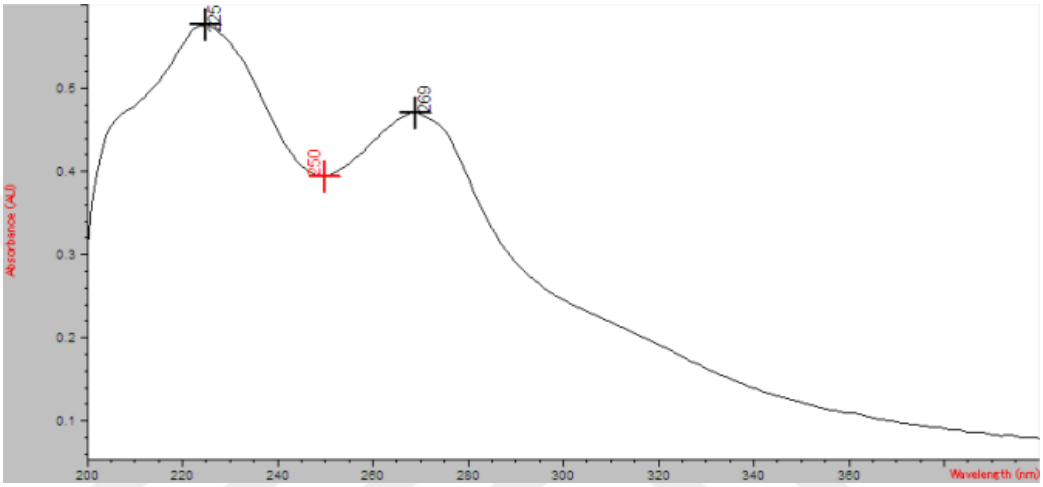


Figure 5.2. UV spectrum of compound **1**

IR spectrum of compound **1** shows aryl C-H stretching frequencies at 3011 cm^{-1}. Amide carbonyl wavenumber corresponds to 1640 cm^{-1}. Other signals are observed as following: 2970 cm^{-1} (C-H; aliphatic), 1601(C=C; aromatic) and 1241 cm^{-1} (C-N).

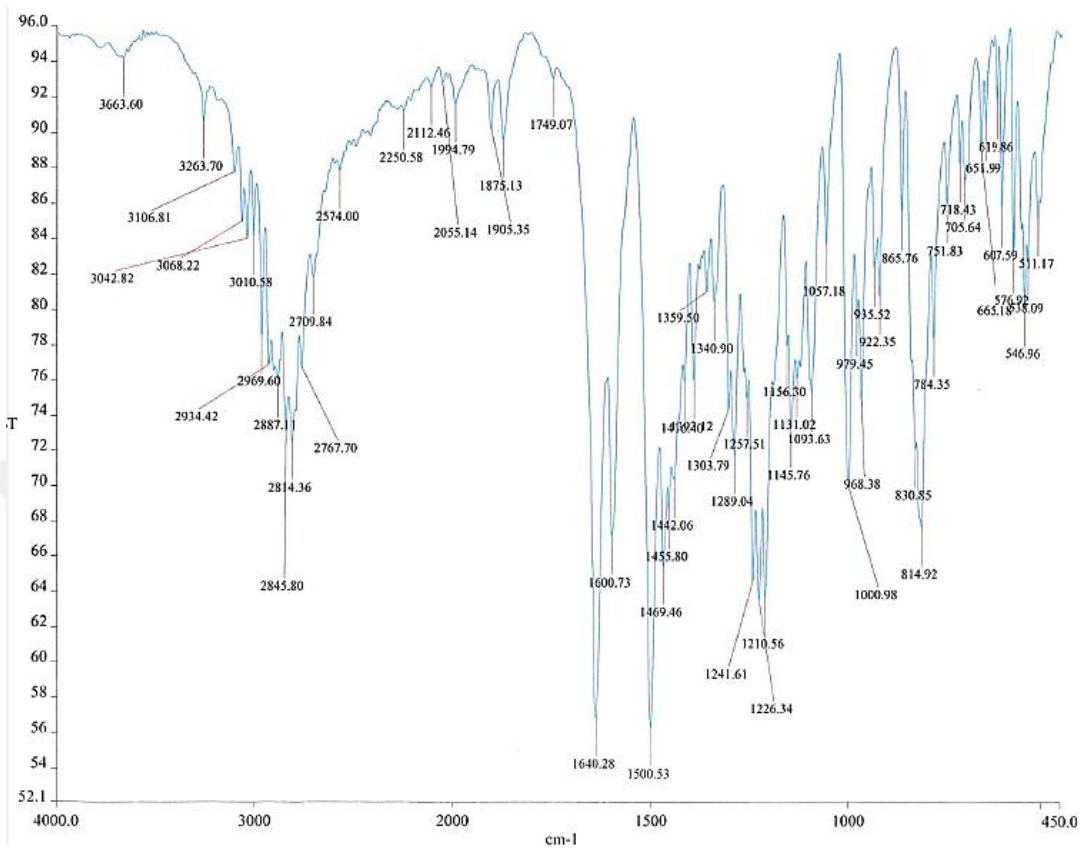
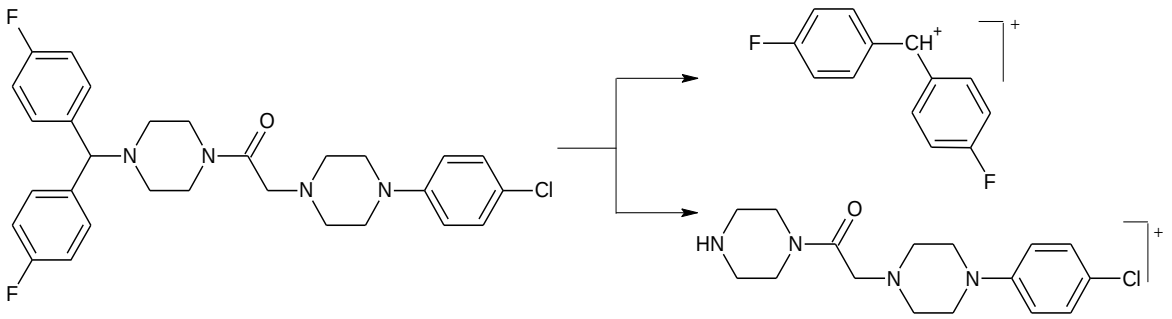


Figure 5.3. IR spectrum of compound **1**

Mass spectra of benzhydrylpiperazine derivatives are illustrated with compound **1.** M^+ peak is observed at 525.6 (m/z) and fragmentation products give peaks at 203 (m/z) (100%) and 323 (m/z). M^{+2} peak is also detected at 527.9 (m/z) which corresponds to chloride isotope. The fragmentation pattern is illustrated in scheme 5.3.



Scheme 5.4. Mass fragmentation pattern of compound **1.**

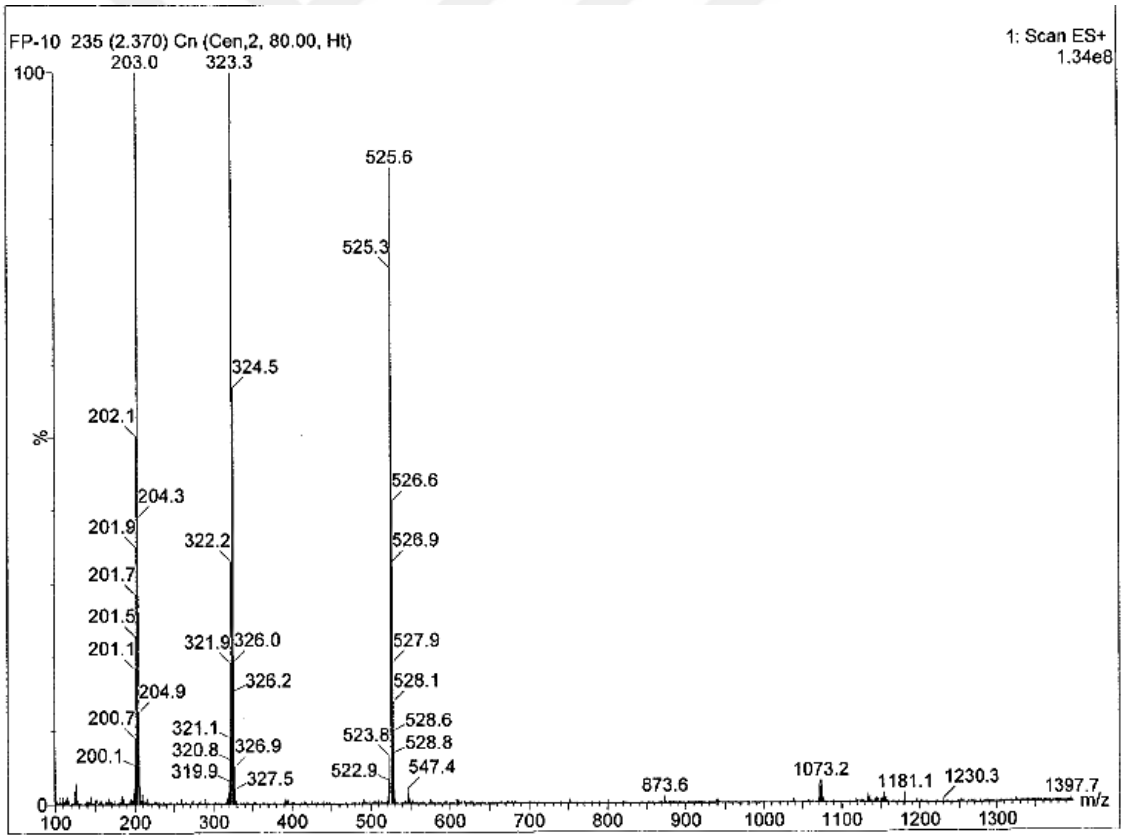


Figure 5.4. Mass spectrum of compound **1**.

^{1}H-NMR spectra of benzhydrylpiperazine derivatives are represented with compound **1.** Protons of piperazine are seen at 2.35 (t, 4H, H^{3}) ppm, 2.63 (t, 4H, H^{1}) ppm, 3.13 (t, 4H, H^{4}), and 3.62 (t, 4H, H^{2}) respectively. Carbonyl methylene gives singlet 3.21 ppm. Diphenylmethyl C-H gives singlet at 4.22 ppm. Aromatic rings give at 6.82-7.35 ppm.

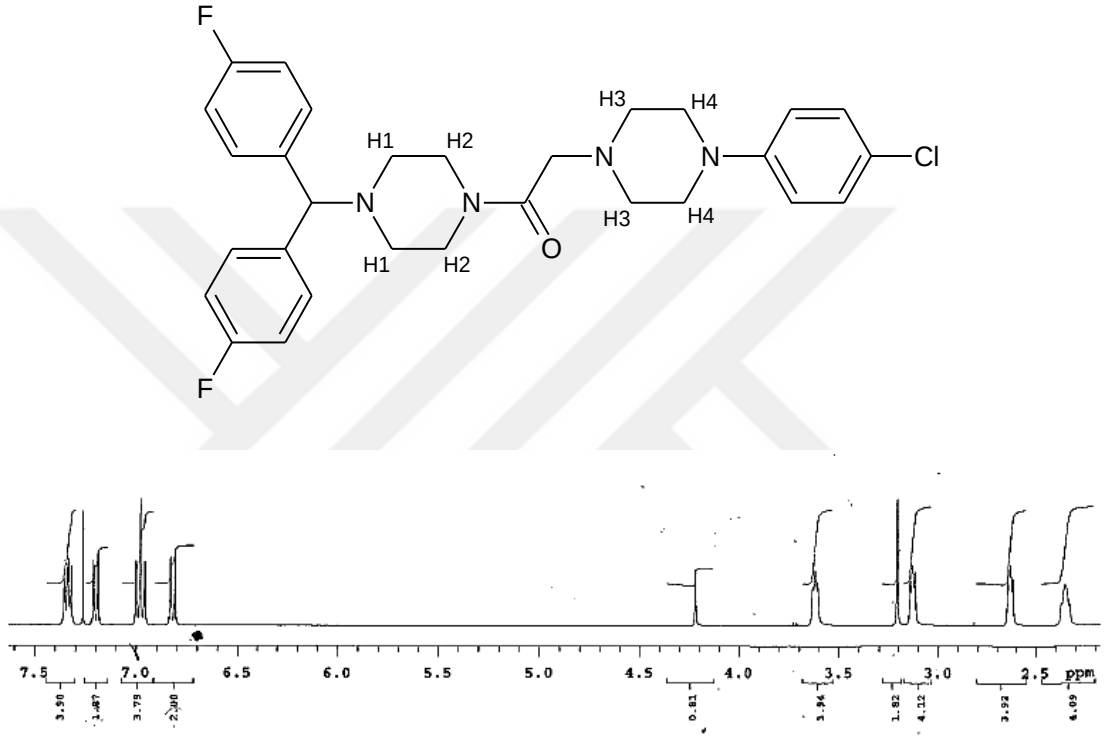


Figure 5.5. ^{1}H-NMR spectrum of compound **1.**

The ^{13}C-NMR spectrum of the compound **1** was taken in $CDCl_3$. Characteristic peaks of benzhydrylpiperazine derivatives were observed at 49, 51.64, and 52.11, 52.91 ppm for piperazine rings, 74.29 ppm for diphenylmethyl carbon and 167.67 ppm for carbonyl group.

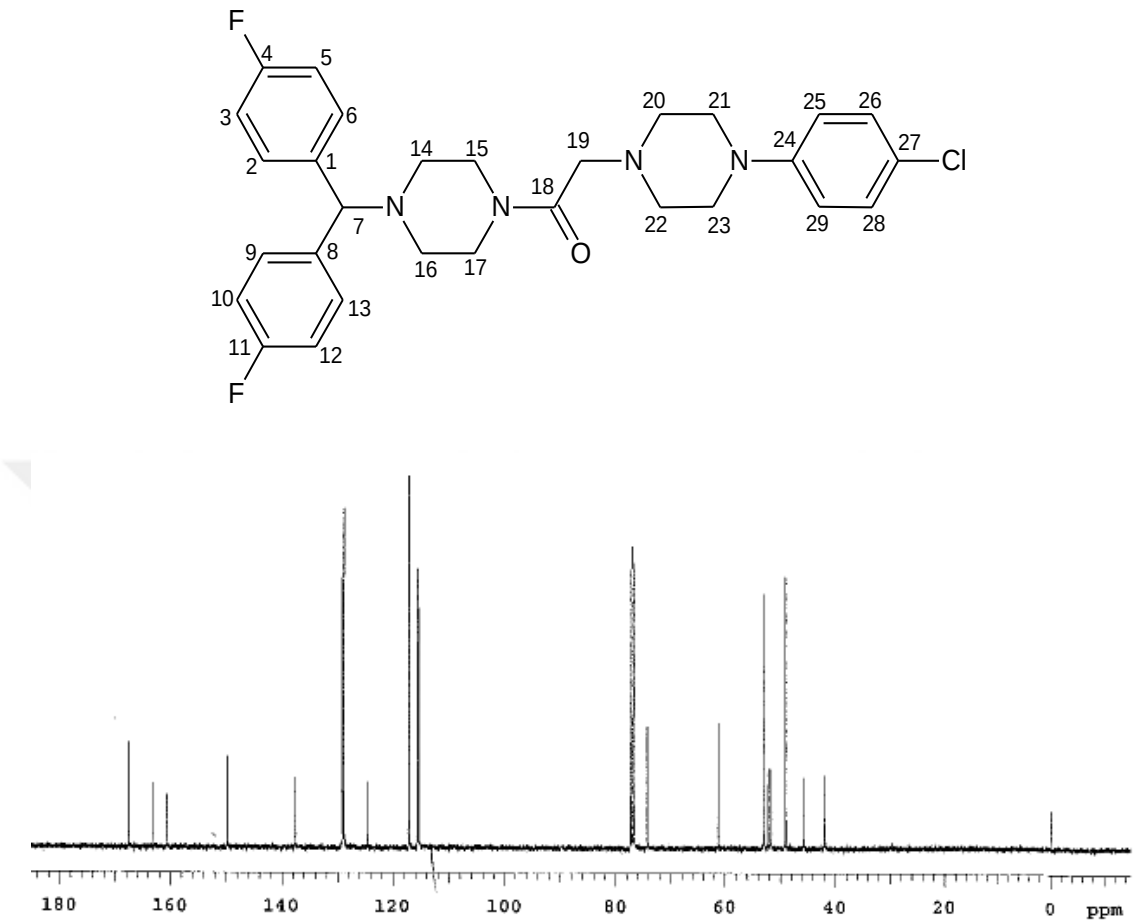


Figure 5.6. ^{13}C-NMR spectrum of compound **1.**

C	Chemical Shift (δ)	C	Chemical Shift (δ)
1	149.78	16	49.0
2	128.92	17	51.64
3	129.15	18	167.67
4	163.12	19	61.23
5	129.15	20	52.11
6	128.92	21	52.91
7	74.29	22	52.11
8	149.78	23	52.91
9	128.92	24	137.6
10	129.15	25	117.9
11	163.12	26	124.59
12	129.15	27	160.67
13	128.92	28	124.59
14	49.0	29	117.19
15	51.64		

All of the synthesized compounds have been evaluated for *in vitro* cytotoxic activity by Sulforhodamine B (NCI-SRB) assay. The results are given in Table 4.1.

In general, most of the compounds are more cytotoxic than 5-fluorouracil against hepatocellular (Huh7) and colorectal (HCT-116) cancer cell lines. However, the activity against breast cancer cell line (MCF-7) is not as good as 5-fluorouracil for none of the compounds.

The most active compound for Huh7 cell line is *m*-methoxyphenyl derivative (compound **5**; GI_{50} = 7.04 μM). Other compounds with GI_{50} values lower than 10 μM have hydroxyphenyl, methylphenyl, dichlorobenzyl groups on piperazine (compounds **2**, **4**, **7**, **8**). Interestingly, *p*-methyl substituted compound **3** (GI_{50} = 15.75 μM) has lower cytotoxicity than *m*-methyl substituted compound **4** (GI_{50} = 9.60 μM).

In breast (MCF-7) cancer cell line, all benzhydrylpiperazine derivatives are less cytotoxic than 5-fluorouracil (5-FU). In similarity with Huh7 results, *m*-methylphenyl derivative (compound **4**; GI_{50} = 15.56 μM) has higher cytotoxicity than *p*-methylphenyl derivative (compound **3**; GI_{50} = 103.69 μM).

In colorectal (HCT-116) cancer cell line, all of the synthesized compounds have higher cytotoxicity activity than 5-fluorouracil, and the most active compound is *p*-chlorophenyl derivative (compound **1**; GI_{50} = 4.36 μM). Other compounds with GI_{50} values lower than 10 μM is benzylpiperazine derivatives (compounds **7**, **8**, **9**) along with *m*-methylphenyl substituted compound **4**. In accordance with previous cell lines, *m*-methylphenyl derivative (compound **4**; GI_{50} = 9.48 μM) has higher cytotoxicity than *p*-methylphenyl derivative (compound **3**; GI_{50} = 14.13 μM).

In summary, we have synthesized nine original benzhydrylpiperazine derivatives which have been tested for their cytotoxic activities on several cell lines of breast (MCF-7), hepatocellular (Huh7), and colorectal (HCT-116) cancer families. In order to obtain a rational structure activity relationship, compound set should be enlarged as a future plan. In addition, the cytotoxicity mechanism will be enlightened for the active compounds.

6. REFERENCES

1) http://www.cancer.gov./cancertopics/cancerlibrary/what-is-cancer/ 6.03.2016
2) http://www.cancer.gov/about-cancer/ causes-prevention/risk/ 6.03.2016
3) http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2016/ 14.11.2016.
4) Callery p, Gannett P. *Cancer and Cancer Chemotherapy*. In: Williams DA, Lemke TL (ed). Foye `s Principles of Medicinal Chemistry, 5th ed.; Lippincott Williams &Wilkins, USA, PP; 2002: 924-951.
5) https://www.scripd.com/ducument/240665690/Chapter-4-Mechanisms-of-Anticancer-Drugs /20.01.2017.
6) Al-Soud YA, Al-Masoudi NA. DNA-directed alkylating agents: synthesis, antitumor activity and DNA affinity of bis-N,N`-trisubstituted 1,2,4-triazolo-piperazines. *II Farmaco*. 2004; 59:41-46.
7) Guo C, Tong R, and Li K. Chloroalkyl piperazine and nitrogen mustard porphyrins: synthesis and anticancer activity. *Bioorg Med Chem*. 2004; 12:2469-2475.
8) Kamal A, Ramu R, Tekumalla V, Khanna G, Barkume M, Juvekar A, Zingde S. Remarkable DNA binding affinity and potential anticancer activity of pyrrolo [2,1-c][1,4]benzodiazepine-naphthalimide conjugates linked through piperazine side-armed alkane spacers. *Bioorg Med Chem*. 2008; 16: 7218-7224.
9) Iqbal N, Iqbal N. Imatinib: A breakthrough targeted therapy in cancer. *Chemother Res Pract*, 2014; 2014.
10) Albro LP, Baltzly R, Phillips AP. Unsymmetrically Disubstituted Piperazines II. Histamine Antagonist. *J Org Chem*, 1949; 14: 771-774.
11) Baltzly R, DuBreuil S, Ide WS, Lorz E. Unsymmetrically disubstituted piperazines III. *N*-methyl-*N'*-benzhydrylpiperazines as histamine antagonists. *J Org Chem***.** 1949; 14: 775-782.
12) Iemura R, Kawashima T, Fukuda T, Ito K, Tsukamoto G. Synthesis of 2-(4-substituted-1-piperazinyl)benzimidazoles as H_1-antihistaminic agents. *J Med Chem*. 1986; 29: 1178-1183.

13) Shaquiquzzaman M, Verma G, Marella A, Akhter M, Akhtar W, Khan MF, Tasneem S, Alam MM. Piperazine Scaffold: A remarkable tool in generation of diverse pharmacological agents. *Eur J Med Chem*, 2015; 102: 487-529.

14) Kumar C, Prasad S, Vinaya K, Chandrappa S, Thimmegowda N, Kumar Y, Swarup S, Rangappaa K. Synthesis and in vitro antiproliferative activity of novel 1-benzhydrylpiperazine derivatives against human cancer cell lines. *Euro J Med Chem*. 2009; 44: 1223-1229.

15) Huang W, Liu M, Li Y, Tan Y, Yang G. Design, synthesis and antitumor activity of novel chromone and aurone derivatives. *Bioorg Med Chem*, 2007; 15: 5191-5197.

16) Huang W, Ding Y, Miao Y, Liu M, Li Y, Yang G. Synthesis and antitumor activity of novel dithiocarbamate substituted chromones. *Eur J Med Chem*. 2009; 44: 3687-3696.

17) Gan L, Fang B, Zhou C. Synthesis of azole-containing piperazines derivatves and evaluation of their antibacterial, antifungal and cytotoxic activities. *B Kor Chem Soc*. 2010; 31: 3684-3692.

18) Gurdal EE, Durmaz I, Cetin-Atalay R, Yarim M. Synthesis and cytotoxicity studies of novel benzhydrylpiperazine carboxamide and thiamide. *J Enzyme Inhib Med Chem*. 2014; 29(2): 205-214.

19) Gurdal EE, Yarim M, Durmaz I, Cetin-Atalay R. Cytotoxic Activities of some Novel Benzhydrylpiperazine Derivatives. *Drug Res*. 2013; 63: 121-128.

20) Naveen S, Ananda Kumar CS , Manjunath HR , Vinaya K, Benaka Prasad SB, Sridhar MA, Rangappa KS, Shashidhara Prasad J. Crystal and molecular stracture of a novel 1-benzhydrylpiperazine derivative: 1-benzhydryl-4-(4-chloro-2-fluoro-benzene-sulfonyl)-piperazine. *Mol. Cryst. Liq. Cryst*. 2009; 503: 151-158.

21) Theophil-Eicher SH. *The Chemistry of Heterocycles Structures*, Reactions, Synthesis and Applications. Wiley-Vch, 2003.

22) Windholz M (ed). The Merck Index, 10th ed.; Merck& Co., Inc.: USA, p 1076, 1983.

23) Kitchen L, Pollard CB. Derivatives of piperazine. XXI. Synthesis of piperazine and C-substituted piperazines. *J Am Chem Soc*. 1947; 69:854-855.

24) Martin WB, Martell AE. Preparation of piperazine. *J Am Chem Soc*.1948; 70:1817-1818.

25) Kyrides LP, Groves W, Manufacture of piperazine, US-2267686, 1941.

26) Katrizky AR (ed). *Advances in Heterocyclic Chemistry, Academic Press*, Vol. 15, 1973.

27) Sebastian S, Patel HV, Thennati R. Method for preparation of piperazine and its derivatives, US-6603003, 2003.

28) http://www.sigmaaldrich.com, 12.12.2016.

29) http://riodb01. ibase.aist.go.jp\sdbs\cgi-bin\direct_frame_top.cgi, 12.12.2016.

30) Kalai T, Khan M, Balog M, Kutala VK, Kuppusamy P, Hideg K. Stracture-activity studies on the protection of Trimetazidine derivatives modifies with nitroxides and their precursors from myocardial ischemia-reperfusion injury. *Bioorg Med Chem*. 2006; 14: 5510-5516.

31) Millan MJ, Hjorth S, Samanin R, Schireiber R, Jaffard R, DeLadonchamps B, Veiga S, Goument B, Peglion J, Spending M, Brocco M. S15535, a novel benzodioxopiperazine ligand of serotonin (5-HT)(1A) receptors. Modulation of hippocampal serotonin release in relation to potential anxiolytic properties. *J pharmacol Exp Ther*. 1997; 282:148-161.

32) Chaki S, Hirota S, Funakoshi T, Suzuki Y, Suetake S, Okubo T, Ishii T, Nakazato A, Okuyama S. Anxiolytic-like and antidepressant-like activites of MCL0129 (1-[(S)-2-(4-fluorophenyl)-2-(isopropylpiperadin-1-yl)ethyl]-4-[4-(2-methoxynaphthalen-1-yl)butyl]piperazine), a novel and potent nonpeptide antagonist of melanocortin-4-receptor. *J pharmacol Exp Ther*. 2003; 304: 818-826.

33) Tandon R, Nasrallah H, Keshavan M, Schizophrenia “just the fact” 5. Treatment and prevention past, present and future. *Schiozphr Res*. 2010; 122:1-23.

34) Srinivas P, Subramanian A, Brust P, Raghavan S, Rangisetty J, Gupta C, Sridhar N, Veeranjaneyulu A, Parimoo P. Synthesis and preliminary pharmacological investigations of 1-(1,2-dihydro-2-acenaphthylenyl)piperazine derivatives as potential atypical antipsychotic agents in mice. *Farmaco*. 1999; 54: 567-572.

35) Saxena M, Gaur S, Prathipati P, Saxena A. Synthesis of some substituted pyrazinopyridoindoles and 3D QSAR studies with related compounds: piprezines, piperidines, pyrazinoisoquinolines, and diphenylhydramine, and its semi-rigid analogs as antihistamines (H-1). *Bioorg Med Chem*. 2006; 14: 8249-8258.

36) Walczynski K, Guryn R, Zuiderweld O, Timmerman H. Non-imidazole histamine H-3 ligands. Part I. Synthesis of 2-(1-piperazinyl)-and 2-(hexahydro-1H-1,4-diazepin-1-yl)benzothiazole derivatives as H-3-antagonists with H-1 blocking activities. *Farmaco*. 1999; 54: 684-694.

37) Park Choo HY, Chung BJ, Chung SH. Synthesis of piperazine derivatives and evaluation of their antihistamine and antibradykinin effects. *Bioorg Med Chem Lett*. 1999; 9: 2727-2730.

38) Kerns RJ, Rybak MJ, Kaatz GW, Vaka F, Cha R, Grucz RG, Diwadkar VU, Ward TD. Piperazinyl-linked fluroquinolone dimers possessing potent antibacterial activity against drug-resistant strains of *staphylococcus aureus. Bioorg Med Chem Lett*. 2003; 13: 1745-1749.

39) Foroumdi A, Soltani F, Moshafi MH, Asharf-Askari R. Synthesis and in vitro antibacterial activity of some *N*-(5-aryl-1,3,4-thiadiazole-2-yl) piperazinyl quinolone derivatives. *II Farmaco*. 2003; 58:1023-1028.

40) Foromudi A, Emami S, Mansouri S, Javidnia A, Saeid-Adeli N, Shirazi F, Shafiee A. Synthesis and antibacterial activity of levofloxacin derivatives with certain bulky residues on piperazine ring. *Eur J Med Chem*. 2007; 42: 985-992.

41) Thomas KD, Adhikari AV, Chowdhury IH, Sumesh E, Pal NK. New quinolone -4-yl-1,2,3-triazoles carrying amides, sulphonamides, and amidopiperazines as potential antitubercular agents. *Eur J Med Chem*. 2011; 46: 2503-2512.

42) Appelbaum PC, Hunter PA. The fluroquinolone antibacterial: past, present, and future perspectives. *Int J Antimicrob Ag*. 2000; 16: 5-15.

43) Upadhayaya RS, Sinha N, Jain S, Kishore N, Chandra R, Arora SK. Optically active antifungal azoles: synthesis and antifungal activity of (2R,3S)-2-(2,4-diflurophenyl)-3-(5-{2-[4-arryl-piperazin-1-yl]-ethyl}-tetrazol-2-yl/1-yl)-1-[1,2,4]-triazol-1-yl-butan-2-ol. *Bioorg Med Chem*. 2004; 12: 2225-2238.

44) Srinivasan S, Beema-Shafreen RM, Nithyanand P, Manisankar P, Pandian SK. Synthesis and *in vitro* antimicrobial evaluation of novel fluroquinolone derivatives. *Eur J Med Chem*. 2010; 45: 6101-6105.

45) Kumar R, Kumar A, Jain S, Kaushik D. Synthesis, antibacterial evaluation and QSAR studies of 7-[4(5-aryl-1,3,4-oxadiazole-2-yl)piperazinyl]quinolone derivatives. *Eur J Med Chem*. 2011; 46: 3543-3550.

46) Ranise A, Spallarossa A, Bruno O, Schenone S, Fossa P, Menozzi G, Bondavalli F, Mosti L, Capuano A, Mazzeo F, Falcone G, Filipelli W. Synthesis of *N*-substituted-*N*-acylthioureas of 4-substituted piperazines endowed with local anesthetic, antihyperlipidemic, antiproliferative activities and antiarrhythmic, analgesic, antiaggregating actions. *II Farmaco*. 2003; 58:765-780.

47) LeeY, Gong Y, Yoon H, Ahn C, Jeon M, Kong J. Synthesis and anticancer activity of new 1-[(5-or 6-substituted 2-alkoxyquinoxalin-3-yl)aminocarbonyl]-4-(hetero)arylpiperazine derivatives. *Bioorg Med Chem*. 2010; 18: 7966-7974.

48) Patel RV, Kumari P, Rajani DP, Chikhalia KH. Synthesis and studies of novel 2-(4-cyno-3-trifluromethylphenylamino)-4-quinoline-4-yloxy)-6-piperazinyl/ piperidinyl)-s-triazines as potential antimicrobial, antimycobacterial and anticancer agents. *Eur J Med Chem*. 2011; 46:4354-4365.

49) Hamlin KE, Weston AW, Fischer FE, Micheals RJ. Histamine antagonists. II.1 Unsymmetrical 1,4-disubstituted piperazines. *J Am Chem Soc*. 1949; 71: 2731-2734.

50) Plobeck N, Delorme D, Wei Z, Yang H, Zhou F, Schwarz P, Gawell L, Gagnon H, Pelcman B, Schmidt R, Yue S, Walpole C, Brown W, Zhou E, Labarre M, Payza K, ST-Onge S, Kamassah A, Morin P, Projean D, Ducharme J, Roberts E. New diarylmethylpiperazines as potent and selective nonpeptidic delta opioid receptors agonists with increased in *vitro* metabolic stability. *J Med Chem*. 2000; 43: 3878-3894.

51) Chern J, shia K, Hsu T, Tai C, Lee C, Lee Y, Chang C ,Tseng S, Shih S. Design, Synthesis, and Structure activity-relation-ships of pyrazolo[3,4-d]pyrimidinies: a novel class of potent enterovirus inhibitors. *Bioorg Med Chem Lett*. 2004; 14:2519-2525.

52) Beck K, Hamlin K, Weston A, Histamine antagonist. IV. C-methyl derivatives of 1,4-disubstituted piperazines. *J Am Chem Soc*. 1952; 74: 605-608.

53) Chen C. Physiochemical, pharmacological and pharmacokinetic properties pf the zwitterionic antihistaminic cetirizine and levocetirizine. *Curr Med Chem*.2008; 15:2173-2191.

54) Song K, Lee S, Chun H, Kim J, Jug M, Ahn K, Kim S, Kim J, Lee J. Design, synthesis and biological evaluation of piperazine analogues as CB1 cannabinoid receptors ligands. *Bioorg Med Chem*. 2008; 16:4035-4051.

55) Vinaya K, Naveen S, Kumar C, Benkaprasad S, Sridhar M, Prasad J, Rangappa K. \Synthesis, characterization, crystal and molecular stracture analysis of a novel 1-benzhydryl piperazine derivative: 1-benzhydryl-4-(2-nitro-benzensulfonyl)-piperazine. *Struct Chem*. 2008; 19: 765-770.

56) Kulig K, Wieckowski K, Wieckowska A, Gajda J, Pochwat B, Hofner G, Wanner K, Malawska B. Synthesis and biological evaluation of new derivatives of 2-substituted 4-hydroxybutanamides as GABA uptake inhibitors. *Eur J Med Chem*. 2011; 46: 183-190.

57) Nelson WL, Antihistamines Related Antiallergic and Antiulcer Agents. In Williams DA, Lemke TL (eds). *Foye's Princibles of Medecinal Chemistry*, Lippincott Williams& Wilkins: USA; 2002: 794-818.

58) Wang L, Wang T, Yang B, Chan Z, Yang H. Design, synthesis, and anti-allergic activities of novel (R)(-)-1-[(4-chlorophenyl)phenylmethyl]piperazine derivatives. *Med Chem Res*. 2010; 21:124-132.

59) Paul R, Brockman JA, Hallett WA, Tarrant ME, Torley LW, Callahan FM, Fabio PF, Johnson BD, Lenhard RH, Schaub RE, Wissner A. Imidazol[1,5-d][1,2,4]triazines as potential antiasthma agents. *J Med Chem*. 1985; 28: 1704-1716.

60) Sasse BC, Mach UR, Leppaenen J, Calmels T, Stark H. Hybrid approach for the design of highly affine and selective dopamine D-3 receptor ligands using privileged scaffolds of biogenic amine GPCR ligands. *Bioorg Med Chem*. 2007; 15: 7258-7273.

61) Jung J, Jung S, Koh H. Asymmetric synthesis of chiral piperazinylpropylisoxazoline ligands for dopamine receptors. *Eur J Chem*. 2007; 42: 1044-1048.

62) Zamponi G, Feng Z, Zhang L, Pajouhesh H, Ding Y, Belardetti F, Pajouhesh H, Dolphin D, Mitscher L, Snutch T. Scaffold-based design and synthesis of potent *N*-type calcium channel blockers. *Bioorg Med Chem Lett*. 2009; 19: 6467-6472.

63) Lee J, Koh H, Seo S, Beak Y, Rhim H, Cho Y, Choo H, Pea A. Synthesis and biological evaluation of oxazole derivatives as T-type calcium channel blockers. *Bioorg Med Chem Lett*. 2010; 20: 4219-4222.

64) Borzenko A, Pajouhesh H, Morrison J, Tringham E, Snutch T, Schafer L. Modular efficient synthesis of asymmetrically substituted piperazine scaffold as potent calcium channel blockers. *Bioorg Med Chem Lett*. 2013; 23: 3257-3261.

65) Bertini S, Ghilardi E, Asso V, Granchi C, Minutolo F, Pineschi M, Di-Bussolo V, Bortolato A, Moro S, Saba A, Macchia M. BACE1 inhibatory activities of enantiomerically pure, variously substituted *N*-(3-(4-benzhydrylpiperazine-1-yl)-2-hydroxypropyl) arylsulfonamides. *Bioorg Med Chem*. 2010; 18: 7991-7996.

66) Meng T, Wang J, Peng H, Fang G, Li M, Xiong B, Xie X, Zhang Y, Wang X, Shen J. Discovery of benzhydrylpiperazine derivatives as CB1 receptor inverse agonist via privileged stracture-based approach. *Eur J Med Chem*. 2010; 45:1133-1139.

67) Gao L, Li M, Meng T, Peng H, Xie X, Zhang Y, Jin Y, Wang X, Zou L, Shen J. Asymmetric synthesis and biological evaluation of *N*-cyclohexyl-4-[1-(2,4-dichlorophenyl)-1-(p-tolyl)methyl] piperazine-1-carboxamide as hCB_1 receptors antagonists. *Eur Med Chem*. 2011; 46:5310-5316.

68) Kucwaj-Brysz K, Warszycki D, Podlewska S, Witek J, Witek K, Gonzalez Izquierdo A, Satala G, Loza MI, Lubelska A, Latacz G, Bojarski A, Castro M, Kiec-Kononowicz K, Handzlik J. Rational design in search for 5-phenylhydantoin selective 5-HT_7R antagonist. Molecular modeling, synthesis and biological evaluation. *Eur J Med Chem*. 2016; 112: 258-269.

69) Tassoni E, Giannessi F, Dell`Uomo N, Gallo G. Inhibitors of CPT in the central nervous system as antidiabetic and\ or antiobesity drugs. WO-2007096251, 2007.

70) Dinakaran M, Senthilkumar P, Yogeeswari P, China A, Nagaraja V, Sriram D. Antimycobacterial activities of novel 2-(sub)-fluro/nitro-5,12-dihydro-5-oxo-

benzothiazolo[3,2-a]quinolone-6-carboxylic acid. *Bioorg Med Chem*. 2008; 16:3408-3418.

71) Punkvang A, Saparpakorn P, Hannongbua S, Wolschann P, Berner H, Pungpo P. Insight into crucial inhibitors-enzyme interaction of arylamides as novel direct inhibitors of the enoyl ACP reducates (InhA) from *mycobacterium tuberculosis*: computer-aided molecular design. *Monatsh Chem*. 2010; 141: 1029-1041.

72) Uapdhayaya R, Vandavasi J, Kardile R, Lahore S, Dixit S, Deokar H, Shinde P, Sarmah M, Chattopadhyaya J. Novel quinoline and naphthalene derivatives as potent antimycobacterial agents. *Eur J Med Chem*. 2010; 45: 1854-1867.

73) Kumar C, Vinaya K, Chandra N, Thimmegowda N, Prasad S, Sadashiva C, Rangappa K. Synthesis and antimicrobial studies of novel 1-benzhydryl-piperazine sulfonamide and carboxamide derivatives. *J Enzyme Inhib Med Chem*. 2008; 23(4): 462-469.

74) Gan L, Fang B, Zhou C. Synthesis of azole-containing piperazine derivatives and evaluation of their antibacterial, antifungal and cytotoxicity activities. *B Kor Chem Soc*. 2010; 31: 3684-3692

75) Burgess S, Kelly J, Shomloo S, Wittlin S, Brun R, Liebmann K, Peyton D. Synthesis, Structure-activity relationship and mode-of-action studies of antimalarial reversed chloroquine compunds. *J Med Chem*. 2010; 53: 6477-6489.

76) Vitorovic-Todorovic M, Juranic I, Mandic L, Drakulic B. 4-Aryl-4-oxo-*N*-phenyl-2-aminobutyramides as acetyl- and butyrylcholinestrase inhibitors. Preparation, anticholinestrase activity, docking study, and 3D structure-activity relationship based on molecular interaction field. *Bioorg Med Chem*. 2010; 18: 1181-1193.

77) Tilley JW, Levitan P, Welton AF, Crowley HJ. Antagonists of slow-reacting substance of anaphylaxis 1. Pyrido[2,1-b]quinazolinecarboxylic acid derivatives. *J Med Chem*. 1983; 26: 1638-1642.

78) Chern J, Shia K, Hsu T, Tai C, Lee C, Lee Y, Chang C, Tseng C, Shih S. Design, synthesis, and structure-activity relationships of pyrazolo[3,4-d]pyrimidines: a novel class of potent enterovirus inhibitors. *Bioorg Med Chem Lett*. 2004; 14: 2519-2525.

79) Curreli F, Zhang H, Zhang X, Pyatkin I, Victor Z, Altieri A, Debnath A. Virtual screening based identification of novel small-molecule inhibitors targeted to the HIV-1 capsid. *Bioorg Med Chem*. 2011; 19: 77-90.

80) Chamoun-Emanuelli AM, Pecheur EI, Chen Z. Benzhydrylpiperazine compounds inhibit cholesterol-dependent cellular entry of hepatitis C virus. *Antiviral research*. 2014; 109: 141-148.

81) Kimura M, Masuda T, Yamada K, Kawakatsu N, Kubota N, Mitani M, Kishii K, Inazu M, Kiuchi Y, Oguchi K, Namiki T. Antioxidative activities of novel diphenylalkyl piperazine derivatives with high affinities for the dopamine transporter. *Bioorg Med Chem Lett*. 2004; 14: 4287-4290.

82) Dhainaut A, Regnier G, Atassi G, Pierre A, Leonce S, Kraus-Berthier L, Prost JF. New triazine derivatives as potent modulators of multidrug resistance. *J Med Chem*. 1992; 35: 2481-2496.

83) Nowaczyk A, Kulig K, Malawska B. 1-(3-(4-Arylpiperazin-1-yl)propyl)-pyrrolidin-2-one derivatives as alpha(1)-adrenoceptor antagonists: A QSAR study. *QSAR Comb Sci*. 2009; 28: 979-988.

84) Santhoshi A, Kumar SN, Sujitha P, Poornachandra Y, Sadhu PS, Kumar CG, RaoVJ. Synthesis of 1-benzhydrylpiperazine derivatives and evaluation of their ACE inhibition and antimicrobial activities. *Med Chem Res*. 2014; 23: 3207-3219.

85) Mavrova A, Anichina K, Vuchev D, Tsenov J, Denkova P, Kondeva M, Micheva M. Antihelminthic activity of some newly synthesized 5(6)-(un)substituted-1H-benzimidazol-2-ylthioacetylpiperazine derivatives. *Eur J Med Chem*. 2006; 41: 1412-1420.

86) Trevor AJ, Katzung BG, Kruidering-Hall MM, Masters SB. Chapter 54. Cancer Chemotherapy. In: Trevor AJ, Katzung BG, Kruidering-Hall MM, Masters SB. eds. *Katzung & Trevor's Pharmacology: Examination & Board Review, 10e*. New York, NY: McGraw-Hill; 2013.

87) Callery P, Gannett P. Cancer and Cancer Chemotherapy. In: Williams DA, Lemke TL (eds). *Foy`s Principles of medicinal chemistry, 5th ed.*; Lippincott Williams &Willkins, USA, 2002; pp 924-951.

88) Calabresi P, Chabner BA. Chemotherapy of Neoplastic Disease. In: Molinoff PB, Ruddon RW (eds). Goodman & Gliman's *The Pharmacological Basis of Therapeutics, 9th ed.*; McGraw-Hill: USA, 1996; pp 1225-1287.

89) Alberts B, Johanson A, Lewis J, Raff M, Roberts K, Walter B, Chapter 18. *Cell Cycle Control and Death. Essential Cell Biology*. pp 571. New York. NY: Garland Publishing; 1998.

90) Margolese RG, Hortobagyi GN, Buchholz TA. Neoplasms of the Breast. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast Jr RC, Gansler TS, Holland JF, Frei III E (eds). *Cancer Medicine-6,* Spain, Vol.2, pp 1879-1997, 2003.

91) Boyle P, Levin B, Karaciğer Kanseri. In: *Dunya Kanser Raporu*. WHO Press: France, pp 350-357, 2008.

92) Engstrom PF, Sigurdson ER, Evans AA, Pingpank JF. Primary Neoplasms of the Liver. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast JRC, Gansler TS, Holland JF, Frei IE (eds). *Cancer Medicine-6,* 6th ed, BC Decker Inc.: Spain, Vol. 2, 2003; pp 1543-1553.

93) Boyle P, Levin B, Kolorektal Kanser. *In Dünya Kanser Raporu*, WHO Press: France, 2008; pp 374-380.

94) Rodriguez-Bigas, MA, Lin EH, Crane CH. Adenocarcinoma of the Colon and Rectum. In: Kufe DW, Pollock RE, Weichselbum RR, Bast JRC, Gansler TS, Holland JF, Feri IE (eds). *Cancer Medicine-6,* 6thed, BC Decker Inc.; Spain, Vol. 2, 2003; pp 1635-1667.

95) Larsen IK, Kastrop JS. Anticancer Agent. In Krogsgaard-Larsen P, Liljefors T, Madsen U, (eds). *Textbook of Drug Design and Discovery*, 3rd ed.; Taylor & Francis, Malta. 2002; pp 511-558.

96) Scudiero AD, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/ formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Research*. 1998; 48: 4827-4833.

97) Houghton P, Fang R, Techatanawat I, Steventon G, Hylands P, Lee C. The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and

derived compounds for activities related to reputed anticancer activity. *Method*. 2007; 42: 377-387.

98) Fotakis G, Timbrell J. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett*. 2006; 160: 171-177.

99) Celis JE, Longo-Sorbello GSA, Saydam G, Banerjee D, Bertino JR. *Cell Biology*: A Laboratory Handbook 3rd ed, chap.38, Elsevier: USA, Vol.1, 2006.

100) Niles A, Moravec R, Riss T. Update on *in vitro* cytotoxicity assays for drug development. *Expert Opin Drug Discovery*. 2008; 3: 655-669.

101) Kim HM, Han SB, Kim MS, Kang JS, Oh JT, Hong DH. Efficient Fixation Procedure of Human Leukemia Cells in Sulforhodamine B Cytotoxicity Assay. *J Pharmacol Toxicol Methods*. 1996; 36: 163-169.

102) Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc*. 2006; 1: 1112-1116.

103) Hamlyn RJ, Huckstep MR, Lynch R, Stokes S, Tickle DC, Patient L. Azacyclic compounds as inhibitors of sensory neurone specific channels. *PCT Int. Appl.* 2005; 125 pp, WO2005005392.

104) Gurdal EE, Durmaz I, Cetin-Atalay R, Yarim M. Cytotoxic activities of some benzothiazole-piperazine derivatives. *J Enzyme Inhib Med Chem*. 2015; 30(4): 649-654.

105) Ergenç N, Gürsoy A, Ateş O. *İlaçların Tanınması ve Kantitatif Tayini*, 4th ed., İstanbul Üniversitesi Yayınları: Turkey, 1989.

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MS Excel	Intermediate