Synthesis of Novel Methotrexate Derivatives for Enhanced Anticancer Activity

Aifa Nemat

00000117028

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Supervised by

Dr.Muddassir Iqbal

Department Of Chemistry

School Of Natural Sciences (SNS)

National University Of Sciences and Technology (NUST), H-12

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We hereby recommend that the dissertation prepared under our supervision by: AIFA NEMAT, Regn No. 00000117028 Titled: Synthesis of Novel Methotrexate Derivatives for Enhanced Anticancer Activity be accepted in partial fulfillment of the requirements for the award of **MS** degree.

Examination Committee Members

1. Name: PROF. MUHAMMAD MAZHAR Signature: 1-10-2018

2. Name: DR. AZHAR MAHMOOD Signature:

External Examiner: DR. WAQAR ASLAM Signature:

Supervisor's Name DR. MUDASSIR IQBAL Signature:

Head of Department

01/10/2018
Date

COUNTERSINGED

Date: 01/10/18

Dean/Principal

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Certified that final copy of MS thesis written by **Ms Aifa Nemat**, (Registration No. **00000117028**), of **School of Natural Sciences** has been vetted by undersigned, found complete in all respects as per NUST statutes/regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of MS/M.Phil degree. It is further certified that necessary amendments as pointed out by GEC members and external examiner of the scholar have also been incorporated in the said thesis.

Signature: _____

Name of Supervisor: Dr. Mudassir Iqbal

Date: 01/10/18

Signature (HoD): _____

Date: 01/10/18

Signature (Dean/Principal): _____

Date: 01/10/18

This Dissertation is dedicated to my Beloved Father (Late), My Mother and Siblings.

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Abstract

Cancer has become a burning issue due to its deadly effects worldwide. Chemist is trying to synthesize drugs with less cytotoxicity, minimum side effects and better efficacy. Methotrexate is a drug having strong anticancer activity, but with serious side effects. Several derivatives have been synthesized by modification at different sites of methotrexate to reduce its side effects and enhance efficacy. Schiff bases achieved a special place due to their biological activities like antitumor activity, antifungal activity, anti-malarial, antivirus, antibacterial and many others. Nine Schiff bases have been synthesized by the reaction of methotrexate with different aldehydes. These schiff base derivatives tested for anticancer activity on gliomas cell lines and compared with the parent drug. Methotrexate derivatives with 2- Chlorobenzaldehyde, 2-Thiocarboxyaldehyde, and 2- pentenal show better results on 100 µM even on lower concentration than Methotrexate, which shows the same result at higher concentration 400 µM. Characterization techniques used are IR, HNMR and CNMR.

Contents

List of Abbreviations

MTX	Methotrexate
AMT	Amethopterin
DNA	Deoxyribonucleaic acid
Nd	Not Determined
EBV	Epstein-Barr Virus
HCC	hepatocellular carcinoma
RNA	Ribonucleaic acid
Cbz	Carboxybenzyl
DHFR	Dihydro folate rductase
FPGS	Folyl polygultamate synthetase
α	Alpha
γ	Gamma
ILS	Increase in life span
DCM	Dichloromethotrexate
uM	micro Molar
IC_{50}	Inhibition Concentration
HCl	Hydrochloric acid
mmol	milimole
Ppt	Precipitate
m.p	Melting point
mg	milligram
TLC	Thin Layer Chromatography
RBF	Round Bottom Flask
UV	Ultraviolet
FTIR	Fourier-transform infrared spectroscopy

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Chapter 1

1.1 Introduction

There are many dreadful and terrible diseases, threatening human being in every era. Cancer is one of the most threatening diseases [1]. Cancer is considered as a major reason of the high mortality rate in the world, first is a myocardial infection [2].

Definition

Cancer is abnormal growth or aberrant proliferation of cells [3]. Cancer is life threatening disease in which uncontrolled growth of cells occurs, might be, there are two or more such cells. This abnormal growth of cells leads to tumor formation which is a solid mass of those cells. First solid mass which is formed is called as the primary tumor. Primary tumor proves to be more disastrous by blocking vessels and organs. *Metastasis* is a process by which primary tumor moves and spread to other body parts [4].

As everyone knows about cell and they are building blocks of the body. In body everything is made up cells, i.e. heart, lung, intestine, brain, bones, and blood [5]. The cell is very tiny complex machine which can communicate with other cells within same organ as well as between different organs. Except blood cells, cell does not leave their origin normally. So, a normal, healthy liver cell will never leave the liver and same with others. But cancerous cell moves and proliferation to other organs in the body and this is a *Metastasis* process [6].

Basically tumors are of two types: *Benign* Tumors don't spread and not life threatening. They can be removed and don't grow again and usually not called as cancer [7]. But *malignant* tumors spread to other tissues and body organ and life threatening. They also destroy healthy cells [8].

1.2 History (Theories and concept about cancer)

Most old writing about cancer was found in Egypt and about 3000BC, although *"cancer"* word was not used in these descriptions.

First time word cancer is used by Greek physician Hippocrates (460-370BC) and so the origin of *"Cancer"* word credited to him. He used *"carcinos and carcinoma"* word to explain tumor formation. Hippocrates, considered as father of medicines [9].

There was several theories explained cancer during different eras of cancer history

(1) Humorat Theory: Physician Hippocrates explained that the body has humors (body fluid).

a) Blood b) Yellow bile

c) Phlegm d) black bile

Unevenness and imbalance between these and particularly high levels of bile, considered as the cause of cancer. This theory was valid through middle age over a period 1300 years.

(2) Lymph Theory: According to lymph theory, it was proposed that lymph causes cancer. This theory was proposed and considered valid in 17^{th}.

(3) Blastema theory: In 1938 Muller proposed that neither lymph nor humors cause cancer. Virchow, a student of Muller found that a cancer cell is formed by other cells, like all normal cells.

(4) Chronic irritation theory: Later Virchow determined and proposed that chronic irritation was a reason of cancer. Thiersch determined that malignant cell spread and metastasis occur. He also found that cancer does not spread by any kind of fluid.

(5)Trauma Theory: Cancer was considered as reason of trauma duriing1800-1920s.

(6) Parasite Theory: up till end of 18^{th} century, it was believed that cancer was infectious, contagious and spread by parasites [9].

1.3 Factors:

There are many factors of cancer, including mutation in genetic material, modified gene expression and epigenetic genes. External factors are viral and bacterial infection, Chemicals, radioactivity, and food.

The detail of these factors is as follows.

1- Mutation: There are many different types of gene mutations in which point mutation and translocation mutation are most common. *Point mutations* are those mutations in which only a single base pair change, and codon forms so can insert false amino acid into specific protein and tumor formation may occur [10]. *Translocation mutations* are those in which of DNA moves from gene to gene or chromosomes to the gene instead of gene to gene. There are two types of protein formed one with loss of DNA part, and another with the extra DNA part. This mutation also causes cancer [11]

Genes involved in translocation mutation also known as *proto oncogenes.Proto- oncogenes*, which do not cause cancer unless it is activated [12].

2- DNA Damage: During different metabolic processes different byproducts are formed, including antioxidant byproducts such as peroxide, superoxide and radical. Studies show that these are same products produced in radiation exposure. These DNA lesion forms, accumulate and has harmful damage to DNA, lipid and protein. This damage modifies DNA sequence and carcinogenic [13].

3- Viral Infection: viral infection was thought to be a reason of cancer but whether a viral infection cause cancer or not depend upon certain other factors as defective or weak immune system, hormonal and genetic factor.Epidermiologic data reveals that Epstein-Barr Virus (EBV)'s DNA invades into genetic information of human stem cells and this cell develop into parent carcinoma cell. Hepatitis B virus is related to serious liver infection and this develops into hepta carcinoma. But later on studies reveal that presence of viral DNA doesn't proves cancer [14].

4- Bacterial infection: it was reported that viral and bacterial infection causes more than 20% of cancer and 2/3 of this linked to viral infection, rest by bacteria.it is practiced that if viral and bacterial infection inhibited, more than 26% cancer can be controlled in developing countries and 7% in most developing countries [15].

Helicobacter pylori is considered carcinogenic since in 1994. This bacteria is a major cause of bacterial infection leads to cancer. It causes gastric

carcinoma as well as reducing risk of esophageal cancer. Streptococus anginosus, treponema denticola are some other bacteria cause human cancer cells [16].

5- Chemicals:First cancer case due to the chemical was reported after the establishment of dye factories in 1938 [17]. Three cases were detected in 45 workers of dye factory. Among carcinogenic chemical benzene, arsenic, cholrnaphazine, 4-aminbiphenyl and asbestos are most prominent. Those chemicals are carcinogenic which effect on cellular constituent as well as lipid, protein and genetic material DNA [18].

1.4 Types of cancer:

There are many different types of cancer, but some important and more common types of cancer as follows:

Breast Cancer

Cancer name is associated with organ having cancer and so does with breast cancer. Breast cancer is the second leading cause of death among women. Heredity causes 5-10% of Breast cancer. There are two types of cancer:-

1- Noninvasive Breast Cancer:

It is a condition in breast cancer when cancer doesn't leave its original location and not spread to other body parts.

2- Invasive Breast Cancer:

In invasive type of cancer, it moves to other location on breast or outside breast [8].

Breast cancer is treated with surgery, chemotherapy and radiation.

Liver Cancer

It is also known as hepatic cancer, or hepatocellular carcinoma (HCC). Its major cause of mortality worldwide and ranked third among the major causes of deaths.

Mortality and incidence are almost equal. Virus, Alcohol, metabolic disorder and heredity are main causes of liver cancer. Liver cancer is more common in man than women[19].

Kidney Cancer

It is also known as renal cancer in which malignant starts to grow in kidney and then invades in other body parts. Renal cancer ranked third in urological carcinoma. Hypertension, smoking, inheritance and obesity are common factors of renal cancer [20].

Bladder Cancer

Bladder cancer stands at fourth among most incident cancers, and ration among female to male is 1:3. Although bladder cancer isn't life threatening but cure is also slow and less rate of progress. Smoking is major cause of bladder Cancer, others are carcinogenic chemicals like aromatic amines, arsenic and medical treatments [21].

Blood Cancer

In case of blood cancer, normal function of blood cells effects. Blood cells are produces abnormally and so prevent functioning like carrying oxygen to the organs, fighting against infections. There are three types of Blood cancer.

1- Myeloma: It is a cancer of plasma cells and weakened immune system of the body.
2- Leukemia: in this large number of white blood cells produced by bone marrow & results in less production of RBC and platelets.
3- Lymphoma: is a cancer associated with the lymphatic system, ultimately weakens the immune system [22].

Prostatic Cancer

It is the most common type of cancer in men and made 13% of all cancer's death. Cancer within the prostate is called as prostate cancer and starts to grow outside and known as cancer.is is hormone sensitive cancer [23].

Obesity is common among the factors of prostatic cancer. Others are diet, hormonal imbalance, and environmental factor [24]. Prostate cancer is treated with hormone therapy and chemotherapy [25].

Colorectal cancer

Cancer of the rectum, colon cancer and colorectal cancer are name of the same type of cancer. Incidence occurs in highly developed countries and among people who migrate towards developing countries, which depicts the lifestyle as major factors of colon cancer, another major factor is heredity [26]. Main colorectal cancer treated with surgery mainly [27].

Lung Cancer

Lung cancer includes 14% of all cancers. There are two types of lung cancer

1- Small cell lung cancer
2- Non-small cell lung cancer [28]
    - Squamous cell lung carcinoma
    - Adenocarcinoma
    - Large cell lung carcinoma

Smoking is the main cause of lung cancer as cigarettes have many toxic compounds causes and promote cancer. Even non-smokers have adenocarcinoma and it is more common in women. It is more common in Asia and south east in young age, in the developed countries rate of adenocarcinoma in young ones and other lung cancer is equal and diagnosis among same age patients [29]. Lung cancer is treated with chemotherapy, radiation, and surgery [30].

1.5 Treatment of Cancer:

Cancer has many types of treatments, and way of treatments depends upon the type and intensity of cancer. Sometimes cancer has only one way of treatments and sometimes combinations of treatments are used again depending on type of cancer.

Cancer is treated in following ways.

- Surgery
- Chemotherapy

- Targeted Therapy
- Immunotherapy
- Radiation Therapy
- Hormone Therapy
- Stem cell Transplant Therapy
- Precision medicine

Cancer Treatment through Surgery

In 1960_s, radiation therapy starts in parts by Curie in France, surgery of breast cancer treatments have started. From 1937- 1953, 100 patients has been treated with surgery. Breast cancer has been studied extensively. Other types of cancer have not been studied through surgery. Mainly those cancers are treated with surgery which is localized [31].

There are several types of surgery of cancer, e.g

- Curative surgery
- Preventive surgery
- Diagnostic surgery
- Staging surgery
- Debulking surgery
- Palliative surgery
- Supportive surgery
- Restorative surgery [31]

Targeted Therapy

This is the type of chemotherapy in which drugs can differentiate between normal, healthy cell and rapidly dividing cancerous cell, and so named as targeted therapy [32].

Targeted therapy requires the exact identification of target molecules. Target is actually that potential molecule which is responsible for proliferation. This target or potential molecule is that site by interruption of which proliferation can be stopped [33].

Target can be of three types, i.e.

Proliferating cell have some physiological properties which can be used as tissue specific differentiation, and these differentiation markers can be used as target e.g CD20 and hormone receptor. Cancerous cell sometimes overexpress some molecule which can be used as target molecule. Genetic modifications and alterations generate novel non physiological protein, which serve as a target molecule[32].

Chemotherapy:

To kill, stop or slow down cancerous cell by use of chemicals, drugs, or a combination of one or more drugs called as chemotherapy. Anticancer drugs work in the opposite way to antibiotics. As cancerous cells are not foreign entity, anticancer drugs should kill cancerous cells without affecting or damaging normal, healthy cell. Cancerous cell are weaker than normal cells so they can easily kill. Drugs pass through all body of patients having capabilities to kill hide cancer. But having some disadvantages simultaneously among which some disadvantages are curable and preventable [34].

Most widely used Drugs [top 30 oncology drugs]

The following drugs are most widely using drugs among 2016-17.

Avastin, Herceptin, Rituxan, Opdivo, Gleevec, Imbruvica,Velcada, Zytiga, Xtandi, Alimata, Gardasil, Ibrance,Tasigna, Xgeva, Afinitor, Jakafi nd Terceva.

[35]

Chapter 2

Literature Review

2.1 Methotrexate

Methotrexate is known as amethopterin which suppresses the immune system as well as chemotherapeutic agents. It was synthesized in 1947 and in 1956 it proved to cure as for metastatic cancer. Methotrexate effects folic acid pathway, as it is closely resembled with folic acid, acting as antimetabolite and used in treatment of various diseases like arthritis and anticancer [36].

Although methotrexate has many applications but it has certain disadvantages, e.g. fast metabolism and low selectivity for tumor cell [37].

Mechanism of inhibition of cancer cells starts from inhibition of DNA protein's precursor synthesis so it inhibit DNA and RNA synthesis particularly in cancerous rapidly dividing cell and act as anticancer[38].

Methotrexate is used to treat Rheumatoid arthritis, psoriasis and cancer. Keeping in view all the uses of methotrexate it has certain disadvantages as it causes life threating side effects on vital organs.

2.2 Synthesized derivatives

Lysine and ornithine derivatives of Methotrexate are synthesized and both derivatives have same binding affinity as Methotrexate and lysine have the same potency as MTX. Reaction mechanism involves protection and de-protection of amino acid and de-protected derivative is 8 times more potent. Lysyl derivative has the same potency in protected and de-protected form. Lysyl derivative is more potent than ornithine derivatives. Presence of Cbz group doesn't affect activity, but removal improves activity 3 folds in ornithine. Inhibition and DHFR binding were tested in liver of Chicken.

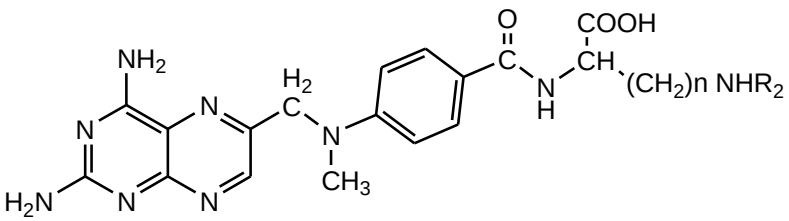


Fig 1: General structure of Lysine and ornithine derivatives

Table 1: Lysine and ornithine derivatives [39]

Comp	**R_1**	**R_2**	**n**	**I_{50} a*10^8 M**
1(MTX)	H	H	2	**9**
2	t-Bu	Cbz	3	310
3	t-Bu	Cbz	4	95
4	H	Cbz	3	33
5	H	Cbz	4	38
6	H	H	3	25
7	H	H	4	13

Shams A. Nadhum et al. *2015* Synthesized a conjugate of silibinin and methotrexate in order to enhance efficacy than parent drugs and to minimize their side effects. Scheme of reaction consists of 6 steps in which firstly methotrexate converted to imine derivative and after 2-3 steps imine conjugate of methotrexate and silibinin. this imine conjugate hydrolyzed to get conjugate of methotrexate and silibinin. Anticancer activity checked against HEP-2 cell lines from human epidermoid larynx carcinoma for 24 hr and 48 hr. General trend shows compound 5 and 6 have a high inhibition rate on high concentration dose and decreased at low concentration. Silibinin shows 33.1 % inhibition methotrexate-silibinin conjugate shows 41.2% [40].

ANDRE et al.*1984* synthesized a novel glutamate derivative. Polygultamate derivative binds as well as methotrexate and leave mammalian cell more slowly. This analogue contains gama- SO_3H instead of COOH and in synthesized in 78% yield. For measuring binding and

anticancer efficacy MTX used as positive control. The binding affinity of analogue is same as of methotrexate. Three experiments were conducted using same quantity and different doses and it revealed that by increasing frequency increase in molar potency of the drug. However this analogue, was unable to form polygultamate derivative [41].

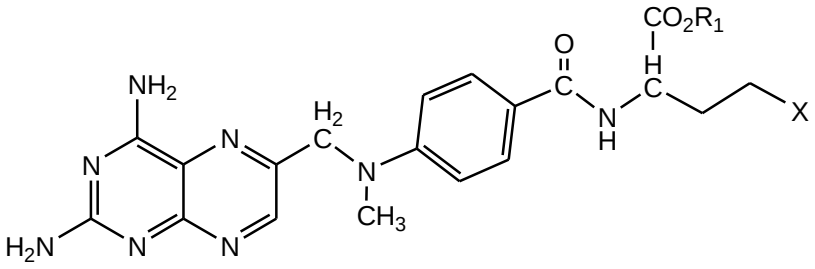


Fig 2: General Structure of Glutamate derivatives of MTX

Table 2:Glutamate derivatives of MTX [41]

Comp	**X**	**L1210DHFR IC_{50} (nM)**	**L.Casei DHFR IC_{50} (nM)**	**L1210 cells IC_{50} (nM)**
MTX	CO_2H	1	17	0.30
mAPA-HCysA	SO_2H	0.95	10	0.01

Gama- sulphonates and gama-phosphonate analogue were synthesized by ANDRE et al. These analogue were tested against folylpolygultamte synthetase (FPGS) and dihydro folate reductase (DHFR) from L1210/R81 murine leukemia cells and L1210/R71 were used R71 line was very less resistant than methotrexate up to 45 fold and R81 was up to 290 fold. And it was also found that gama phosphonate has 3 times less potency than gama sulphonate and has less binding with DHFR and FPGS.

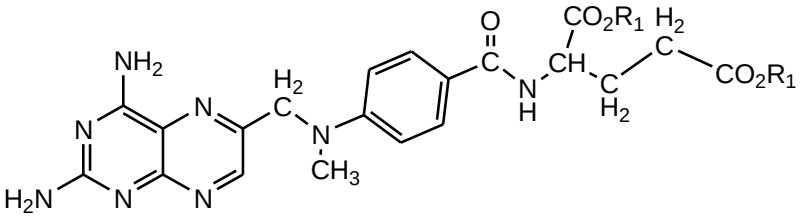


Fig 3: : Analogues of Methotrexate

Table 3: Analogues of Methotrexate [42]

Comp	R_1	R_2	L.Casei	L.Casei(Binding affinity)	L1210 (Binding Assay)	L1210
1	H	O-t-Bu	0.025	0.012	0.0029	0.0029
2	H	$NHNH_2$	0.0021	0.013	0.012	0.012
3	H	NH-n-Bu	0.553	0.036	0.0033	0.0033
4	H	$NHCH_2C_6H_5$	0.055	0.022	0.0030	0.0030
6	CH_3	-t-Bu	0.33	3.9	0.011	0.011
7	-t-Bu	CH_3	0.20	2.2	0.019	0.019
8	-t-Bu	$NHNH_2$	0.17	2.2	ND	ND
9	-t-Bu	H	0.036	0.21	0.035	0.035
10	CH_2Ph	NH-nC_4H_9	ND	0.25	ND	ND

Gamma- hydrizde, gamma-n-butylamide, gamma-benzylamide and gamma –tert butyl ester analogues of MTX has been synthesized. Binding affinity of synthesized derivatives tested for DHFR from L1210 mouse leukemia cells and lactobacillus casei. It has been found that gamma terminal region of MTX is site for modification. Binding affinity has been tested to DHFR of L.Casei. It was found that gamma substituted compound binds effectively, then α-Substituted.

Gamma-tert-butyl ester shows 1.9 times higher binding affinity than MTX. Gamma-tert-butyl ester and gamma hydrazide shows same affinity as MTX. Gamma-n- Butyl amide shows slightly same activity as MTX. Gamma-Benzyl amide shows less activity [42].

Several MTX derivatives have been synthesized by modification of glutamyl moiety with peptide side chain. Nine different amino acids were used for modification of the side chain. Various intermediate peptides were also separated. Biological activity was tested for L1210 leukemia and W25 carcinoma in mouse and rat respectively. It has been found that α-COOH is more important for activity than gamma(γ)-COOH. All derivatives are inactive, which shows glutamyl moiety is necessary for activity. If α-COOH is present, an increase in the length of he alkyl chain restores activity.

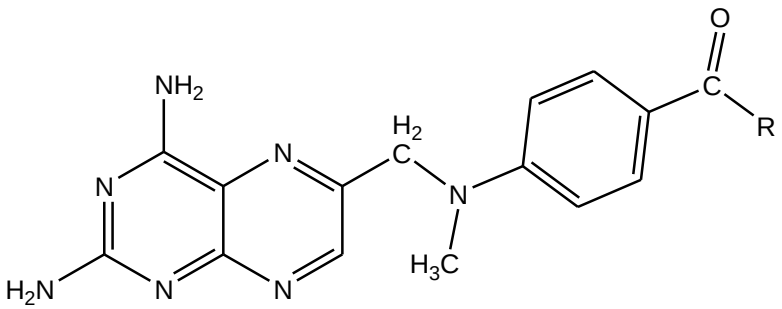


Fig 4: Analogues of methotrexate

Table 4: Analogues of methotrexate [43]

Comp	**R**	**LD $_{5u}$ mg/kg**	**LD $_{5u}$ mmol/kg**
1a	Glycine	250	0.65
1b	DL-alanine	349	0.81
1c	B-alanine	250	0.58
1d	Sarcosine	400	0.92
1e	DL-α-aminobutyric acid	925	2.25

1f	γ-aminobutyric acid	106	0.25
1g	DL-Valine	200	0.47
1h	L-leucine	710	0.42
1i	L-phenylalanine	500	1.02

Different derivative of MTX has been synthesized by alkylation of the side chain. DHFR affinity and anti-proliferating activity was tested against L1210 in mice. It was found that those derivatives have a high inhibition rate, which are structurally closed to MTX. Minute changes in Structure such as derivatization of pyridine ring, Chlorination give good result. But substitution of the aliphatic group decreases the activity greatly. Introducing the carbon around the benzene ring decrease binding affinity but between –COOH enhances the binding affinity but none of the synthesized derivatives shows significant anti-proliferative activity [44].

In this study α and γ- monoesters of MTX were synthesized and anticancer activity checked against lymphoblastic leukemia cell lines. γ –monoesters are more inhibitory than α-isomer and difference is about 10 folds, but with the increase in chain length this difference reduces to 2.5 folds. Monoesters show the less inhibitory effect than diesters. Diethyl esters are 516 times more active than α-monoethyl ester and 48 times than γ –monoester. Dibutyl esters shows same result as diethyl esters [45].

In this work numbers of alkyl esters derivatives of MTX were prepared by the direct esterification method. But with 2º alcohols or 1º alcohols, reaction at room temperature gives a poor yield. So, reaction mixture heated at 55-60 ºC. In vitro growth inhibitory activity was also studied with two murine leukemia L1210 in hybrid mice and p1534 leukemia in inbred mice. Dibutyl ester shows 25% increase in life span. Binding affinity was tested with DHFR of Lactobacillus casei ATCC 7469 and it shows 1000 times less tightly than MTX [46].

Several derivatives of MTX have been synthesized by substitution of alkyl group at 7-position. Inhibitory activity against streptococcus faecium ATCC 8043 shows that synthesized derivatives 1000 times less potent than the parent drug. The inhibitory action against p388 murine leukemia shows similar results. The lack of activity of both derivatives

against DHFR shows that it might be due to steric effect of $–CH_3$.In vivo studies against L1210 leukemia in mouse shows that these derivatives are inactive [47].

Numbers of MTX derivatives were synthesized by MTX diethyl esters and various amines. Procedure involves use of excess of amines without solvent. Inhibitory activity against lymphoblastic leukemia CCRF-CEM shows that bis-amides derivatives less active than MTX or MTX esters. Bis (benzylamide) shows higher activity in vivo against L1210 in mice, and this activity was considered as bis (benzylamide) derivative release free MTX at site other than serum [48].

Number of derivatives of MTX were synthesized having general formula 8-alkyl-7,8-dihydromethotrexate. Synthetic pathway includes alkylation of 7,8-dihydromethotrexate. In vitro studies tested against lactobacillus casei, thmidylate synthetase, and DHFR. All derivatives are less potent for DHFR than MTX but more potent for thymidylate synthetase. In vitro inhibitory activity tested against CCRF-CEM show all derivatives have less inhibitory activity than MTX. Derivative having H at 8-poistion has the same activity as MTX but it was inactive for l1210 leukemia [49].

MTX γ-L-glutamate diethyl ester, MTX α-L-glutamate ester and α- γ-bis(L-glutamate tetraethyl ester). Major product obtained is γ-isomer. Further esterifications of both isomers give triethyl esters. Anti-proliferating activity tested against L 1210 leukemia. The increase in life span due to γ and the α diethyl ester is 40% and 10% respectively while MTX has +60%. Growth inhibition activity again CCRF-CEM shows γ-isomer 10 times more activity but α-isomer doesn't shows the same result. L isomer shows higher activity than D isomer [50].

Aza derivative of MTX has been synthesized by additional N-atom between phenyl and carbonyl of the side chain. Photochemical method was used to insert additional N-atom. Inhibitory activity was tested against DHFR and thymidylate synthetase of Lactobacillus casei and CCRF-CEM and found to be less cytotoxic. In vivo inhibitory was tested against L-1210 leukemia (mice) and shows significant activity than MTX in the order of 55% and 88% respectively [51].

Various Derivatives of MTX have been synthesized by modification of γ-COOH like MTX-N(idoacetyl) L-lysine, another with Cbz, and NH_2 Group. Bonding Affinity for DHFR tested against L-Casei and L1210. N(idoacetyl) L-lysine shows the best activity for L.casei than

MTX, But less activity for L1210. All other derivatives exhibit less activity and possible reason is the presence of charged groups at the end of chain lesson binding affinity[52].

Tripepetide derivatives of methotrexate have been synthesized by reacting with four different aminoacid. These synthesized derivatives have been tested for anti-proliferative activity against W256 (rat) and L 1210 (mouse) leukemia. In this work an extra amino acid added between glutamic acid and aminobenzoyl portion. This insertion of aminoacid made these derivatives to shows borderline activity [53].

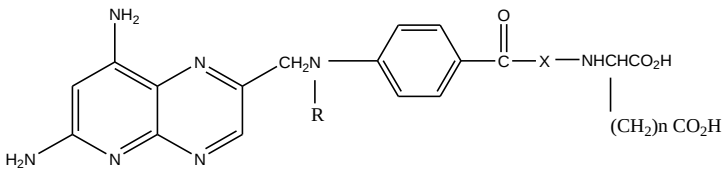


Fig 5: Tripeptide derivatives of MTX

Table 5: Tripeptide derivatives of MTX

comp	R	X	n	L 1210 mg/kg Dose (mg/kg*)no. Of admin	ILS %
1a	H	Gly	1	40*10	69
1b	H	Gly	2	33*6	14
1c	CH_3	Gly	1	100*8	40
1d	CH_3	Gly	2	100*8	0
1e	CH_3	DL-ala	2	50*6	19
1f	CH_3	Sar	2	100*6	0
1g	CH_3	L-Leu	2	50*8	0
1h	CH_3	L-phe	1	100*7	0
1i	CH_3	L-phe	2	50*10	25

Various diesters of MTX and DCM have been prepared by the reaction of acid catalyzed esterification. Neutral esterification also carries out using Cs_2CO_3. In vitro anticancer activity was tested against L1210 in mice. Different doses injected to check the increase in median life span. Generally an increase in dose increase %ILS [54].

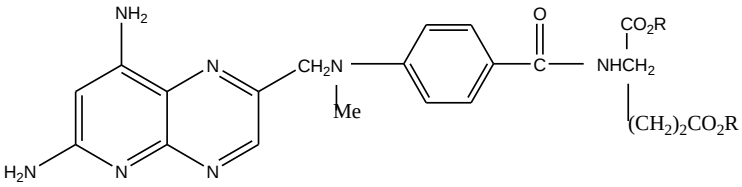


Fig 6: Disters Analougues of MTX

Table 6: Disters Analougues of MTX

Comp	R	Dose, mg/kg	% ILS
1	n-propyl	20	+44
		40	+100
2	n-butyl	15	+77
		30	+66
		60	+88
3	n-pentyl	10	+33
		20	+55
4	3-methylbutyl	10	+33
		20	+55
		40	+88
5	n-octyl	7.5	+44
		15	+66
6	2-ethoxyethyl	20	+44
		40	+55
7	n-dodecyl	30	+33
		60	+100
8	Benzyl	15	+33
		30	+100
9	tert-butyl	400	+36
10	2,5-dimethylbenzyl	10	+88
		20	+111
		40	+155
11	2,4,6-trimethylbenzyl	20	+44
		40	+88
12	2,6-dichlorobenzyl	10	+66
		20	+167
13	6-chloropiperonyl	10	+88
		20	+66
		40	+167
14	3-picolyl	10	+88

		20	+77
		40	+144
15	α-n-butyl, γ-3-picolyl	20	+50
		40	+75
		80	+137
16	n-butyl (DCM)	45	+64
		90	+82
17	2-methlpropyl	40	+44
		80	+111
18	3-methylbutyl	20	+67
		40	+100
		80	+55
19	1-methylbutyl	20	+55
		40	+66
		80	+133
		120	+145
		140	+155
20	n-octyl	45	+44
		90	+66
		180	+122
MTX		15	+77
		30	+88
		60	+100
DCM		120	+22
		160	+44
		200	+78
		240	+67
		280	+89

Lysyl derivatives of methotrexate have been synthesized by reaction of γ-COOH with amine group of lysine. These derivatives vary due to the number of lysyl groups attached. It was found that synthesized derivatives show less affinity to DHFR from 2-3 folds as well as decrease in cytotoxic activity by 30-120 folds. However increase in number of lysyl derivative does not affect activity [55].

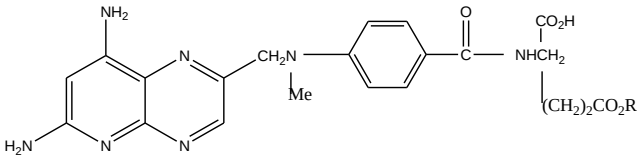


Fig 7: Lysyl derivatives of MTX

MTX: R= OH

1: [MTX(γ-e)-Lys], R= $NH_2CH[(CH_2)_4\,NH]\,COOH$

2: [MTX(γ-e)-$(Lys)_2$], R= $NH_2CH[(CH_2)_4\,NH]\,CO\,NHCH[(CH_2)_4\,NH_2]\,COOH$

3:[MTX(γ-e)-$(Lys)_3$],R= $NH_2CH[(CH_2)_4NH]CONHCH[(CH_2)_4NH_2]CO\,NHCH[(CH_2)_4\,NH_2]$

Table 7: Lysyl derivatives of MTX

Comp	L1210 DHFR IC_{50} nM	L1210 cells IC_{50} uM	H35 Cells IC_{50} uM
MTX	50	0.024	0.010
1	87	0.76	0.40
2	86	1.8	0.50
3	140	2.9	0.56

Various γ-monoamides of AMT and MTX have been synthesized by modification of coupling method of mixed carboxylic-carbonic anhydride method. All derivatives have been tested in vitro against L1210 leukemia, wild type L1210 and subline (CEM/MTX). In vivo all derivatives have been synthesized against L1210 in mice. In vitro γ-N-arylalkyl and γ-N-aryal derivatives show more potency than γ-N-tert-alkyl analogues. Results show that AMT derivatives have more potency than MTX derivatives and all MTX derivatives shows more potency against subline L1210/R81than MTX. It was also found that all derivatives show more activity against cell lines than human cells. In vivo study shows γ-N-tert-alkyl analogues inactive and significant activity showed by γ-N-arylalkyl and γ-N-aryal derivatives [56].

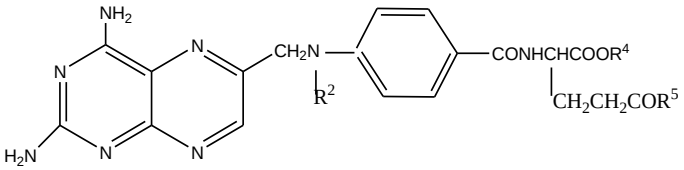


Fig 8: γ-monoamides Derivatives of MTX

Table 8: γ-monoamides Derivatives of MTX

Comp	R^2	R^4	R^5	DHFR IC_{50} um	L1210	L1210/ R81
3a	H	H	t-BuNH	0.044	0.12	22
3b	H	H	(1-admantyl)NH	0.10	0.68	25
3c	H	H	$C_6H_5CH_2NH$	0.087	0.005	17
3d	H	H	3,4-Cl_2 $C_6H_3CH_2NH$	0.13	0.046	32
3e	H	H	2,6-$Cl_2C_6H_3$ CH_2NH	0.080	0.0037	61
3f	H	H	C_6H_5NH	0.060	0.0035	10
3g	H	H	3,4(OCH_2O)C_6H_3NH	0.045	0.0032	28
3h	Me	H	3,4-(OCH_2O) C_6H_3NH	0.042	nd	25
3i	H	H	3,4-$(OH)_2$ C_6H_3NH	0.057	0.037	23
3j	Me	H	3,4-$(OH)_2$ C_6H_3NH	0.031	0.030	38
AMT	H	H	H	0.020	0.003	84
MTX	Me	H	H	0.020	0.01-0.03	220

Various derivatives of MTX and AMT have been synthesized by replacing glutamate moiety with DL-2-aminoalkanedioic acid having up to 10 CH_2 alkyl group. Al derivatives have been tested against L1210 leukemia, CEM leukemia (human), CEM/MTX, and L1210/R81. Derivative with 9 CH_2 alkyl group shows highest activity with CEM, and with 6 CH_2 alkyl group against L1210 cell lines [57].

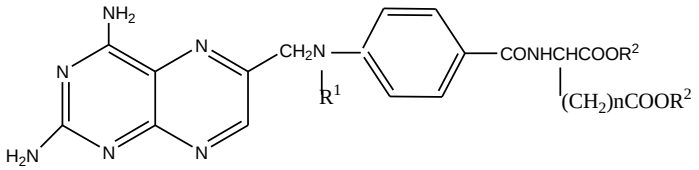


Fig 9: Derivatives of MTX

Table 9: Derivatives of MTX

comp	R^1	n	R^2	DHFR IC_{50} um	CEM	L1210	L1210/R81
2	Me	6	H	0.023	0.15	0.0012	68
3	Me	7	H	0.032	0.062	0.0042	73
4	Me	8	H	0.029	0.056	0.0031	78
5	Me	9	H	0.034	0.016	0.0071	58
6	Me	10	H	0.026	0.64	0.026	56
7	H	6	H	0.54	nd	0.020	>218
8	H	9	H	0.081	nd	0.00065	110
9	H	10	H	0.067	nd	0.0011	215
MTX	Me	2	H	0.025	0.032	0.0046	197
AMT	H	2	H	0.025	0.001	0.002	84

γ –tert-butyl esters of AMT and MXT have been synthesized with the new synthetic scheme. Affinity for DHFR and inhibitory activities tested against L1210, CEM, and several other cell lines of human carcinoma [58].

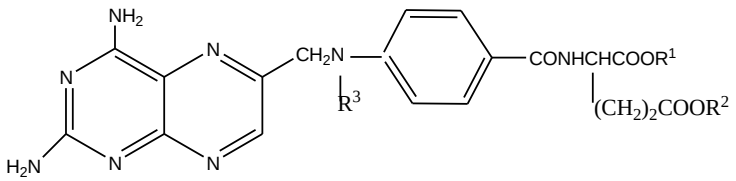


Fig 10: γ –tert-butyl esters derivatives of MTX

Table 10: γ –tert-butyl esters derivatives of MTX

Comp	R^1	R^2	R^1
MTX	H	H	Me
AMT	H	H	H
1(γ-tBMTX)	H	t-Bu	Me
2(γ-tBAMT)	H	t-Bu	H

Table 10 a: activity of γ –tert-butyl ester derivatives of MTX

						Human Squamous Cell Carcinoman				
Comp	CEM	L1210	L1210/R71	L1210/R81	SSC25	SSC68	SSC78	SSC25/R1	SSC68/R1	SSC78/R1
MTX	0.62	0.056	40	25	0.40	0.37	0.48	0.78	1.4	1.4
AMT	0.45	0.023	3.5	6.5	0.066	0.19	0.080	1.8	3.5	0.43
1(γ-tBMTX)	0.032	0.0020	19	220	0.014	0.032	0.013	0.15	0.25	0.071
2(γ-tBAMT)	0.0010	0.0020	7.9	84	0.0016	0.0037	0.0025	0.043	0.29	0.014

In this article antibodies coupled with MTX by two different methods. First one is water soluble carbiimide coupling and other is a modification of anhydride coupling and later method was found to be more effective. In vivo studies of antibody MTX conjugate shows

that antibodies associated with cytotoxic drug are more potent than the drug alone. Drug, mixture, and γ- globulin-MTX conjugate were used as a control group [59].

A fluorescent derivative of MTX has been synthesized by lysine derivative of MTX to dansyl analogue and this analogue show higher activity against DHFR of L.casei.

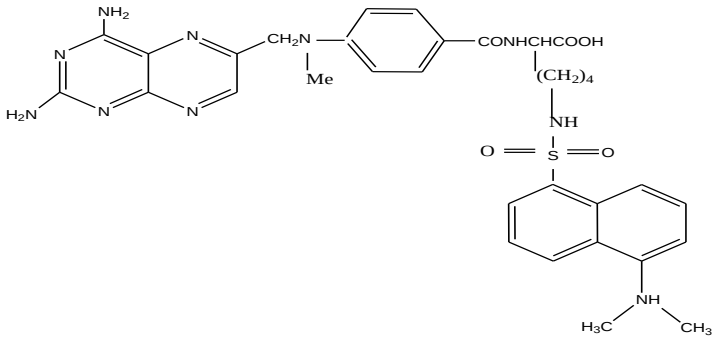


Fig 11: fluorescent derivative of MTX

[60]

A poly (γ-L-glutamate) derivative of methotrexate has been synthesized in 4 steps, and this derivative contains 2-3 glutamate units more than MTX. Synthetic route involves peptide coupling, Blocking groups removed by catalytic hydrogenolysis. A coupling reagent used was Diphenylphosphoryl azide.

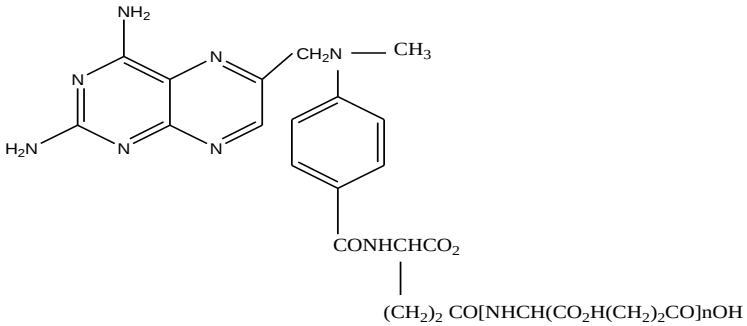


Fig 12: poly (γ-L-glutamate) derivative of methotrexate

[61]

5,6,7,8-tetrahydromethotrexate (3) and di-hydro-methotrexate (2) were synthesized and affinity was checked against DHFR. It was found that tetrahydro-MTX shows more potency than dihydro-MTX for mice, S.faecalis, P.cerevisiae, dogs and chicks. It was also found that both reduced derivatives are less potent against DHFR and more potent than MTX against

thymidylate synthetase. Results show dihydro-MTX is more potent than 5,6,7,8-tetrahydromethotrexate [62].

Table 11. tetra and dihydro derivatives of Methotrexate

Comp	DHFR	Thyimdylate synthetase	S.faecalis	S.faecalis	L.casei
MTX	9	45000	0.15	60	0.01
(3)	16	1125	0.011	24	0.008
(2)	46	2250	0.047	68	0.056

Anilides of MTX and AMT have been synthesized and their anti-proliferative activity and binding affinity were tested against DHFR, L1210, and W1-L2 and it was found that the presence of a hydrophobic ring with an acid group enhances potency. All the anilides found to be potent, However *γ-amide containing* -(BOH_2) founds to be most potent. It was proposed that CONH group of these derivatives involved in hydrogen bonding and enhances affinity for DHFR [63].

Table 12. Anilides derivatives of MTX

Comp	R	X	Y	DHFR inhibition IC_{50}, nM	L1210	W1-L2
1	H	$(CH_2)_3NHCO$	o-COOH	52	0.75	48
2	H	$(CH_2)_2CONH$	m-COOH	32	25	6.1
3	Me	$(CH_2)_2CONH$	m-COOH	35	1.6	2.2
4	Me	$(CH_2)_2CONH$	m-(BOH_2)	30	0.70	nd

Several derivatives of methotrexate have been synthesized by modifying glutamyl moiety and two oxide analogues. These derivatives have been tested for DHFR affinity against L.casei, chicken liver, L 1210- FR8, inhibitory activity tested against S.facium. All derivatives show inhibitory activity except oxide derivatives, rather these showed deleterious effect. This detrimental effect may be due to decrease in basicity of pteridine ring [64].

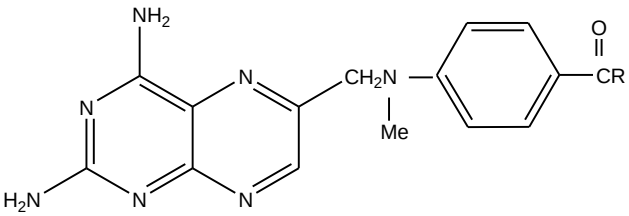


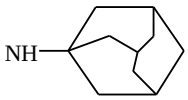
Fig 13: Oxide analougues of Methotrexate

6a: R= $NHCH(CO_2Et)(CH_2)_2\ CO_2Et$

6b: R= $NHCH(CO_2H)(CH_2)_2\ CO_2H$

6c: R=$NHCH(CH_3)\ (CH_2)_2CH_3$

6d: R=

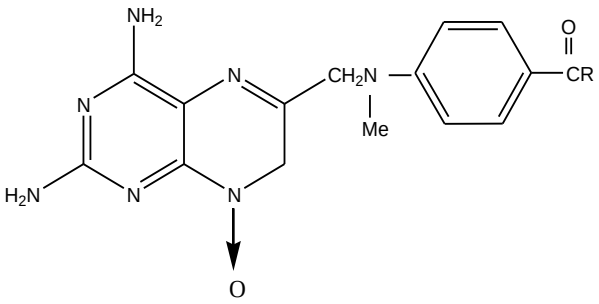


6e: R= OH

6f= OEt

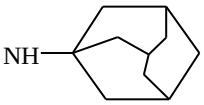
Table 13. Oxide analougues of Methotrexate

comp	S.facium ID $_{50}$, ug/ml
6a	0.017
6b	0.37
6c	0.002
6d	0.003
6e	0.002
6f	0.001
7a	1.0
7d	1.0



7a: R= $NHCH(CO_2Et)(CH_2)_2\ CO_2Et$

7d:



MTX bisamide derivatives have been synthesized with different aryl, alkyl aryl, and alkyl amines. MTX-dianilide was also prepared. These derivatives have been tested for DHFR inhibit and anti-proliferative for L1210 leukemia. Results showed that methyl group on benzylic carbon decrease activity. Most active compound is that having R group aryl amine having no methyl group [65].

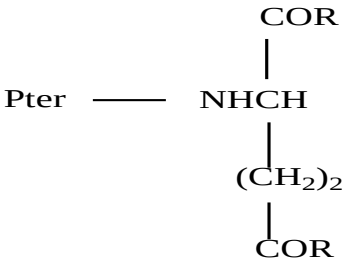
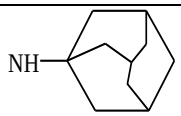


Fig 14: General structure of Bisamides Derivatives of MTX

Table: 14 Bisamides Derivatives of MTX

Comp	R	CEM	L1210
1		>10	3.4
2	n-$C_5H_{10}N$		>10
3	4-$ClC_6H_4CH_2N$	>10	6.6
4	4-$MeOC_6H_4CH_2N$	>10	8.2

5	$C_6H_5CH_2N(CH_3)$	6.4	9.4
6	$(C_6H_5CH_2)_2$ N	7.6	0.69
7	C_6H_5 CH (CH3)NH	>10	>10
8	C_6H_5NH	3.3	0.41
9	H_2 NNH	7.5	0.95
10	CH_3 NHNH		
11	c- $(CH_2\ CH_2)_3$ NNH		
	MTX	0.003	0.01

α and γ esters of MTX have been synthesized having 8, 12, and 16 Carbon chain length. These synthesized derivatives have been tested for DHFR affinity and inhibitory activity against CEM. It was found that all derivatives have less inhibitory activity than MTX and γ esters have more inhibitory activity than α esters. It was found that by increasing chain length there is an increase in cytotoxicity but decrease in DHFR affinity [66].

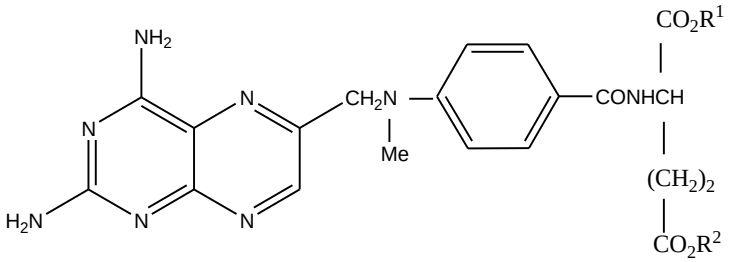


Fig 15: General structure of α and γ esters Derivatives of MTX

Table 15: α and γ esters Derivatives of MTX

Comp	R^1	R^2	DHFR inhibition, IC_{50},uM	CEM inhibition, IC_{50},uM
MTX	H	H	0.0033	0.025
1 (α)	n-C_8H_7	H	0.36	3

2(γ)	H	n-C_8H_7	0.0054	0.92
3(α)	n-$C_{12}H_{25}$	H	0.44	2.1
4(γ)	H	n-$C_{12}H_{25}$	0.034	0.37
5(α)	n-$C_{16}H_{33}$	H	1.2	0.25
6(γ)	H	n-$C_{16}H_{33}$	0.037	0.11

Stretched MTX derivatives having different numbers of (Gab) as spacer between glutamate and MeAPA moiety have been synthesized. The DHFR affinity and inhibitory activity have been evaluated. DHFR inhibition is directly related to number of Gab spacers, but growth inhibitory potency lost slightly [67].

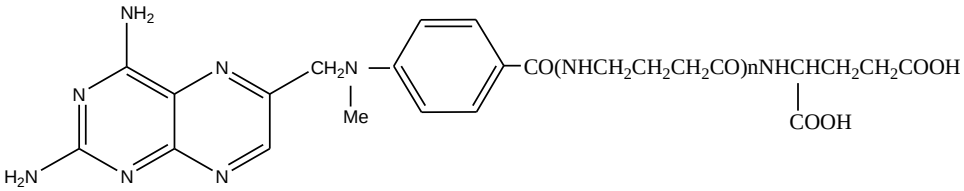


Fig 16: Stretched MTX derivatives

Table 16: Stretched MTX derivatives

Compound	n	L1210 DHFR IC_{50}um	L1210 cells IC_{50}um	L.casei TS IC_{50}um
mAPA-Gab_1-Glu (1a)	1	0.082	0.82	0.53
mAPA-Gab_2-Glu (1b)	2	0.090	1.3	5.6
mAPA-Gab_3-Glu (1c)	3	0.31	4.4	29
mAPA-Gab_4-Glu (1d)	4	0.54	7.7	>100
mAPA-Gab_5-Glu (1e)	5	0.84	12	>100
mAPAGlu		0.035	0.50	0.02

(MTX)				

Dihydro-2H-1,4-benzothiazine and dihydro-2H-1,4-benzoxazine MTX derivatives have been synthesized and tested for anti-proliferative activity against hSC and hPBMC in vitro. In vivo activity tested against rat arthritis. 3c is found to be more potent than MTX [68].

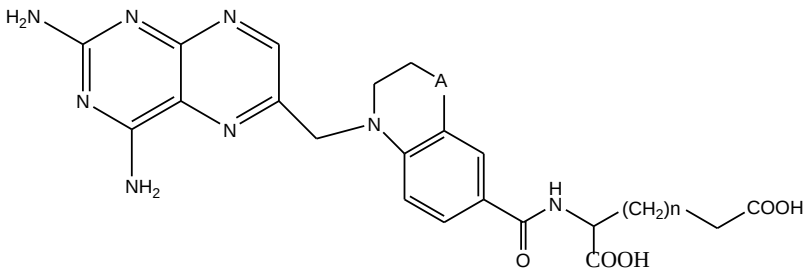


Fig 17: General Structure of Benzothiazine and Benzoxazine derivatives

Table 17: Benzothiazine and Benzoxazine derivatives

Comp	A	n	hSC, IC_{50} nM	hPBMC, IC_{50} nM
3a	O	1	0.52	1.0
3b	O	2	1.4	1.9
3c	S	1	0.30	0.50
3d	S	2	0.77	1.3
MTX-33	C	1	3.8	0.83
MTX			1.0	1.0

Various derivatives of MTX have been synthesized and few were studied for anti-proliferative activity against L1210 was tested. 9Aa and 9Ab shows higher activity than MTX [69].

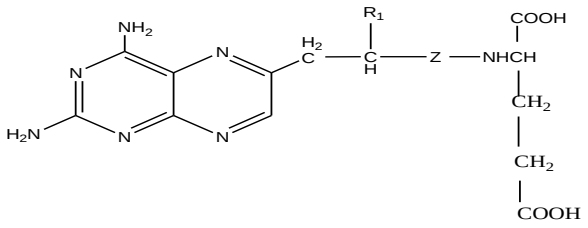
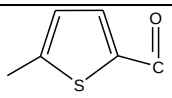
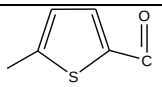
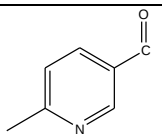
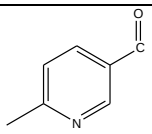


Fig 18: Derivatives of MTX

Table 18: Derivatives of MTX

Comp	R_1	Z	L1210 IC_{50} nM	Chang Liver
9Aa	H		8.1	3.1
9Ab	Et		3.7	1.6
9Ba	H		88	36
9Bb	Et		268	35
MTX	H		20	14

Various MTX derivatives have been synthesized having a different group by replacing γ-COOH and only a few were tested for anti-proliferative activity. These derivatives have been tested against DHFR, FPGS, L1210 and L1210/R81. Ornithine derivative was found to be more potent against DHFR and FPGS [70].

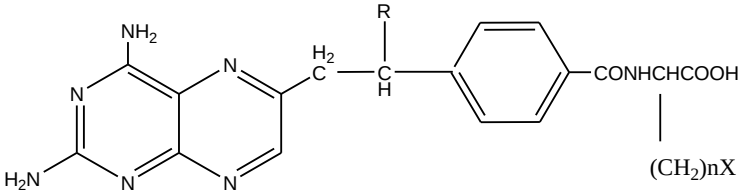


Fig 19: Derivatives of MTX by replacement of γ-COOH

Table 19: Derivatives of MTX by replacement of γ-COOH

Comp	R	n	X	DHFR IC_{50} uM	FPGS K_i uM	L1210 IC_{50} uM	L1210/ R81
2	Me	4	NH_2	0.065		0.40	220
3	Me	3	NH_2	0.160	20.4	1.30	86

4	Me	2	NH_2	0.120		2.42	290
5	Me	1	NH_2	0.180		0.44	405
6	H	3	NH_2	0.072	0.15	1.30	32
MTX	Me	2	COOH	0.035		0.002	220
AMT	H	2	COOH	0.035		0.002	84

Derivatives of MTX have been synthesized by using a different number of Carbon chain length and N-haloacetylation and tested for DHFR affinity and anti-proliferative against L1210 and L1210/R81. N-bromoacetyl-L-ornithin found to be more potent than other synthesized activity [71].

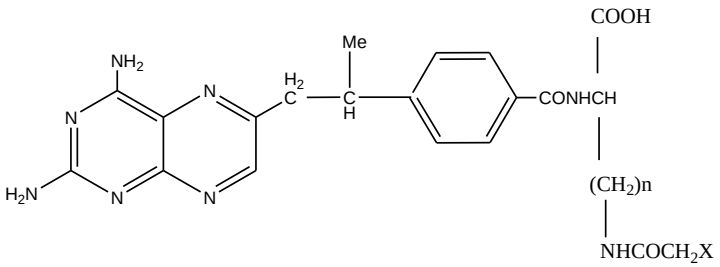


Fig 20: N-haloacetyl derivatives of MTX

Table 20: N-haloacetyl derivatives of MTX

Comp	n	X	DHFR IC_{50}, nM	L1210 Cells IC_{50}, uM	L1210/R81
1	4	I			
2	4	Br	72	0.096	240
3	4	Cl	32	0.033	93
4	3	Br	46	0.126	155
5	3	Cl	32	0.062	105
MTX			25	0.005	200

Aminoalkanephosphonic, aminophosphonoalkanoic and aminoalkanesulfonic MTX derivative have been synthesized instead of glutamate moiety. All derivatives have been tested for enzyme affinity against FPGS and inhibitory activity was tested against MTX, MTX resistant cells. The maximum number of CH_2 for optimal activity was found to be two

and less than this is detrimental. Removal of α- COOH was found to lose the anti-proliferative activity, and reason behind is α- COOH involved in binding activity [72].

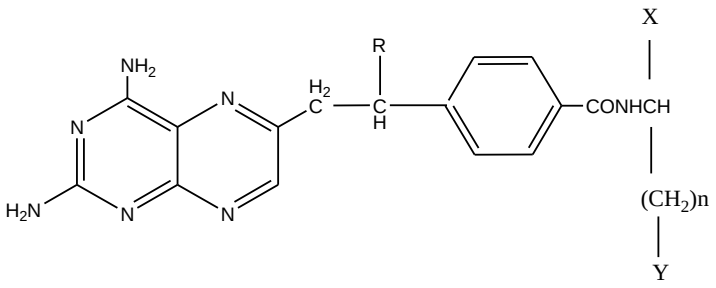


Fig 21: Derivatives of MTX by replacement of α and γ COOH

Table 21: Derivatives of MTX by replacement of α and γ COOH

Comp	R	X	n	Y	% inhibition
3	Me	COOH	2	SO_2H	59
4	H	COOH	2	SO_2H	77
5	Me	COOH	2	$PO(OH)_2$	53
6	H	COOH	2	$PO(OH)_2$	100
7	H	COOH	1	$PO(OH)_2$	22
8	H	COOH	3	$PO(OH)_2$	52
9	H	COOH	4	$PO(OH)_2$	44
10	Me	H	0	SO_2H	19
11	Me	H	1	SO_2H	22
12	Me	H	2	SO_2H	18
13	Me	H	3	SO_2H	26
14	Me	H	1	$PO(OH)_2$	22
15	Me	H	2	$PO(OH)_2$	16
16	Me	H	3	PO(OH)(OEt)	9

2.3 Method of Synthesis:

MTX and benzaldehyde in excess was refluxed with 0.5 moles of 10 % NaOH for 3 hrs. Absolute ethanol was used as solvent. As reaction proceed and complete acidified with 5% HCl to maintain pH at 5. Product was washed with absolute ethanol and dried [40].

2.4 Schiff Bases

Novel schiff bases of 4-Haloaniline and 2,3-Dihydroxybenzaldehyde were synthesized by refluxing in ethanol for 2 hrs. These schiff bases were complexes with Zn and Co metals. A synthesis confirmed by FTIR, H^1NMR, C^{13}NMR, and crystal diffraction. Schiff bases tested for antibacterial activity, cytotoxic analysis, and antidiabetic activity. It was found that Zn complex schiff base has higher cytotoxicity [73].

In this article schiff based of 11-chloro-6H-indole was synthesized by using DMF as solvent at 150℃ for 24 hours. Triethylamine was used as catalyst. Schiff Bases were complex with metal ligand. These schiff bases and metal complexes assayed for anticancer activity and it showed complexes show better activity than ligand alone depending metal used for complexation [1].

In this article hetero schiff bases were synthesized using different amine and aldehydes using ethanol as solvent and glacial acetic acid as catalyst and refluxed for 4hr. Anticancer activity tested for acetylcholinesterase and butyrylcholinesterase. Synthesized schiff bases show better activity [74].

Schiff bases of 4,4'-diaminodiphenyl sulphide has been synthesized by dissolving aldehyde and adding drop wise to 4,4'-diaminodiphenyl sulphide. Reaction mixture was refluxed for 3 hours in absolute ethanol. These compounds were tested for antibacterial, anticancer and antifungal activity. Generally, all compound show better efficacy than parent compound [74]. A tridentate schiff base of *S*-benzyldithiocarbazate has been synthesized and then complexes with Cu (II), Zn(II) and Cd(II) metals. Ethanol was used as solvent and the mixture was stirred for 15 min. Antimicrobial, cytotoxic, antioxidative were tested for schiff base and complexes [75].

Chapter 3

Experimental

3.1 Chemicals

Benzaldehyde, 2-Chlorobenzaldehyde, 3-nitrobenzaldehyde, 5-chloro-2-hydroxybenzaldehyde, 2- hydroxy-5-nitrobenzaldehyde, 2-thiophenecarboxyaldehdye, Glutar aldehyde and trans-2-pentenal were purchased from sigma aldrich and alfa aesar. All chemicals used without further purification.

3.2 Solvents

All solvents ethanol, toluene, chloroform, hexane, ethyl acetate, and dichloromethane were of analytical grade. Ethanol was used after distillation before every reaction. All other reagents were used without further purification.

3.3 Instrumentation

The compounds were weighted in electronics analytical balance ATY224. Progress of the reaction was monitored through TLC. A spot of aldehydes is UV active so observed in UV lamp. Organic solvents were dried under vacuum by using rotary evaporator R-210. Open capillary tubes were used to record melting point, which was determined on melting point apparatus SMP10 and are uncorrected. To identify the functional group of synthesized compounds, FT-IR with ATR model ALPHA 200488 was used.

3.4 General procedure for synthesis of Schiff base from Aldehydes

Condensation reactions of amine with carbonyl compound generate schiff bases also known as imine. Reaction occurs in the presence of acid or basic catalyst or by heating. General scheme of synthesis as follows:

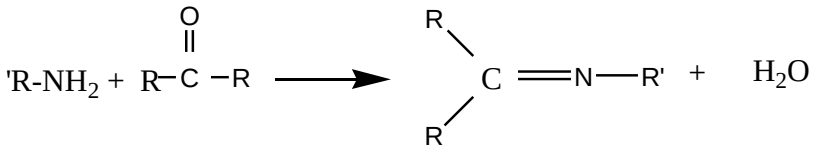


Fig 21: General Scheme of synthesis

Mechanism of reaction as follows

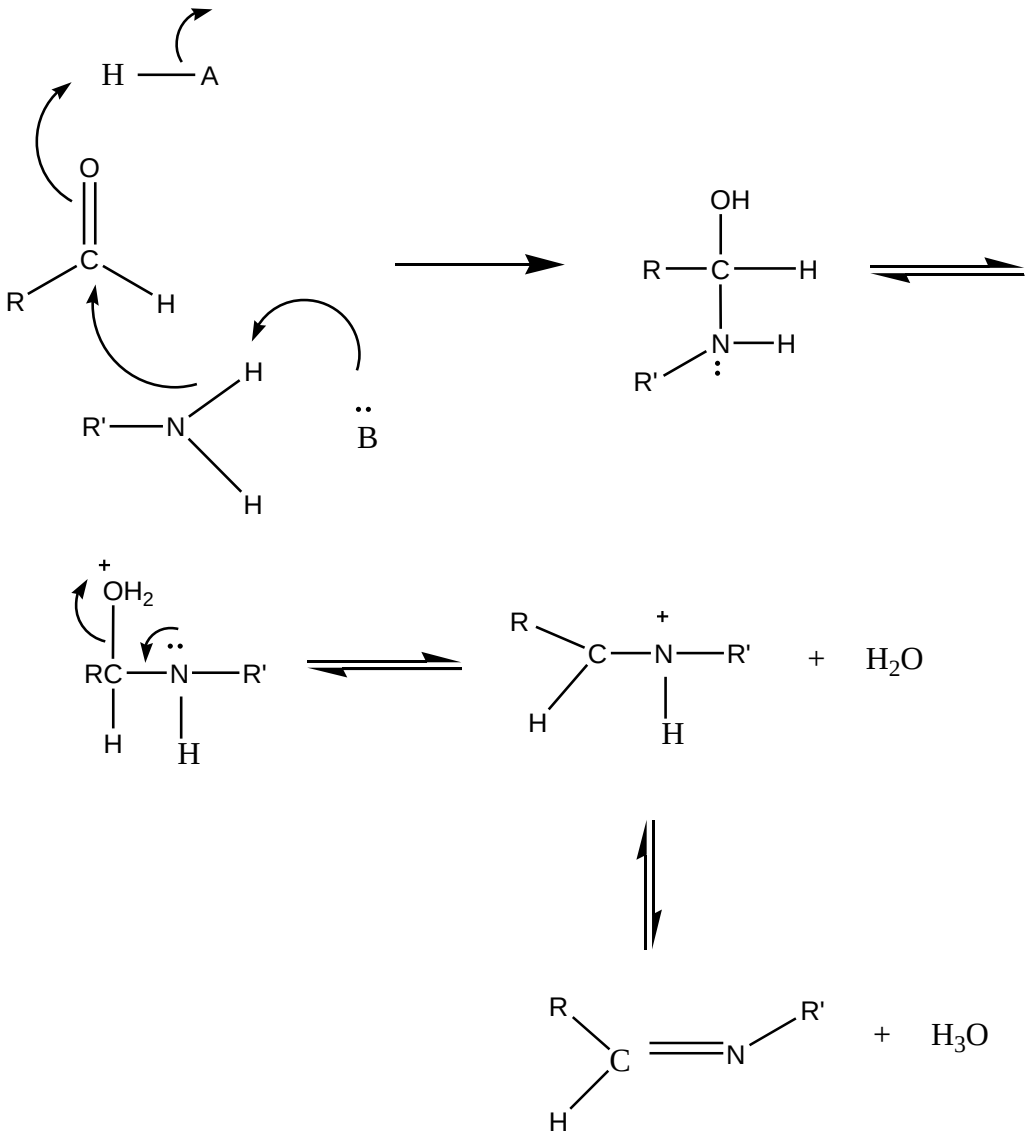


Fig 23: Mechanism of synthesis of Schiff Bases

Methotrexate has two primary amine groups at a pteridine ring which can react with the carbonyl group of aldehyde and schiff base or imine formed. The general reaction of methotrexate with aldehyde to form imine as follow:

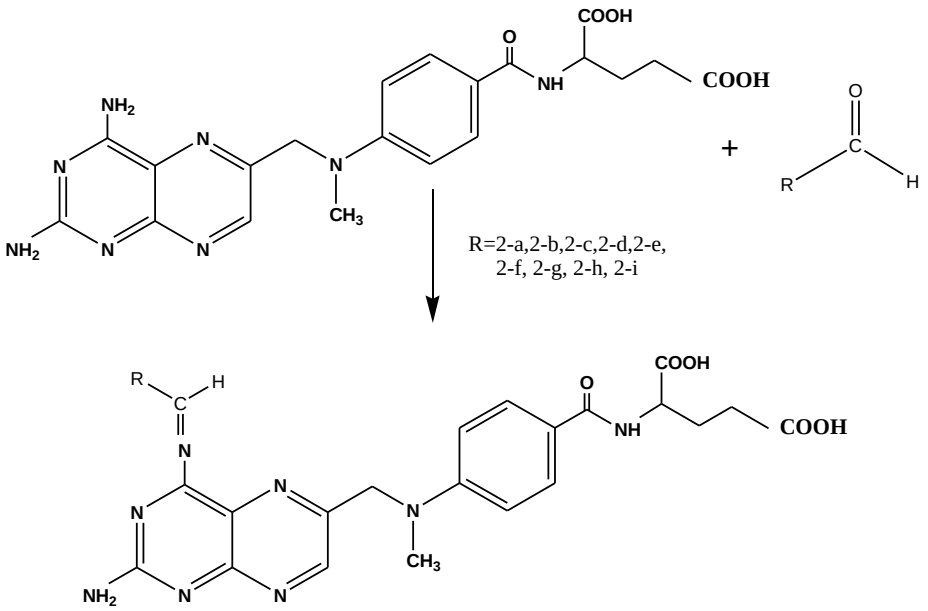


Fig 24: General reaction of MTX with aldehyde

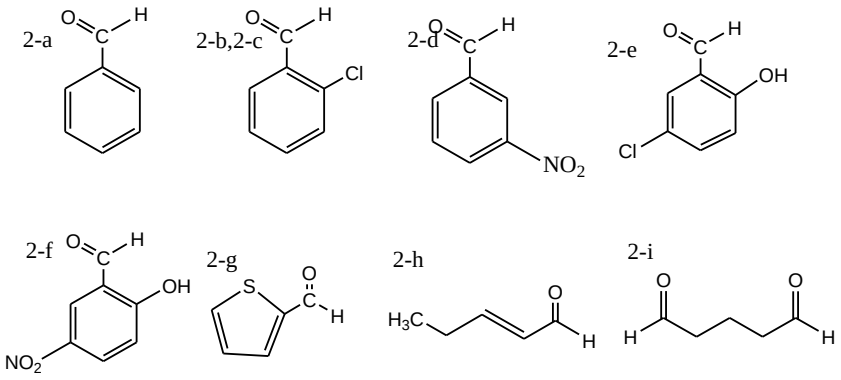


Fig 25: Structure of different Aldehydes

As there are two amino groups present on pteridine ring, two imine compounds can be synthesized depending on equivalents of aldehydes. MTX (0.066 mmol, 30 mg) dissolved in 20 ml of solvent in 100 ml round bottom flask. NaOH (0.066 mmol, 2.64 mg), and aldehyde (0.066 mmol) was added and set on reflux. Reaction monitored by TLC, 15%$CHCl_3$in hexane was used as the solvent system. After completion of reaction pH maintained at 5, ppt washed with hexane and dried.

3.5 Synthesis of 2-(4-(((2-amino-4-(benzylideneamino)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3-a) 10

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, benzaldehyde (0.066 mmol, 7.00 mg) also added to the RBF and reflux at 78 ℃ for 18hr. Reaction was monitored with TLC. After completion of the reaction, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 94%, m.p 283 ^{o}C., dark brown,

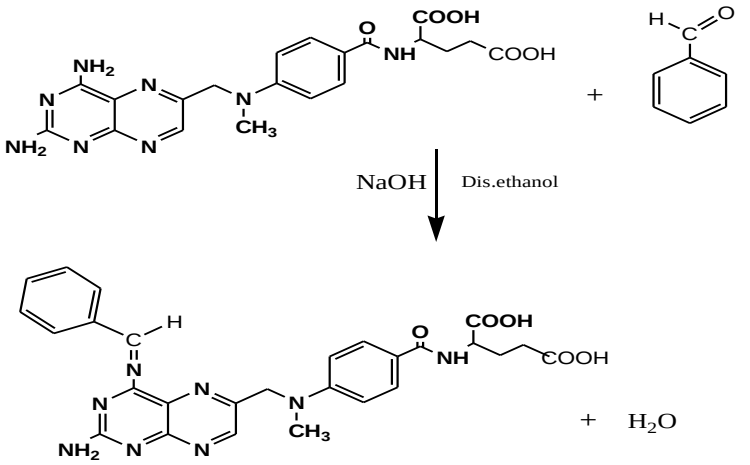


Fig 26: Scheme of synthesis of MTX with Benzaldehyde

3.6 Synthesis of 2-(4-(((4-(2-chlorobenzylideneamino)-2-aminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3-b) 1

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, 2-chlorobenzaldehyde (0.066 mmol, 9.27 mg) also added to the RBF and reflux at 78 ℃ for 19hr. Reaction was monitored with TLC. As spot of aldehyde disappears from the reaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove

unreacted aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 92 %, m.p. 240 ^{o}C, Dark brown

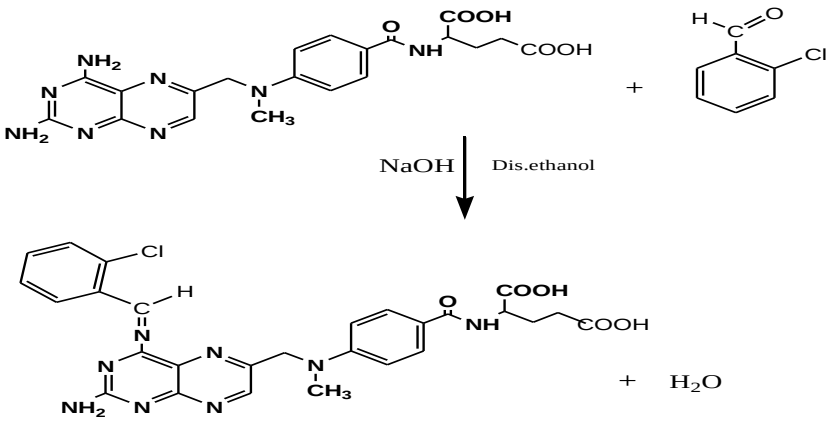


Fig 27: Scheme of synthesis of MTX with chlorobenzaldehyde

3.7 Synthesis of (Z)-2-(4-(((2,4-bis(2-chlorobenzylideneamino)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3-c) 4

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, 2-chlorobenzaldehyde (in excess) also added to RBF and reflux at 78 ℃ for 19hr. As aldehyde was used in excess so reaction monitored through TLC by comparing with a MTX spot in polar mobile phase. TLC showed a different spots than MTX, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 77 , m.p. 250 ^{o}C, Brown.

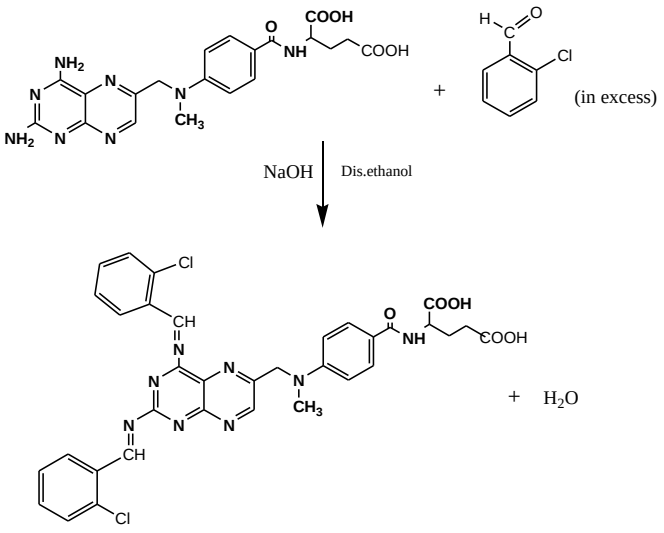


Fig 28: Scheme of synthesis of MTX with 2 equivalent of Chlorobenzaldehyde

3.8 Synthesis of 2-(4-(((4-(3-nitrobenzylideneamino)-2-aminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3-d) 5

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, 3-nitrobenzaldehyde (0.066 mmol, 9.97 mg) also added to RBF and reflux at 78 ℃ for 48hr. Reaction was monitored with TLC. A spot of aldehyde disappears from the reaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 93 ^{o}C , m.p. 257 ^{o}C, dark brown

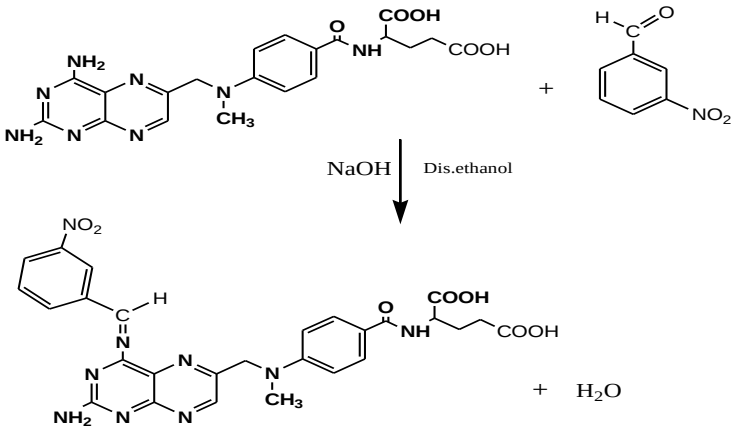


Fig 29: Scheme of synthesis of MTX with 3-NO_2benzaldehyde

3.9 Synthesis of 2-(4-(((4-(5-chloro-2-hydroxybenzylideneamino)-2-aminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (2-e) 6

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, 5-chloro-2-hydroxybenzaldehyde (0.066 mmol, 8.98 mg) also added to the RBF and reflux at 78 ℃ for 48hr. Reaction was monitored with TLC. A spot of aldehyde disappears from the reaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 78 %, m.p. 233^{o}C, yellow

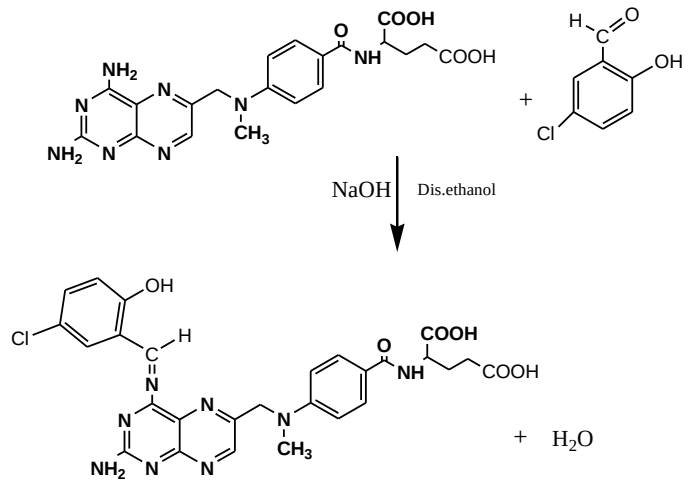


Fig 30: Scheme of synthesis of MTX with 5-Chloro-2-hydroxybenzaldehyde

3.10 Synthesis of 2-(4-(((4-(2-hydroxy-5-nitrobenzylideneamino)-2-aminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3-f) 7

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, 2-hydroxy-5-nitrobenzaldehyde (0.066 mmol, 11.02 mg) also added to the RBF and reflux at 110℃ for 40hr. Reaction was monitored with TLC. A spot of aldehyde disappears from the reaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. Toluene evaporated with rotavapor, pat dried and collected. Yield 92 % , m.p. 270 ^{o}C dark yellow

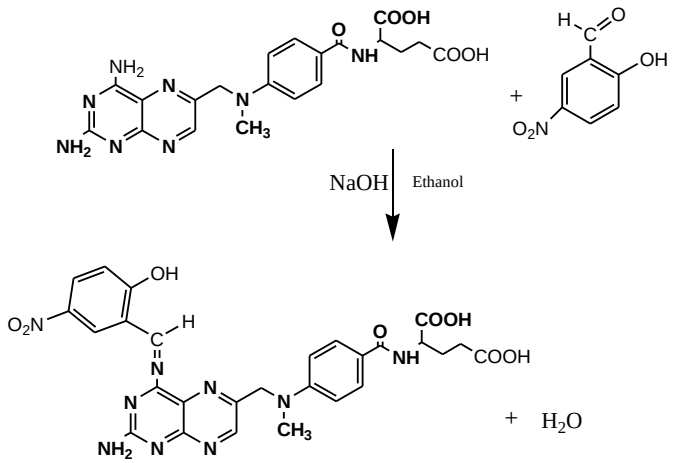


Fig 31: Scheme of synthesis of MTX with 2-hydroxy-5-NO_2benzaldehyde

3.11 Synthesis of 2-(4-(((2-amino-4-(thiophen-2-ylmethyleneamino)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (3-g)

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol 30 mg) add to RBF, 2-thiophencarboxyaldehyde (0.066 mmol, 7.40 mg) also added to the RBF and reflux at 78 ℃ for 22hr. Reaction was monitored with TLC. A spot of aldehyde disappears from the reaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 82 %, m.p. 260 ^{o}C, Yellow brown

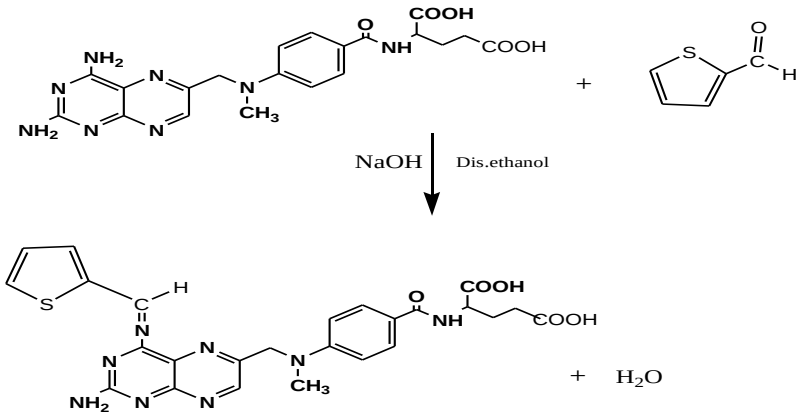


Fig 32: Scheme of synthesis of MTX with 2-Thiocarboxybenzaldehyde

3.12 Synthesis of 2-(4-(((2-amino-4-(Z)-pent-2-enylidene)amino)pteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid (2-h)

NaOH (0.066 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066 mmol, 30 mg) add to RBF, 2-transpentenal (0.066 mmol, 5.55 mg) also added to RBF and reflux at 78 ℃ for 18hr. Reaction was monitored with TLC. As spot of aldehyde disappears from reaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted aldehyde if present. Solvent evaporated with rotavapor, ppt dried and collected. Yield 80%, m.p. 273 ^{o}C, brown

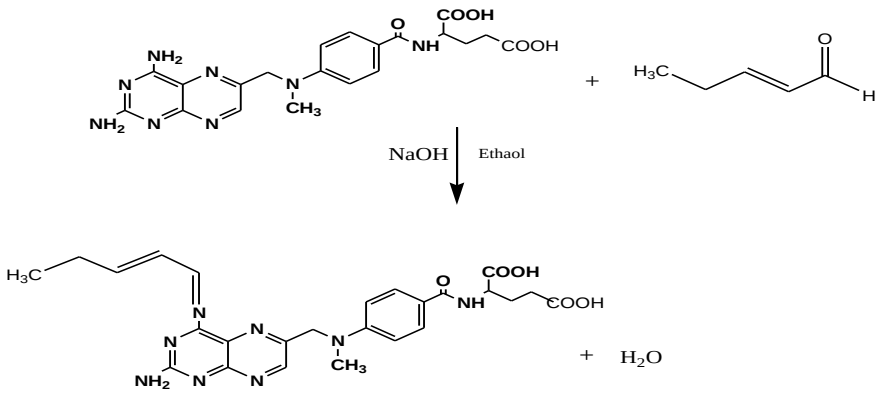


Fig 33: Scheme of synthesis of MTX with Trans-2-pentenal

3.13 Synthesis of imine derivative of MTX with Glutaraldehyde (3-i) 11

NaOH (1 mmol, 2.64mg) dissolved in 10 ml of freshly distilled ethanol in 100 ml round bottom flask. MTX (0.066mmol, 30 mg) add to RBF, Glutaraldehyde (0.033 mmol, 3.3 mg) also added to the RBF and reflux at 78 ℃ for 18hr. Reaction was monitored with TLC. A spot of aldehyde disappears from thereaction mixture on TLC, pH maintained by 5% HCl at 5 and ppt washed with ethanol. Ppt of product also washed with hexane to remove unreacted

aldehyde if present. The solvent evaporated with rotavapor, ppt dried and collected. Yield 77%, m.p.246 ^{o}C, Bright yellow

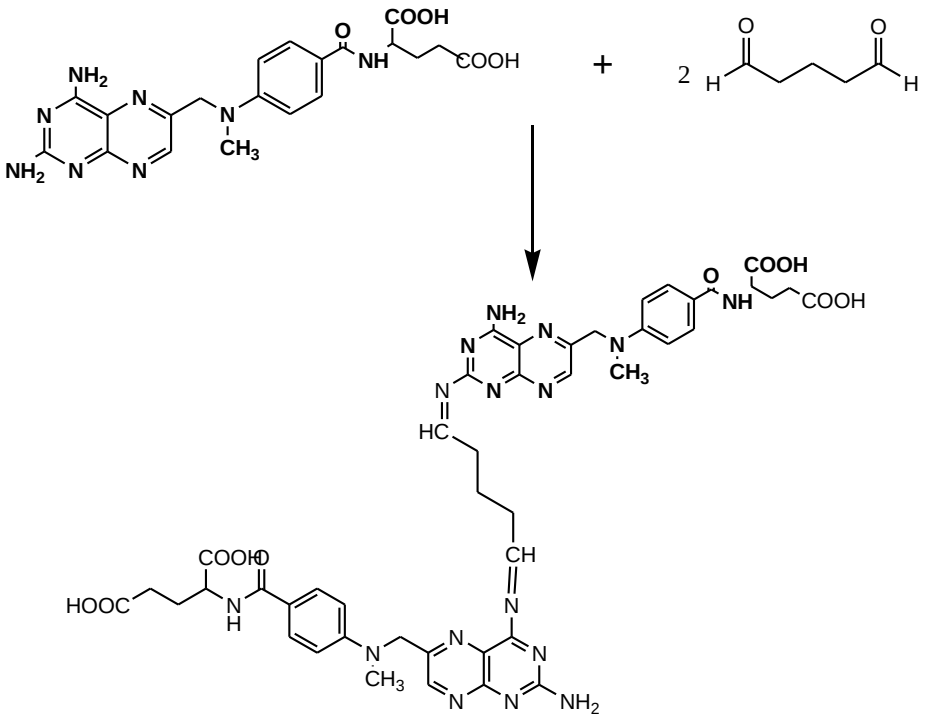


Fig 34: Scheme of synthesis of MTX with Glutaraldehyde

Chapter 4

Results and Discussion

General Discusssion

This chapter deals with the results and discussion of synthetic schemes used to complete research plan. There were two reaction schemes used to synthesize schiff bases used. The first synthetic scheme is as reported [40]. Another scheme is to use toluene as solvent, Na_2SO_3 to absorb water and overnight reflux at 110 ℃.

Aldehydes used Benzaldehyde, 2-Chlorobenzaldehyde, 3-Nitrobenzaldehyde, 5-Chloro-2-Hydroxybenzaldehyde, 2-Hydroxy-5Nitrobenzaldehyde, 2-Thiophenecarboxyaldehyde, Trans-2-Pentenal, and Glutaraldehyde. All aldehydes are of use as it is without further purification.

Reaction was continuously monitored thorough TLC and both reactant are UV active. After completion of the reaction, product was washed with ethanol and dried. Imine bond was identified through FTIR. The Band 1690-1640 medium shows the presence of imine bond.

Scheme of reaction is as follows.

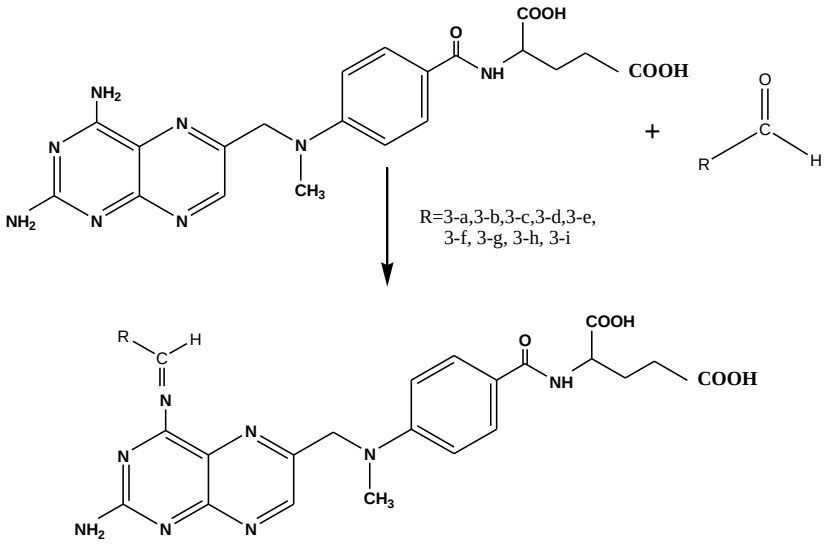


Fig 35: Scheme of General reaction of MTX and aldehyde

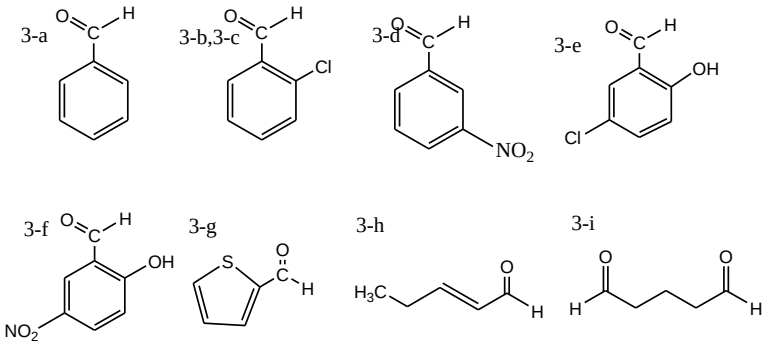


Fig 36: Structure of Aldehydes use in Synthesis of Schiff bases

Table 22: Physical Properties

Compound	**Physical Appearance**	**Molecular weight (g/mol)**	**Melting Point (°C)**	**Yield (%)**
3-a	Dark brown powder	542.521	Stable up to 283 °C	92
3-b	Dark brown powder	577.007	Stable up to 240 °C	77
3-c	Brown	699.574	Stable up to 250 °C	93
3-d	Dark brown powder	587.56	Stable up to 257°C	78
3-e	Yellow	595.005	Stable up to 233°C	92
3-f	Dark Yellow	603.56	Stable up to 270 °C	82
3-g	Yellow Brown	548.586	Stable up to 260 °C	80
3-h	Brown	520.558	Stable up to 273 °C	94
3-i	Bright Yellow	972.99	Stable up to 246 °C	88

Synthesized compounds have different melting points than parent drug which is an indication that schiff base ligands may synthesize.

FT-IR Spectroscopy:

In the FT-IR spectra of the compounds (3a-3i), the characteristic band for C=N appeared at 1575-1602 cm^{-1}. The C=N is the main functional group of the Schiff base. The appearance of the band for C=N in the spectra indicated the Schiff base's formation.

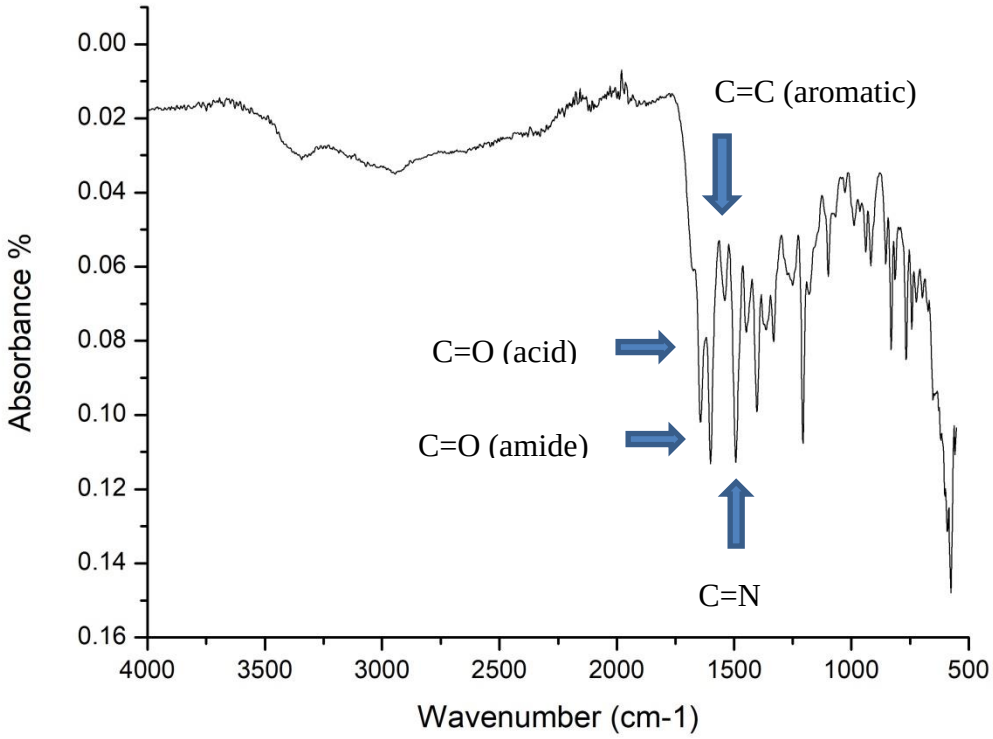


Fig 37: FT-IR spectrum synthesized Schiff base derivatives of Methotrexate

Table 23: FT-IR values of derivatives

Comp	**C=N (ν cm^{-1})**	**C=O (amide) (ν cm^{-1})**	**C=C (aromatic) (ν cm^{-1})**	**C=O (acid) (ν cm^{-1})**
MTX	1500	1600	1492	1688
MTX-Cl	1590	1596	1485	1680
MTX-Cl(2.0)	1600	1610	1480	1670
MTX-NO_2	1620	1650	1478	1730
MTX-Cl OH	1600	1620	1520	1720
MTX-Cl NO_2	1580	1595	1495	1640
MTX-Thio	1610	1590	1530	1690

MTX-Pentenal	1620	1600	1510	1635
MTX-Benz	1605	1600	1555	1620
MTX-Glu	1630	1590	1560	1690

Elemental Analysis:

Elemental analysis shows the percentage content of particular element in a compound. Table below shows the percentage content of Nitrogen, Carbon, and Hydrogen.

	C		H		N	
	Experimental	Calculated	Experimental	Calculated	Experimental	Calculated
MTX-Cl	57.06	56.20	3.92	4.37	18.61	19.42
MTX-Cl (2.0)	56.92	58.32	4.22	4.03	15.53	16.02
MTX-NO_2	54.81	55.19	5.10	4.29	21.21	21.46
MTX-Cl OH	57.13	54.69	4.97	4.25	18.51	18.90
MTX-$OHNO_2$	54.10	53.79	5.21	4.18	21.15	20.89
MTX-Thio	56.17	54.74	3.92	4.41	19.96	20.43
MTX-Pent	56.82	57.62	4.60	5.42	20.92	21.43
MTX-Benz	58.63	59.77	5.25	4.83	21.23	20.65
		55.55	4.45	4.97	23.25	23.03

4.4 Biological activity

The anticancer activity of synthesized derivatives was assayed against malignant glioma cell lines. All synthesized derivatives were tested for six different ratios: 12.5 μM, 25 μM, 50 μM, 100 μM. 200 μM, 400 μM in triplicate. All analogues were potentially active against malignant glioma cell lines after 24 hr treatment. Methotrexate was also used in all concentration for the same cell line. Six fold of dilution was used

- 1^{st} dilution contains 400 μM
- 2d dilution contains 200 μM
- 3^{rd} dilution contains 100 μM
- 4^{th} dilution contains 50 μM
- 5^{th} dilution contains 25 μM
- 6th dilution contains 12.5 μM

The synthesized derivatives show % survival rate between 53.92-56.43 for first dilution. It is in the range of 74-79 %, 76-78%, 93-98%, 94-98% and 94-98% for 2^{nd},3^{rd}, 4^{th},5^{th}, and 6^{th} dilution respectively. It is obvious from a graph that percent survival rate for cancerous cell is maximum for most diluted concentration that is 12.5 μM and it goes on increasing for higher concentration. It is highest on for 400 μM or first dilution. Three of synthesized derivative namely Thio-MTX, Cl-MTX and 2-Pentenal-MTX shows 50-55 % survival rate on 200 μM concentrations as compared to MTX which gives the same results on 400 μM concentration. MTX –Cl shows 52 % survival rate on 100 μM, which is the lowest among all synthesized analouges and parent drug. MTX-Cl (2.0) gives the 57% survival rate on 100 μM but later it increases in higher concentration. This enhanced efficacy in synthesized analogues is due to imine linkage.

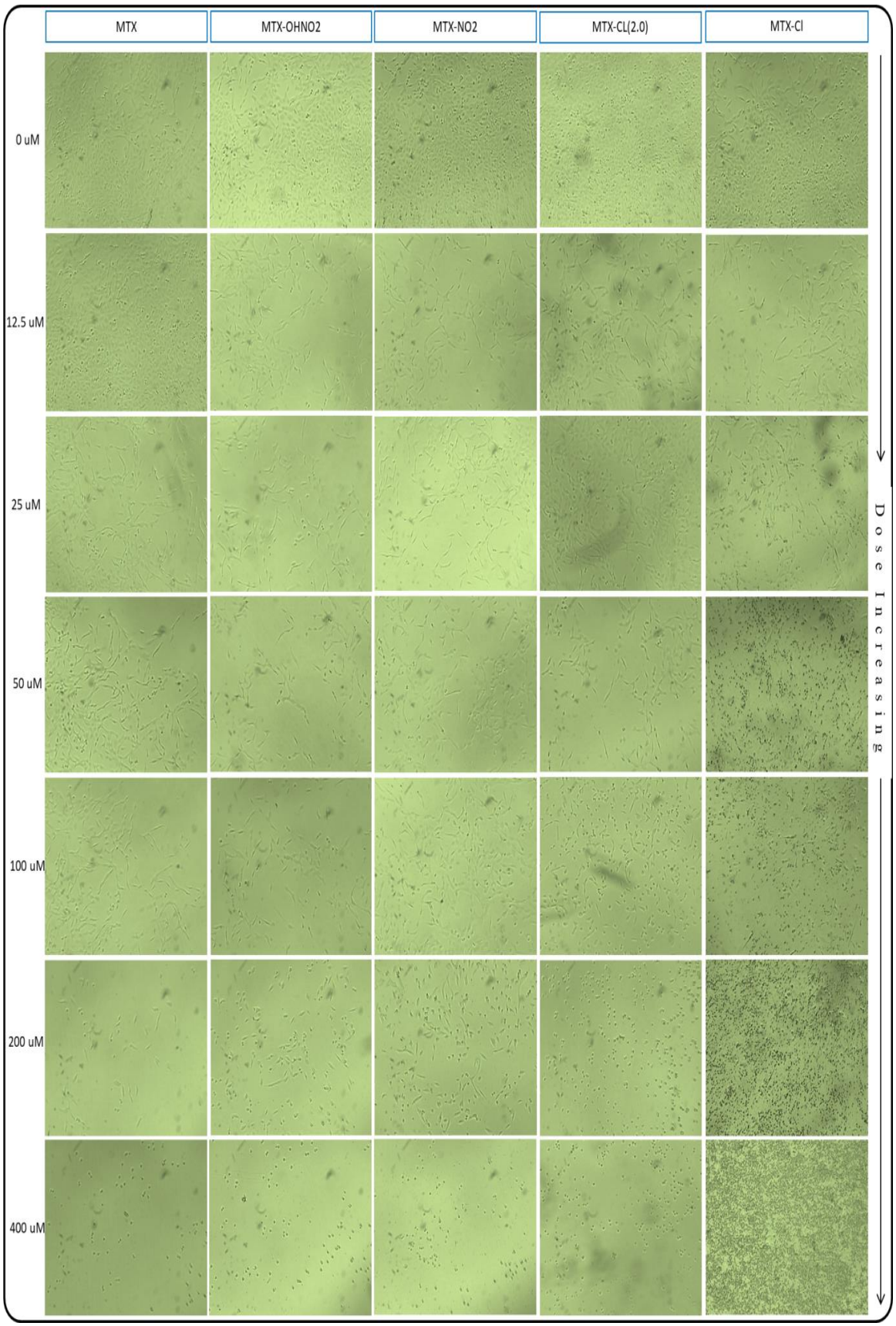

MTX
MTX-OHNO2
MTX-NO2
MTX-CL(2.0)
MTX-Cl
0 uM
12.5 uM
25 uM
50 uM
100 uM
200 uM
400 uM
Dose Increasing

Fig 38:

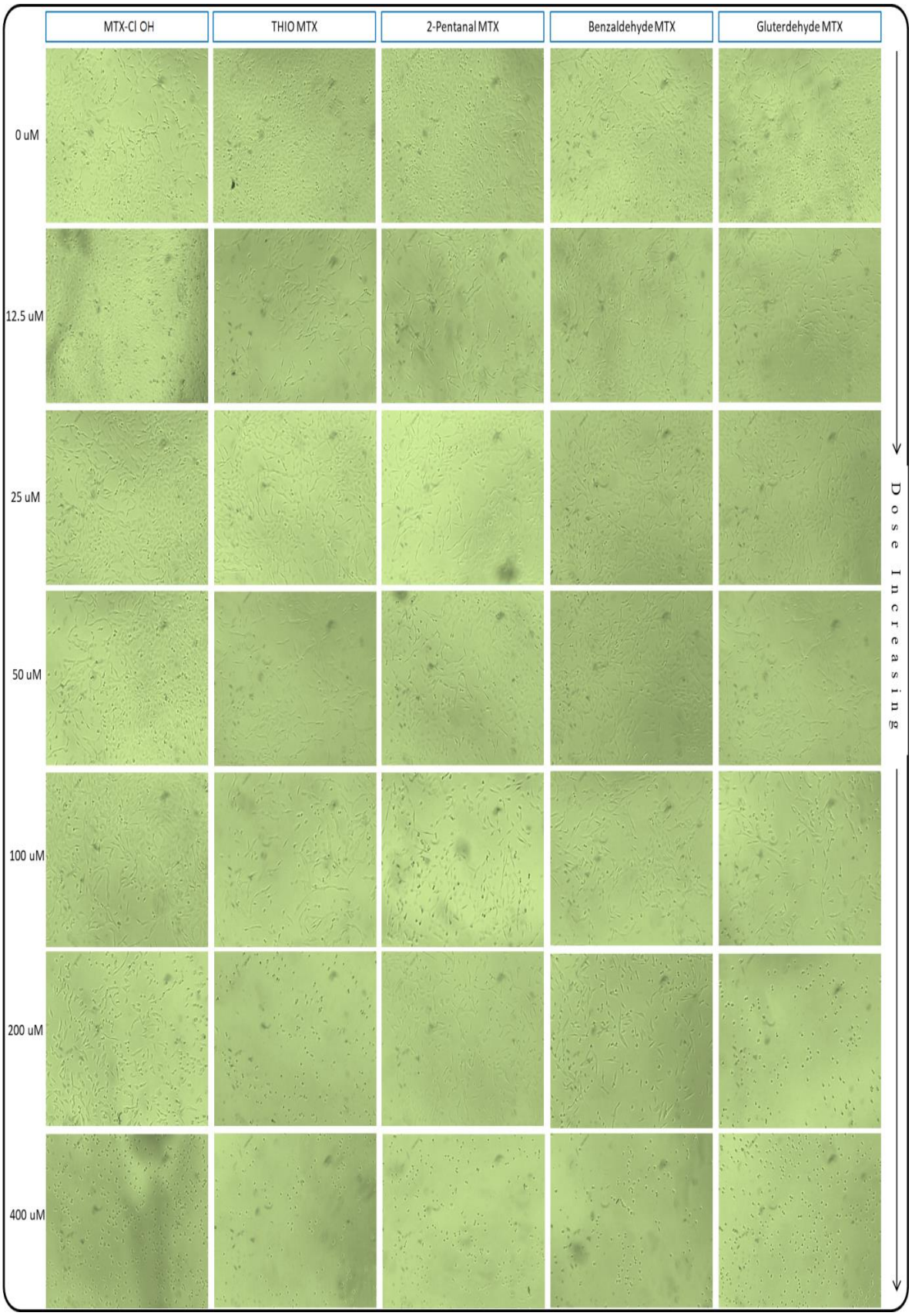

MTX-Cl OH
THIO MTX
2-Pentanal MTX
Benzaldehyde MTX
Gluterdehyde MTX
0 uM
12.5 uM
25 uM
50 uM
100 uM
200 uM
400 uM
Dose Increasing

Fig 39:

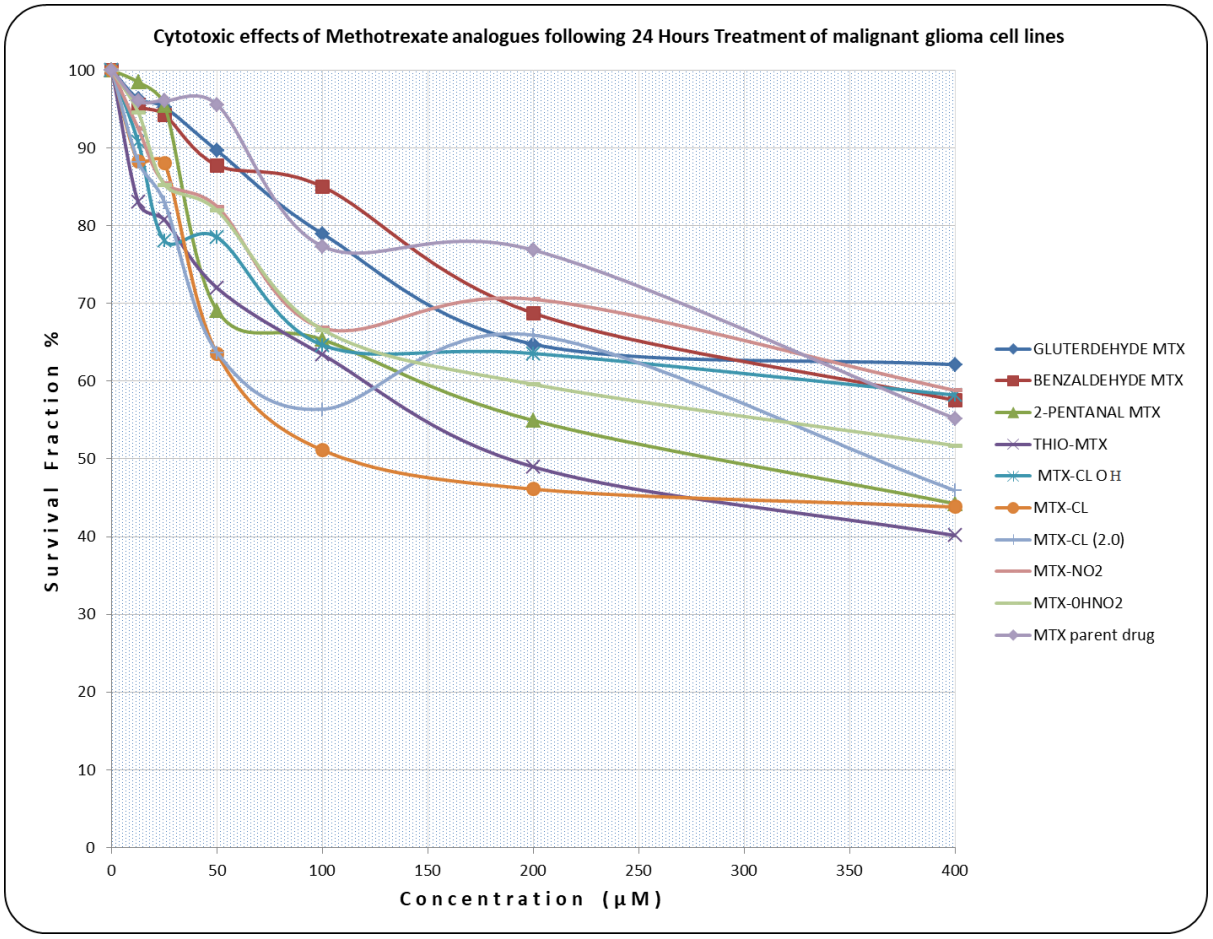


Fig 40: Graph of Survival fraction rate % of cytotoxic effects of MTX analogues

4.5 Computational Studies:

The results of these studies can serve as a starting point for further theoretical and experimental studies. Novel compounds were evaluated for malignant Glioma cell lines *in vitro*. The current work describes the development of active MTX derivatives better against malignant Glioma cell lines.

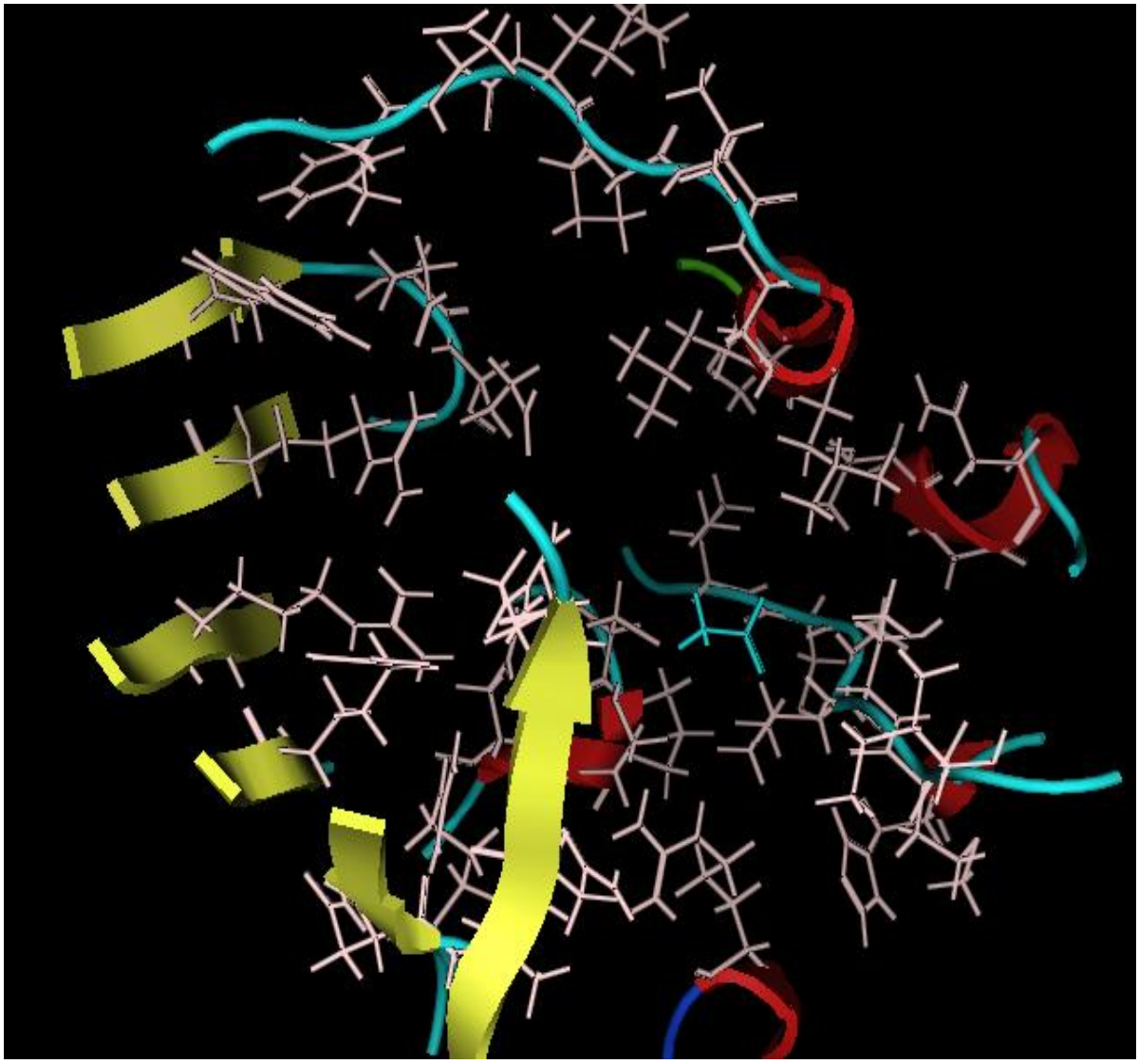


Fig 42: Active site of brain tumor protein 1QH4.

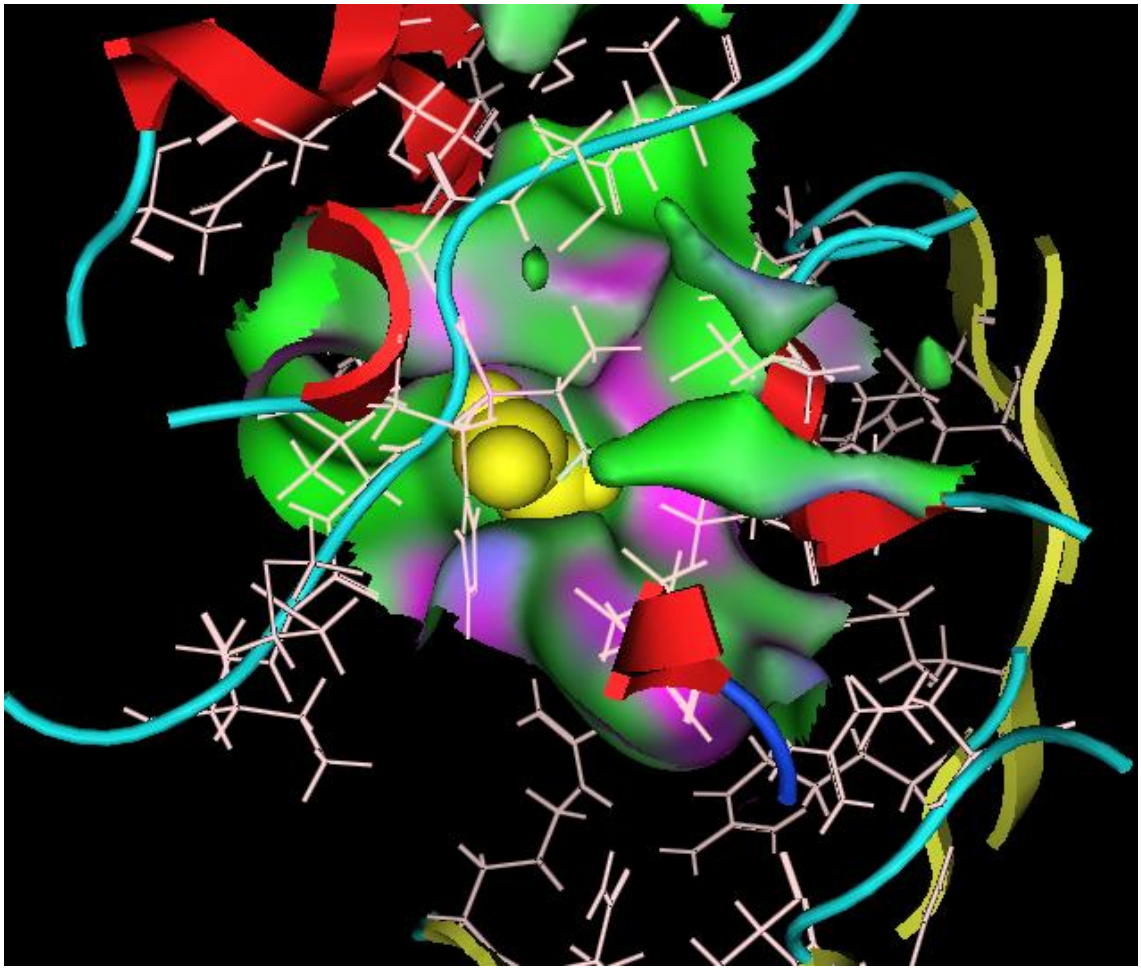


Fig. 43: Surface and map representation of Protein complex, the Co - crystallized ligand is shown in a yellow space filling form.

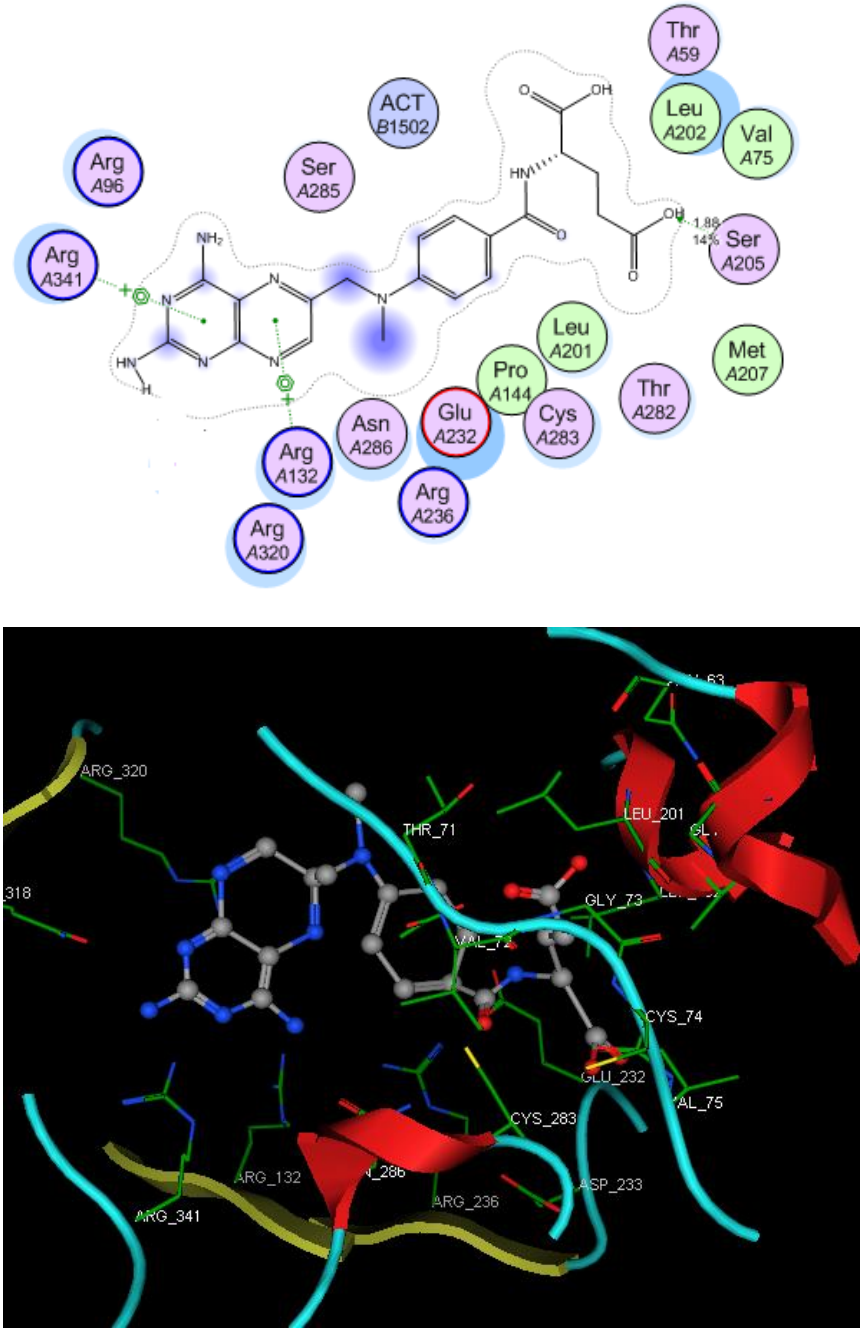


Figure 44: MTX Drug 2D & 3D docked binding mode

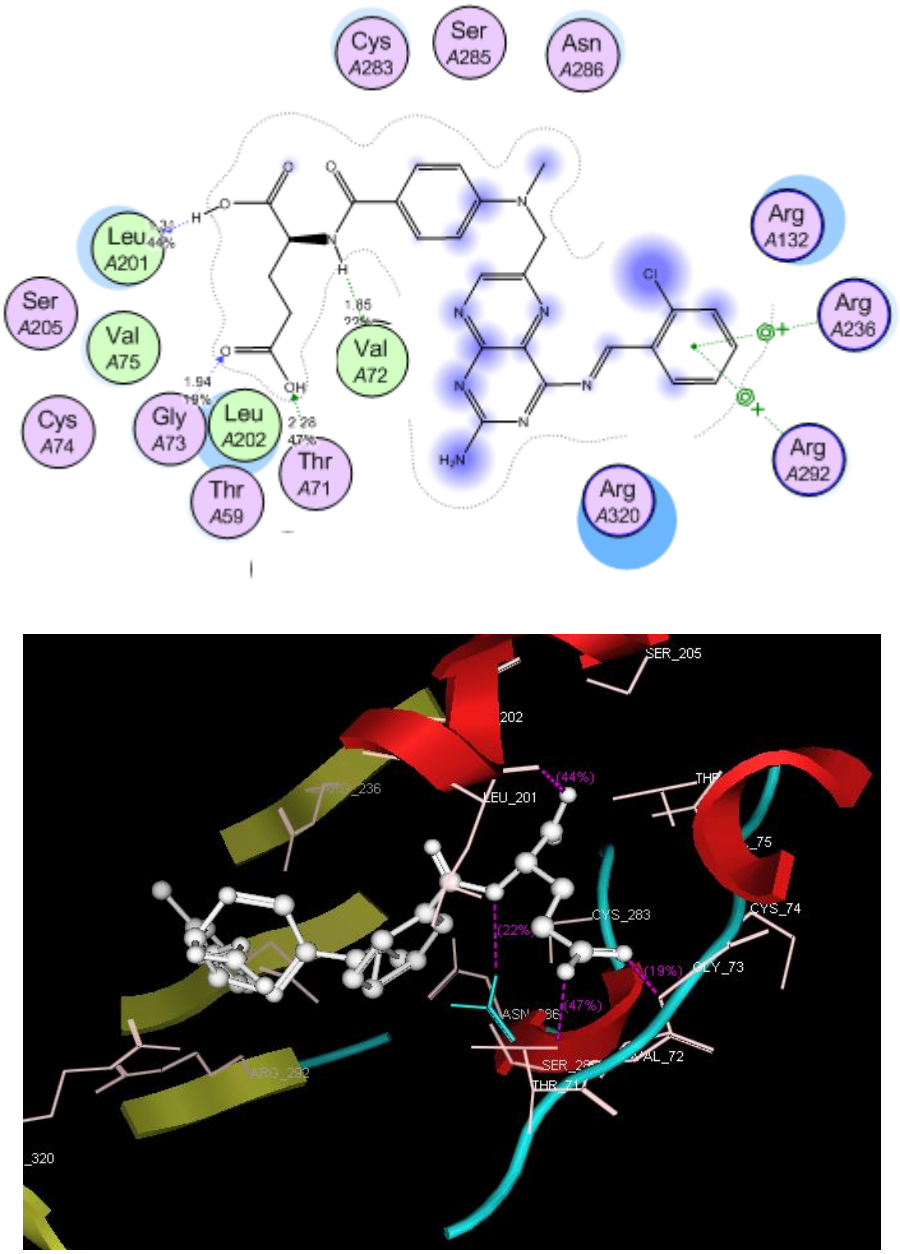


Figure 45: MTX- Cl(2.0) 2D & 3D docked view in the active site of protein complexes, H-binding shown by purple dotted line.

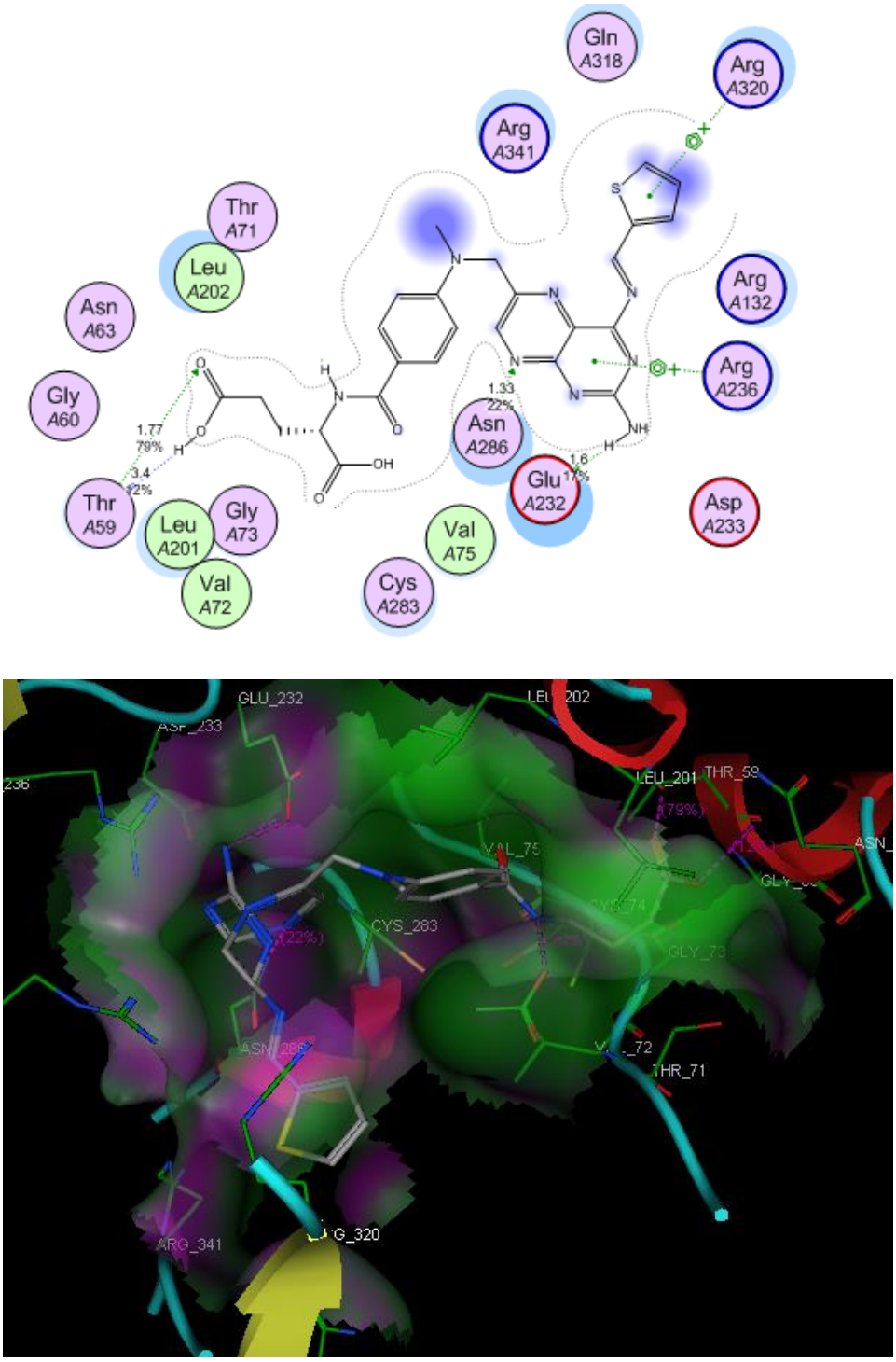


Fig 46: MTX-NO2 2D & 3D docked view in the active site of protein complexes

Table 24 : Dockedbinding interaction of MTX derivatives in active site of 1QH4.

Drug +derivatives	Survival fraction %	**H-bonding**			**Arene-π interactions**	**Binding Energy(kcal/mol)**
		Distance (°A)	Score (%)	Amino acids	Amino acids	
MTX (parent	78	1.88	14	SerA205	ArgA132 ArgA 341	-11.23
MTX-Cl	46	2.28, 1.31,1.85, 1.94	47,44, 19, 21	Thr71, Leu201, Gly73, ValA72	ArgA236, ArgA292	-12.52
MTX-Thio	48	1.77,3.4, 1.33, 1.6	79,12, 22, 17	ThrA59, AsnA286 ,GluA232	ArgA236, ArgA320,	-12.00
MTX-2-pent	56	3.11,1.9 2.64, 2.03	10,65, 58,24	CysA74, GluA232 ,Asn286	ArgA341	-11.83
MTX-OHNO2	59.50	3.0, 1.97. 2.75	54, 17.7, 4.75	GlyA73, CysA74, AsnA63	ArgA320	-11.78
MTX-ClOH	65	2.01, 2.1	45, 28	ArgA236 , SerA286	ArgA132	-11.77
MTX-Glutaraldehyde	66	2.53, 1.9	40,22	GlyA73, CysA74,	ArgA132	-11.34
MTX-Cl(2.0)	67	2.20, 1.9	29, 32	GlyA73, CysA74,	ArgA132	-11.62
MTX-Benzaldehyde	69.5	2.56, 2.1	30,19	ArgA236 , SerA286	ArgA132	-10.43
MTX-NO2	70	3.0, 2.3	25, 19	SerA206	ArgA341	-10.37

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