A comprehensive review of synthesized derivatives of methotrexate in relation to their anticancer potential.

Nemat A[1], Iqbal M[1]*, Mehmood T[2]

[1]Department of Chemistry, School of Natural Sciences (SNS), National University of Sciences and Technology (NUST), Islamabad, Pakistan
[2]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences-UVAS, Lahore, Pakistan

Abstract

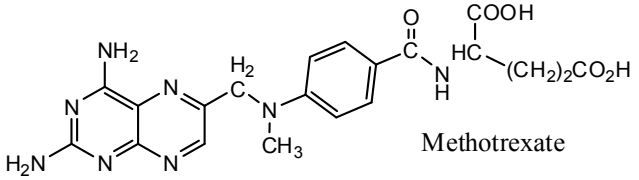
Methotrexate (MTX) is a chemotherapeutic agent with certain side effects. Efforts were made to synthesize analogues of MTX to enhance the activity and reduce side effects. Various derivatives were synthesized by structural modification of parent drug and mainly assayed for anticancer activity and binding affinity with dihydrofolatereductase (DHFR) and folylpolyglutamate synthetase (FPGS). *In vitro* study was carried out on cell lines CCRF. CEM, ATCC8, *L. Casei*, P388, L1210, L1210/R81, and H35. These synthesized derivatives have been assayed for human squamous cell carcinoma namely SSC25, SSC68, SSC78, SSC25/R1, SSC68/R1, and SSC78/R1. Some modification proved to enhance anticancer activity while others are detrimental. Some of derivatives were also tested for other biological activity like anti-malarial, anti-bacterial and anti-diabetic and show better activity. This article deals with a comprehensive overview of the synthesized derivatives by structural modifications and the impact of these modifications on its activity.

Keywords: Methotrexate, Derivatives, Anticancer activity, Review.

Accepted on February 11, 2020

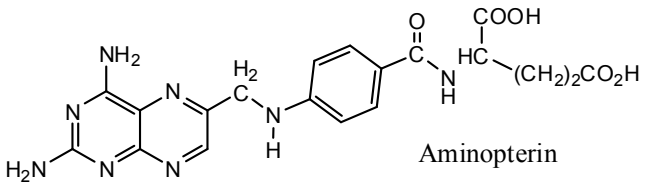
Introduction

Methotrexate is known as amethopterin which suppresses the immune system and used as chemotherapeutic agents. It was synthesized in 1947 and in 1956 it proved to cure as for metastatic cancer [1].



Methotrexate

There is another analogue of folic acid and methotrexate known as aminopterin. It is also used in chemotherapy as it supresses the production of folic acid. It has comparable activity with methotrexate.

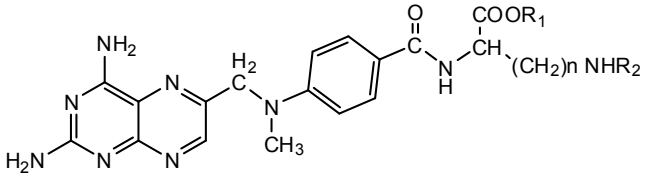


Aminopterin

Methotrexate effects folic acid pathway [2], as it closely resembles folic acid, acting as antimetabolite and used in the treatment of various diseases like arthritis and cancer [3]. Although methotrexate has many applications but it has certain disadvantages, e.g. fast metabolism and low selectivity for tumor cell [4]. The mechanism of inhibition of cancer cells by MTX starts from inhibition of DNA and RNA synthesis, particularly in rapidly dividing cancerous cells thus acting as anticancer drug [5]. Dihydrofolatereductase (DHFR) is used to catalyse the dihydrofolate (DHF) to tetrahydrofolate (THF). THF is necessary for folate reduction and hence its needed for DNA synthesis [6]. Methotrexate is used to treat rheumatoid arthritis [7], psoriasis [8], cancer [9] and ectopic pregnancy [10]. Keeping in view all the uses of methotrexate it has certain disadvantages as it causes life threatening side effects on vital organs [11]. There are a number of derivatives synthesized by modification of MTX showing different activity and binding affinity.

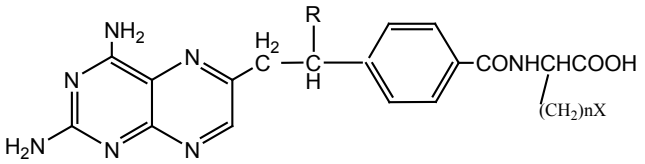
Literature Review

Lysine and ornithine derivatives of MTX were synthesized and both derivatives have same binding affinity as MTX and lysine have the same potency as MTX. Reaction mechanism involved protection and de-protection of amino acid and de-protected derivative is eight times more potent. Lysyl derivative has the same potency in protected and de-protected form. Lysyl derivative was more potent than ornithine derivatives. Presence of Cbz group didn't affect activity but removal improved activity 3 folds in ornithine. Inhibition and DHFR binding were tested in liver of chicken. Results are summarized in (Table 1).

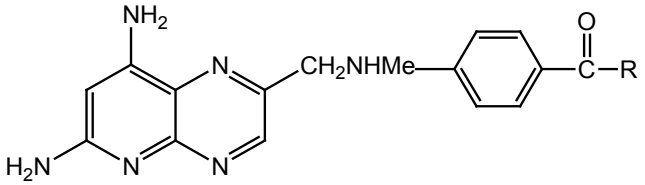


Various MTX derivatives have been synthesized having a different group by replacing γ-COOH and only a few were tested for anti-proliferative activity. These derivatives have been tested

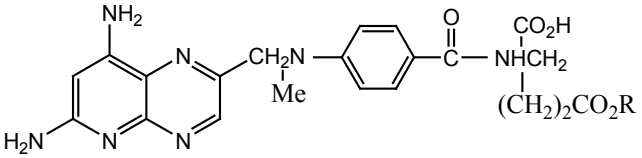
against DHFR, FPGS, L1210 and L1210/R81. Ornithine derivative was found to be more potent against DHFR and FPGS (Table 2).



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 lessens binding affinity results mentioned in (Table 3).



Lysyl derivatives of methotrexate have been synthesized by reaction of γ-COOH with the 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. Minimum activity was shown by derivative with three Lysyl groups; moreover, an increase in the number of Lysyl derivative does not affect activity. Activity was tested against L1210 and H35 cell line and these showed same result (Table 4).



MTX: R= OH

1 [MTX (γ-e)-Lys], R= $NH_2CH[(CH_2)_4NH]$ COOH

2 [MTX $(\gamma\text{-e})\text{-}(Lys)_2$], R= $NH_2CH[(CH_2)_4NH]$ $CONHCH[(CH_2)_4NH_2]$ COOH

Table 1: *Lysine and ornithine derivatives and their I_{50} valμes [12].*

Comp	R_1	R_2	n	$I_{50} \times 10^8$M[a]
1(MTX)	H	H	2	9
2	t-Bμ	Cbz	3	310
3	t-Bμ	Cbz	4	95
4	H	Cbz	3	33
5	H	Cbz	4	38
6	H	H	3	25
7	H	H	4	13
a=The enzyme concentration was 18.4 × 10^{-8} M				

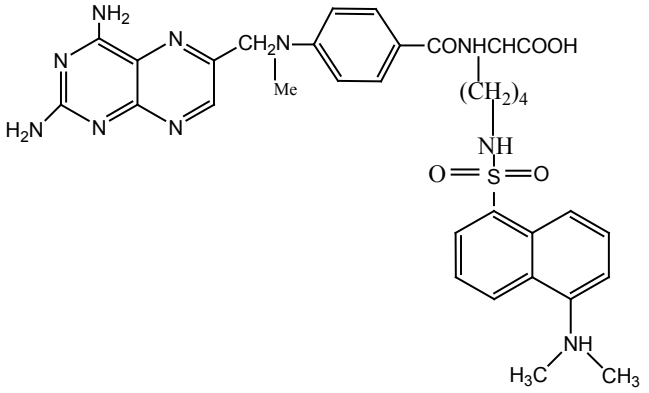
Table 2: *Analogμes of MTX and their IC_{50} valμes [13].*

Comp	R	n	X	DHFR IC_{50} μM	FPGS Ki μM	L1210 Ki μM	L1210/R81 IC_{50} μM
MTX	Me	2	COOH	0.035	-	0.002	220
AMT	H	2	COOH	0.035	-	0.002	84
2	Me	4	NH_2	0.065	-	0.4	220
3	Me	3	NH_2	0.16	20.4	1.3	86
4	Me	2	NH_2	0.12	-	2.42	290
5	Me	1	NH_2	0.18	-	0.44	405
6	H	3	NH_2	0.072	0.15	1.3	32

Table 3: *Analogμes of MTX and their binding affinity [14].*

Comp	R	*L. casei* (ID_{50})[b]	L1210 (ID_{50})[b]
MTX	$NHCHCO_2H$ $(CH_2)_4NH_2CO_2H$	6.2	2.7
1	$NHCHCO_2H$ $(CH_2)_4NHCOCH_2I$	4.5	31
4	$NHCHCO_2H$ $(CH_2)_4NHCbz$	14	11
5	$NHCHCO_2H$ $(CH_2)_4NH_2$	23	11
b=Competitive [3H] MTX binding assay affinity for DHFR			

3 [MTX (γ-e)-(Lys)$_3$],R= $NH_2CH[(CH_2)_4NH]CONHCH[(CH_2)_4NH_2]CONHCH[(CH_2)_4NH_2]$

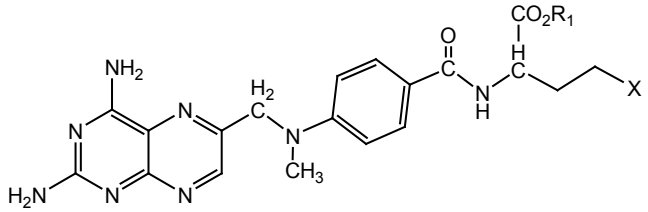


A fluorescent derivative of MTX has been synthesized by the lysine derivative of MTX to dansyl analogue and this analogue show higher activity against DHFR of *L. Casei* [16].

Protected dilysine and trislysine analogues of methotrexate have been synthesized. Two schemes were used to synthesize lysine derivatives. In Scheme (I) the substituted L-glutamyl-L-lysine intermediate **6** was formed stepwise from dilysine, while in the scheme (II) synthesis was done from two N-terminal lysine joined in 6 steps [17].

Shams A. Nadhum et al *2015* synthesized a conjugate of silibinin and MTX in order to enhance efficacy than parent drugs and to minimize their side effects. Scheme of reaction consists of 6 steps in which firstly imine derivative formed. This imine derivative is converted into imine-MTX-cysteine and after 2-3 steps imine conjugate of MTX and silibinin. Imine conjugate hydrolyzed to get conjugate of MTX and silibinin. Anticancer activity was tested 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 MTX-silibinin conjugate shows 41.2% [17].

Andre et al *1984* synthesized a polygultamate derivative bind as well as MTX and leave mammalian cell more slowly. This analogue contained gama-SO_3H instead of COOH and was synthesized in 78% yield. Binding affinity is measure of interaction by which a molecule (protein) binds with drug and in turn potency of drug can be found which actually drugs activity, for anticancer activity cell lines of humane lymphoblastic leukemia cells were used for measuring binding and anticancer efficacy, MTX used as positive control. The binding affinity of analogue was same as of MTX. Three experiments were conducted using same quantity and different doses and it revealed that by increasing frequency increase in molar potency of the drug shown in (Table 5) [18].



γ-Hydrazide, γ-n-butylamide, γ-benzylamide and γ-tert-butyl ester analogues of MTX have been synthesized. The binding affinity of synthesized derivatives was tested for DHFR from L1210 mouse leukemia cells and *Lactobacillus casei*. It has been found that γ-terminal region of MTX is site for modifications. Binding affinity has been tested to DHFR of *L. Casei* and L1210 by spectrophotometric assay and radio-ligand assay respectively. It was found that gamma substituted compound binds effectively than α-Substituted. γ-tert-butyl ester shows 1.9 times higher binding affinity than MTX. γ-tert-butyl ester and γ hydrazide showed same affinity as MTX. γ-n-butyl amide showed slightly same activity as MTX. γ-benzyl amide showed less activity (Table 6).

Table 4: *Dihydrofolate reduμctase inhibitory activity and cytotoxicity of lysyl derivatives of MTX [15].*

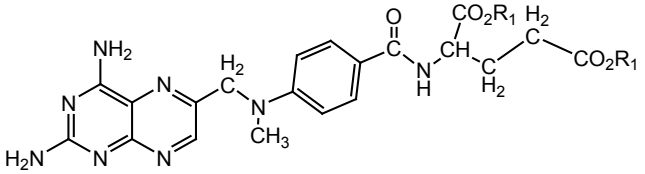
Comp	L1210 DHFR IC_{50} nM	L1210 cells IC_{50} μM	H35 Cells IC_{50} μM
MTX	50	0.024	0.01
1	87	0.76	0.4
2	86	1.8	0.5
3	140	2.9	0.56

Table 5: *Dihydrofolate reduμctase (DHFR) binding and cytotoxicity of glμtamate derivatives of MTX [19].*

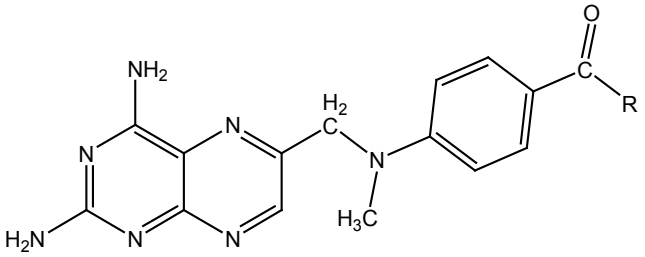
Comp	X	L1210DHFR IC_{50} (nM)	*L. Casei* DHFR IC_{50} (nM)	L1210 cells IC_{50} (nM)
MTX	CO_2H	1	17	0.3
mAPA-HCysA	SO_2H	0.95	10	0.01

Table 6: *Binding affinity of side chain modified MTX derivatives for analogμes of methotrexate [20].*

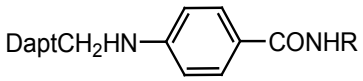
Comp	R_1	R_2	*L. Casei* ID_{50} μm	*L. Casei* (Binding affinity) ID_{50} μm	L1210 (Binding Assay) ID_{50} μm	L1210 ID_{50} μm
1	H	O-t-Bμ	0.025	0.012	0.0029	0.0029
2	H	$NHNH_2$	0.0021	0.013	0.012	0.012
3	H	NH-n-Bμ	0.053	0.036	0.0033	0.0033
4	H	$NHCH_2C_6H_5$	0.055	0.022	0.003	0.003
6	CH_3	-t-Bμ	0.33	3.9	0.011	0.011
7	-t-Bμ	CH_3	0.2	2.2	0.019	0.019
8	-t-Bμ	$NHNH_2$	0.17	2.2	ND	ND
9	-t-Bμ	H	0.036	0.21	0.035	0.035
10	CH_2Ph	$NH\text{-}nC_4H_9$	ND	0.25	ND	ND

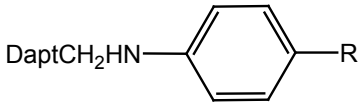


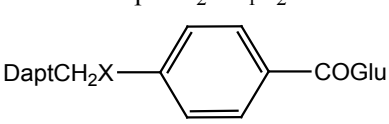
Several MTX derivatives have been synthesized by modification of the glutamyl moiety with peptide side chain. Nine different amino acids were used for modification of the side chain. Various intermediate peptides were also separated. Biological activity was tested for L1210 leukemia and W25 carcinoma in mouse and rat respectively. It has been found that α-COOH is more important for activity than gamma(γ)-COOH. All derivatives were inactive, which show glutamyl moiety is necessary for activity. If α-COOH is present, an increase in length of the alkyl chain restores activity (Table 7).

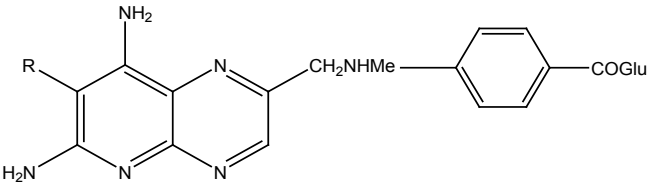


Different derivatives of MTX have been synthesized by alkylation of the side chain. DHFR affinity and anti-proliferating activity was tested against L1210 in mice. It was found that those derivatives have a high inhibition rate, which was structurally closed to MTX. Minute changes in Structure such as derivatization of pyridine ring, chlorination gave good results. But the substitution of aliphatic group decreased the activity greatly. Introducing the carbon around the benzene ring decreased binding affinity, but between –COOH enhances the binding affinity, but none of the synthesized derivatives show significant anti-proliferative activity (Tables 8a-8e).









In a study [23] α and γ-monoesters of MTX were formed and anticancer activity checked against lymphoblastic leukemia (CCRF-CEM) cell lines. γ–monoesters were inhibitory than α-isomer and difference was about 10 folds, but with increase in chain length this difference reduced 2 to 2.5 folds. Monoesters showed a less inhibitory effect than diesters. Diethyl ester was 516 times more activity than α-monoethyl ester and 48 times

Table 7: *Biological data of analogµes of methotrexate [21].*

Comp	R	L1210 Dose (mg/kg) × no. of administration	L1210 %ILS
1a	Glycine	23 × 7	0
1b	DL-alanine	33 × 6	0
1c	B-alanine	25 × 6	19
1d	Sarcosine	50 × 6	0
1e	DL-α-aminobµtyric acid	78 × 9	16
1f	γ- aminobµtyric acid	10 × 8	0
1g	DL-Valine	20 × 7	0
1h	L-leµcine	90 × 9	24
1i	L-phenylalanine	45 × 10	14

Table 8a: *Biological data for derivatives of MTX [22].*

No	R	Inhibition of DHFR I_{50} µm	Cytotoxicity to KB cells ED_{50} µg/µL	Inhibition of leµkemia cells L1210
1	-NMe- (MTX)	0.026	0.004	1.5
2	$CH(CO_2H)(CH_2)_2CO_2H$	0.026	0.003	0.67
26	$CH(CO_2H)(CH_2)_4CO_2H$	0.013	0.025	20
27	$(CH_2)_3CO_2H$	0.38	>100	25
28	CH_2CO_2H	1.1	88	80

Table 8b: *Biological data for derivatives of MTX [22].*

No	R	Inhibition of DHFR I_{50} µm	Cytotoxicity to KB cells ED_{50} µg/µL	Inhibition of leµkemia cells L1210
29	CH2COGlµ	0.38	41	500
30	CH2COAsp	1	>100	200
31	(CH2)2COGlµ	0.46	37	500
32	SO2Glµ	1.1	45	100

Table 8c: *Biological data for derivatives of MTX [22].*

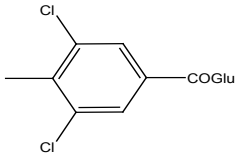
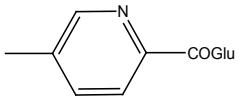
No	R	Inhibition of DHFR I_{50} µm	Cytotoxicity to KB cells ED_{50} µg/µL	Inhibition of leµkemia cells L1210
33	Et		0.050	0.022
34	H		0.030	0.006
35	Me	-(CH2)4COGlu	20	>100

Table 8d: *Biological data for derivatives of MTX [22].*

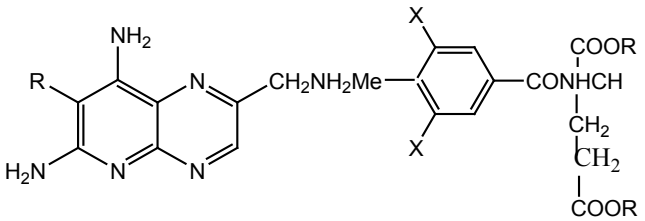
No	R	Inhibition of DHFR I_{50} µm	Cytotoxicity to KB cells ED_{50} µg/µL	Inhibition of leµkemia cells L1210
2	-NH-	0.026	0.003	0.67
1	-NMe-	0.026	0.004	1.5
36	-NEt-	0.045	0.005	10
37	-NBn-	14	>100	80
38	-NPhe-	0.28	26	80
39	$-NMeCH_2-$	0.75	0.043	50
40	-O-	0.51	3.5	1.3

Table 8e: *Biological data for derivatives of MTX [22].*

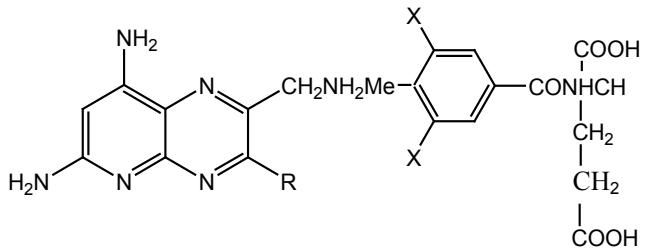
No	R	Inhibition of DHFR I_{50} µm	Cytotoxicity to KB cells ED_{50} µg/µL	Inhibition of leµkemia cells L1210
41	H	0.63	0.05	100
42	Br	2.5	76	200
43	NO_2	38	>100	200

than γ-monoester. Dibutyl esters show the same result as diethyl esters (Table 9).

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 55-60ºC. *In vitro* growth inhibitory activity was also studied with two murine leukemia L1210 in hybrid mice and p1534 leukemia in mice. Dibutyl ester showed a 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 (Table 10).



Several derivatives of MTX have been synthesized by substitution of alkyl group at 7-position. Inhibitory activity against *Streptococcus faecium* ATCC 8043 showed that synthesized derivatives were 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 showed that it might be due to steric effect of $-CH_3$. Studies against L1210 leukemia in mouse showed that these derivatives were inactive (Table 11).



Many MTX derivatives were synthesized by MTX diethyl esters and various amines. The procedure involves the use of an excess of amines without solvent. Four of synthesized derivatives have been tested for CCRF-CEM and three for RBL (Rat basophilic leukemia) cells to make a comparison between two cell lines. Inhibitory activity against lymphoblastic leukemia CCRF-CEM showed that bis-amides derivatives were less active than MTX or MTX esters. Bis (benzylamide) showed higher activity *in vivo* against L1210 in mice, and this activity considered as bis (benzylamide) derivative release free MTX at site other than serum. 1h show most significant activity and IL%. Derivatives show better result for RBL than CCRF-CEM (Table 12).

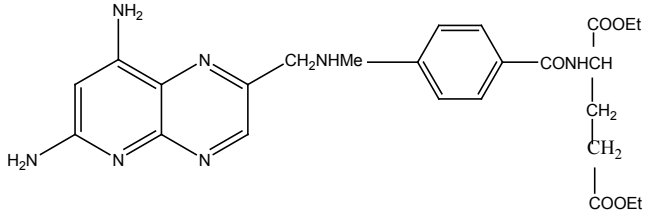


Table 9: *In vitro biological data for ester derivatives of MTX [23].*

Comp	CCRF-CEM ID_{50} mol/L
MTX	0.006×10^{-6}
MTX- γ-monomethyl ester	0.43×10^{-6}
MTX-dimethyl ester	0.40×10^{-6}
MTX- α-monoethyl ester	6.2×10^{-6}
MTX- γ-monoethyl ester	0.58×10^{-6}
MTX-diethyl ester	0.012×10^{-6}
MTX- α-monobµtyl ester	2.0×10^{-6}
MTX- γ-monobµtyl ester	0.76×10^{-6}
MTX-dibµtyl ester	0.057×10^{-6}

Table 10: *In vitro growth inhibitory activity alkyl ester derivatives of MTX [24].*

Comp	R	X	Sµrvival in L1210
			ID_{50} µM
1	C_2H_5	H	51%
5	n-C_4H_9	H	25%
6	n-C_4H_9	Cl	66%
12	n-C_8H_{17}	H	Inactive
13	n-C_8H_{17}	Cl	Inactive

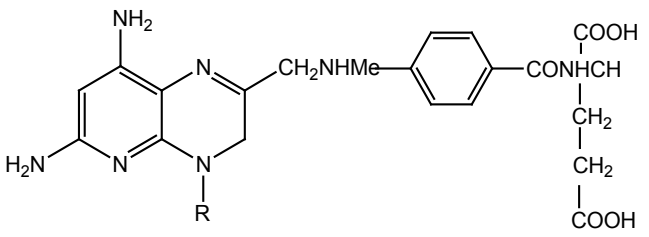
Table 11: *Biological data for alkyl derivatives of MTX [25].*

R	X	*S. faeciµm*	L1210-FR8	*L. case*	P388	CCRF-CEM
		ATCC8043 ID_{50}	ID_{50} mol/l	ID_{50} mol/l	ID_{50} mol/l	ID_{50} mol/l
H(MTX)	H	0.002	1.5×10^{-9}	3×10^{-9}	0.01	0.018
CH_3	H	0.17	7×10^{-6}	1×10^{-5}	0.1	0.1
H	Cl	0.01	1×10^{-9}	3×10^{-9}	0.007	0.009
CH_3	Cl	1	4×10^{-6}	9×10^{-6}	0.1	0.1

Table 12: *Growth inhibitory activity of selected bisamide derivatives of MTX [26].*

Comp	R	CCRF-CEM	RBL
		ID_{50}	ID_{50}
MTX	H	0.003	0.003
1a	NH_2	-	-
1b	NH-n-C_3H_7	7	-
1c	NH-n-C_4H_9	-	-
1d	NH-s-C_4H_9	-	-
1e	NH-s-C_6H_{13}	10	-
1f	NH-c-C_6H_{11}	1	0.22
1g	c-NC_4H_8	-	-
1h	$NHCH_2C_6H_5$	7.6	2.5
1i	NH $CH_2CH_2C_6H_5$	-	-
1j	$NHCH_2CH_2C_6H_3$-3,4 $(OMe)_2$	-	-
1k	$NHCH_2CH_2CH_2OH$	-	-
1l	$NHCH_2CH_2NMe_2$	-	-
1m	$NHCH_2CH_2CH_2NMe_2$	-	-

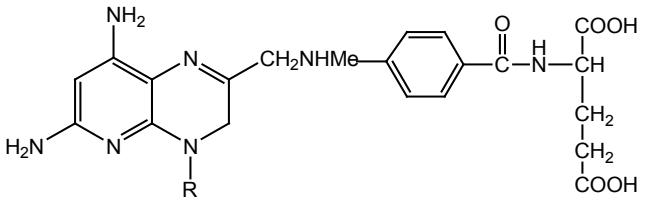
Various derivatives of MTX were synthesized having general formula 8-alkyl-7,8-dihydromethotrexate. Synthetic pathway includes alkylation of 7,8-dihydromethotrexate. *In vitro* studies tested against *Lactobacillus casei*, thymidylate synthetase, and DHFR. All derivatives were less potent for DHFR than 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-position has the same activity as MTX but it was inactive for l1210 leukemia (Table 13).

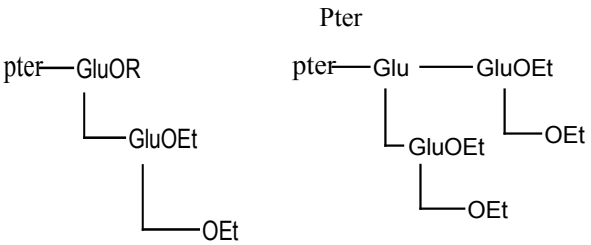


MTX γ-L-glutamate diethyl ester, MTX α-L-glutamate esters and α- γ-bis (L-glutamate tetraethyl esters) derivatives were

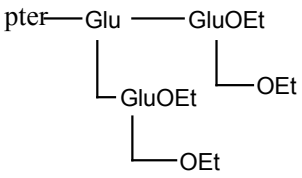
synthesized. Major product obtained was γ-isomer. Further esterification of both isomers gave triethyl esters. Anti-proliferating activity tested against L1210 leukemia. The increase in life span due to γ and α diethyl ester is 40% and 10% respectively while MTX has +60%. Growth inhibition activity again CCRF-CEM show γ-isomer 10 times more activity but α-isomer didn't show same result. L isomer show higher activity than D isomer (Table 14).

Pter= 4-amino-4-deoxy-N-methylpteroyl



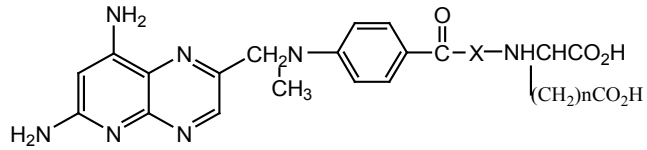


1: R=H, 4: R=Et 2: R=H, 5: R=Et 3

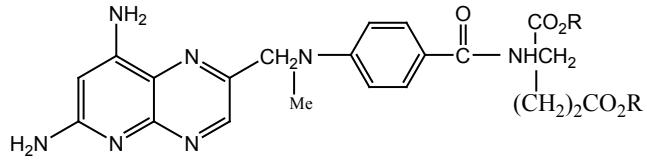


Aza derivative of MTX has been synthesized by additional N-atom between phenyl and carbonyl of the side chains. Photochemical method was used to insert additional N-atom. Inhibitory activity was tested against DHFR and thymidylate synthetase of *Lactobacillus casei* and CCRF-CEM and found to be less cytotoxic. *In vivo* inhibition was tested against L-1210 leukemia (mice) and show significant activity than MTX in the order of 55% and 88% respectively. (Table 15) summarizes the obtained results.

Tripepetide derivatives of methotrexate have been synthesized by reacting with four different amino acids. 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 amino benzoyl portion. This insertion of amino acid made these derivatives to shows borderline activity. Generally, an increase in dose increase in life spam (%ILS) (Table 16).



Various diesters of MTX and DCM have been prepared by the reaction of acid catalysed 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 [31].



Various γ-monoamides of the AMT and MTX have been synthesized by modification of coupling method of mixed carboxylic- anhydride method. All derivatives have been tested in vitro against L1210 leukemia, wild type L1210

Table 13: *In Vitro biological data of MTX analogμes [27].*

Comp	R	*L. casei* (×10^{-11})a	DHFR (×10^{-11})a	Thymidylate Synthetase (X 10^{-6})a	CCRF-CEM ID_{50} μg/mL
-	MTX	4	0.3	>100	0.018
7	H	2	2	1	0.021
8	Methyl	10	14	4	0.23
9	Ethyl	35	90	20	0.27
10	n-Bμtyl	120	38	19	0.23
11	n-hexyl	27	180	43	0.58
12	Cyclohexylmethyl	350	87	17	0.14
13	Benzyl	35	74	8	0.78
14	3,4 dichlorobenzyl	1940	360	13	>1.0
15	1-Naphthylmethyl	47	450	16	>1.0
a=Molar conc. for 50% inhibition					

Table 14: *Growth inhibitory activity of glμtamate ester derivatives of MTX [28].*

Comp	Hμman Lymphoblastic Leμkemia Cells (CCRF-CEM)ID_{50} μg/mL
MTX	0.003
1	0.88
2	10+
3 (LLL)	3.8
3(DLL)	9
4	1.8
5	0.85

Table 15: *Inhibition of L. casei (ATCC 7469) growth and DHFR and thymidylate synthetase by MTX derivatives [29].*

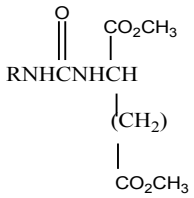
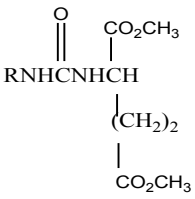
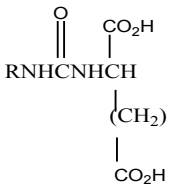
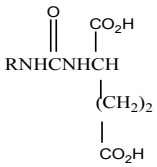
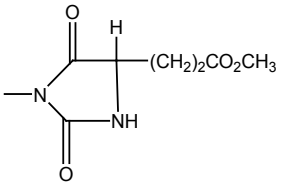
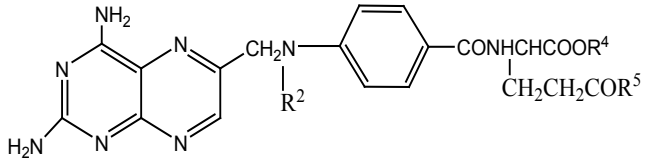
No.	Comp	*L. casei* ng/mL	DHFR M X (10^{-8})	Thymidylate synthetase M X (10^{-6})
MTX	-	0.01	0.3	100
5		6	60	-
6		6	30	-
7		150	5	190
8		8	8	250
10		104	400	-

Table 16: *Anti proliferative activity of peptide derivatives of MTX [30].*

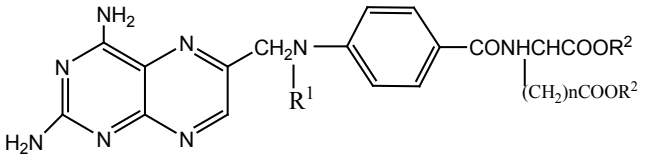
Comp	R	X	n	L 1210 mg/kg	%ILS
				Dose (mg/kg) × no. of admin	
1a	H	Gly	1	40×10	69
1b	H	Gly	2	33×6	14
1c	CH_3	Gly	1	100×8	40
1d	CH_3	Gly	2	100×8	0
1e	CH_3	DL-ala	2	50×6	19
1f	CH_3	Sar	2	100×6	0
1g	CH_3	L-Leμ	2	50×8	0
1h	CH_3	L-phe	1	100×7	0
1i	CH_3	L-phe	2	50×10	25

and sublime (CEM/MTX). *In vitro* γ-N-aryl alkyl and γ-N-aryl derivatives show higher potency than γ-N-tert-alkyl analogues. Results show that AMT derivatives have more potency than MTX derivatives and all MTX derivatives show more potency against sublime L1210/R81 than MTX. It was also found that all derivatives show more activity against cell lines than human cells. In vivo studies showed γ-N-tert-alkyl analogues inactive however significant activity was shown by γ-N-aryl alkyl and γ-N-aryl derivatives. The results are shown in (Table 17).

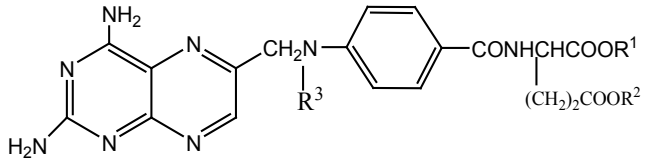


Various derivatives of MTX and AMT have been synthesized by replacing glutamate moiety with DL-2-aminoalkanedioic acid having up to 10 CH_2 alkyl group. All derivatives have been

tested against L1210 leukemia, CEM leukemia (human), CEM/MTX, and L1210/R81. Derivative with 9 CH_2 alkyl group show higher activity with CEM, and with 6 CH_2 alkyl group against L1210 cell lines (Table 18).



γ-tert-butyl esters of the 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 (Tables 19a-b).



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 show that antibodies associated with cytotoxic the drug were more potent than drug alone. Drug, mixture, and γ-globulin-MTX conjugate were used as a control group [35].

A poly (γ-L-glutamate) derivative of methotrexate has been synthesized in 4 steps, and this derivative contains

Table 17: *In Vitro biological data for monoamides derivatives of MTX [32].*

Comp	R^2	R^4	R^5	DHFR	L1210	L1210/R81
				IC_{50} µm	IC_{50} µm	IC_{50} µm
MTX	Me	H	H	0.02	0.01-0.03	220
3a	H	H	t-BµNH	0.044	0.12	22
3b	H	H	(1-admantyl)NH	0.1	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_2 C_6H_3 CH_2NH	0.08	0.0037	61
3f	H	H	C_6H_5NH	0.06	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	0.03	38
AMT	H	H	H	0.02	0.003	84

Table 18: *Biological data for aminoalkanedioic acid derivatives of MTX [33].*

Comp	R^1	n	R^2	DHFR	CEM	L1210 IC_{50} µM	L1210/R81 IC_{50} µM
				IC_{50} µm	IC_{50} µM		
MTX	Me	2	H	0.025	0.032	0.0046	197
AMT	H	2	H	0.025	0.001	0.002	84
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	0.02	>218
8	H	9	H	0.081	nd	0.00065	110
9	H	10	H	0.067	nd	0.0011	215

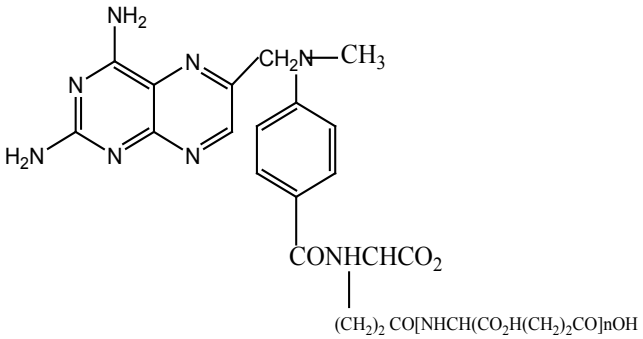
Table 19a: *Ester derivatives of MTX [34].*

Comp	R^3	R^2	R^1
MTX	H	H	Me
AMT	H	H	H
1(γ-tBMTX)	H	t-Bµ	Me
2(γ-tBAMT)	H	t-Bµ	H

Table 19b: *DHFR inhibition and cell growth of MTX derivatives.*

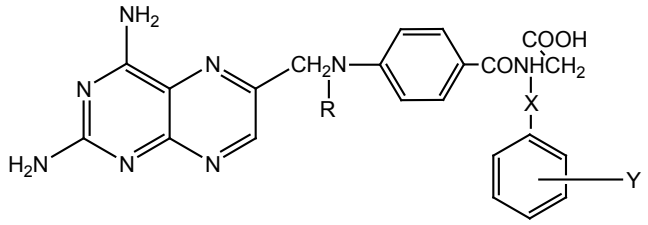
Comp	Hµman Sqµamoµs Cell Carcinoma									
	CEM	L1210	L1210/R71	L1210/R81	SSC25 IC_{50} µm	SSC68 IC_{50} µm	SSC78 IC_{50} µm	SSC25/R1 IC_{50} µm	SSC68/R1 IC_{50} µm	SSC78/R1 IC_{50} µm
	IC_{50} µm	IC_{50} µm	IC_{50} µm	IC_{50} µm						
MTX	0.62	0.056	40	25	0.4	0.37	0.48	0.78	1.4	1.4
AMT	0.45	0.023	3.5	6.5	0.066	0.19	0.08	1.8	3.5	0.43
1(γ-tBMTX)	0.032	0.002	19	220	0.014	0.032	0.013	0.15	0.25	0.071
2(γ-tBAMT)	0.001	0.002	7.9	84	0.0016	0.0037	0.0025	0.043	0.29	0.014

2-3 glutamate units more than MTX. Synthetic rout involved peptide coupling, blocking groups removed by catalytic hydrogenolysis. A coupling reagent used was diphenylphosphoryl azide [36].

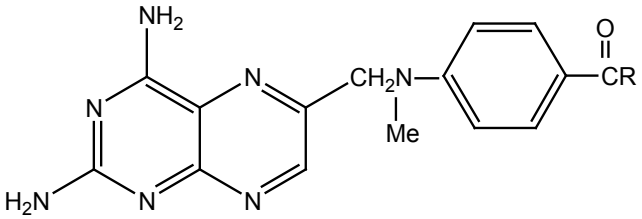


5,6,7,8-tetrahydromethotrexate (3) and di-hydro-methotrexate (2) were synthesized and affinity was checked against DHFR. It was found that tetra hydro-MTX shows more potency than dihydro-MTX for mice, *S. faecalis*, *P. cerevisiae*, dogs and chicks. It was also found that both reduced derivatives were 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 (Table 20).

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) found to be most potent. It was proposed that CONH group of these derivatives involved in hydrogen bonding and enhances affinity for DHFR (Table 21).



Several derivatives of methotrexate have been synthesized by modifying glutamyl moiety along with 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 (Table 22) [39].

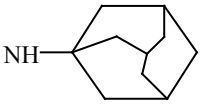


6a: R= $NHCH(CO_2Et)(CH_2)_2CO_2Et$

6b: R=$NHCH(CO_2H)(CH_2)_2CO_2H$

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

6d: R=



6e: R= OH

6f= OEt

Table 20: *DHFR affinity dihydro and tetra hydro derivatives of MTX [37].*

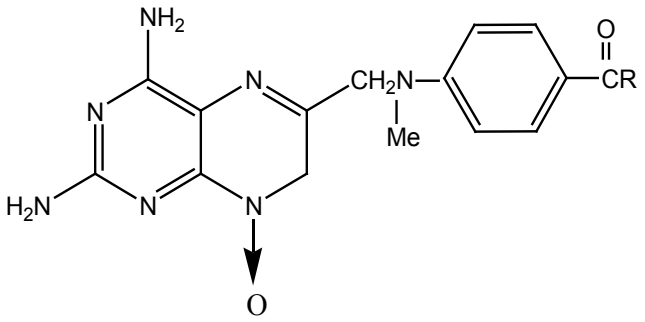
Comp	DHFR mg/mla	Thyimdylate synthetase mg/ml	*S. faecalis* mg/ml	*L. casei* mg/ml
MTX	9	45000	0.15	0.01
-3	16	1125	0.011	0.008
-2	46	2250	0.047	0.056
a=These are the concentration for 50% inhibition.				

Table 21: *DHFR affinity and anti-proliferative activity of anilides of MTX [38].*

Comp	R	X	Y	DHFR, IC_{50}, nM	L1210 IC_{50}, nM	W1-L2 IC_{50}, nM
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	0.7	nd

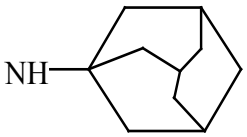
Table 22: *Inhibitory activity of derivatives of MTX.*

Comp	*S. faciμm* ID 50, μg/ml
6a	0.017
6b	0.37
6c	0.002
6d	0.003
6e	0.002
6f	0.001
7a	1
7d	1

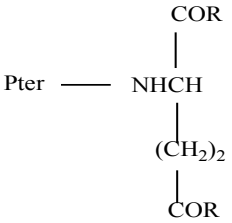


7a: R= $NHCH(CO_2Et)(CH_2)_2CO_2Et$

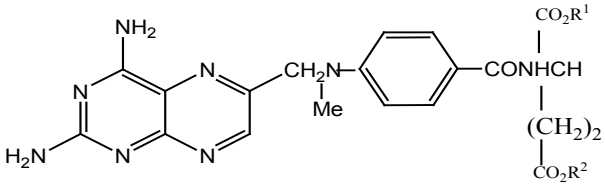
7d:



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 compounds was that having R group aryl amine having no methyl group (Table 23).



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 (Table 24).



Stretched MTX derivatives having different numbers of (Gab) as a spacer between glutamate and MeAPA moiety have been synthesized. The DHFR affinity and inhibitory activity have been evaluated. DHFR inhibition was directly related to number of Gab spacers, but growth inhibitory potency lost slightly (Table 25).

Table 23: *Biological activity of anilides and dianilide of MTX [40].*

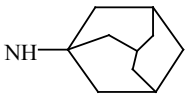
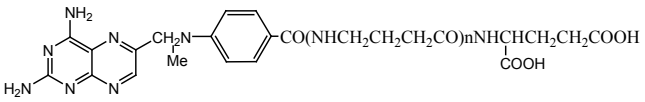
Comp	R	CEM ID_{50} µM	L1210 ID_{50} µM
-	MTX	0.003	0.01
1		>10	3.4
2	n-$C_5H_{10}N$	-	>10
3	4-$ClC_6H_4CH_2N$	>10	6.6
4	4-$MeOC_6H_4CH_2N$	>10	8.2
5	$C_6H_5CH_2N(CH_3)$	6.4	9.4
6	$(C_6H_5CH_2)_2$ N	7.6	0.69
7	C_6H_5 CH (CH3)NH	>10	>10
8	C_6H_5NH	3.3	0.41
9	H_2 NNH	7.5	0.95

Table 24: *DHFR affinity and inhibitory activity against CEM of esters of MTX [41].*

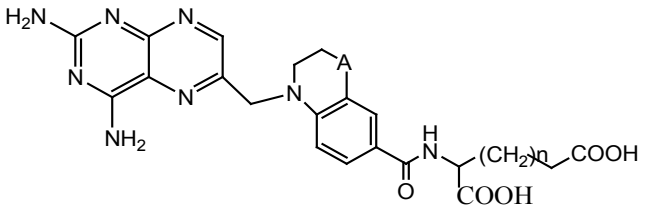
Comp	R^1	R^2	DHFR, IC_{50}, µM	CEM, IC_{50}, µM
MTX	H	H	0.0033	0.025
1 (α)	n-C_8H_7	H	0.36	3
2(γ)	H	n-C_8H_7	0.0054	0.92
3(α)	n-$C_{12}H_{25}$	H	0.44	2.1
4(γ)	H	n-$C_{12}H_{25}$	0.034	0.37
5(α)	n-$C_{16}H_{33}$	H	1.2	0.25
6(γ)	H	n-$C_{16}H_{33}$	0.037	0.11

Table 25: *DHFR affinity and inhibitory activity of derivatives of MTX [42].*

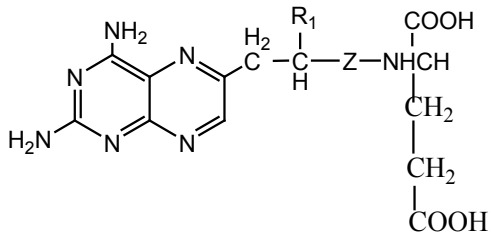
Comp	n	L1210 DHFR IC_{50} µm	L1210 cells IC_{50} µm	*L. casei* TS IC_{50} µm
mAPA-Gab_1-Glµ (1a)	1	0.082	0.82	0.53
mAPA-Gab_2-Glµ (1b)	2	0.09	1.3	5.6
mAPA-Gab_3-Glµ (1c)	3	0.31	4.4	29
mAPA-Gab_4-Glµ (1d)	4	0.54	7.7	>100
mAPA-Gab_5-Glµ (1e)	5	0.84	12	>100
mAPAGlµ (MTX)	-	0.035	0.5	0.02



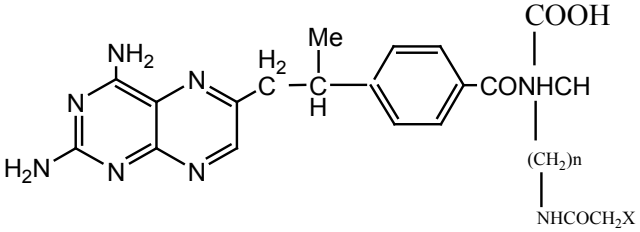
Dihydro-2H-1,4-benzothiazine and dihydro-2H-1,4-benzoxazine MTX derivatives have been synthesized and tested for anti-proliferative activity against hSC and hPBMC *in vitro*. *In vivo* activity checked against rat arthritis. 3c was found to be more potent than MTX (Table 26).



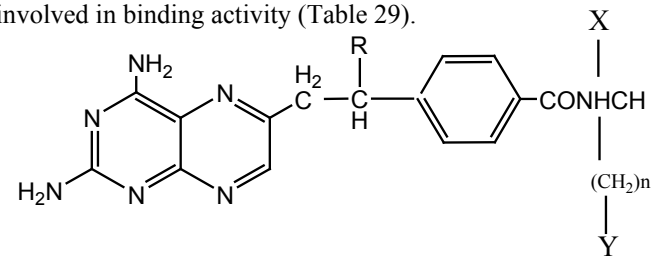
Various derivatives of MTX have been synthesized and few were studied for anti-proliferative activity against L1210 was tested. 9Aa and 9Ab showed higher activity than MTX (Table 27).



Derivatives of MTX have been synthesized by using a different number of Carbon chain length and N-halo acetylation. These derivatives have been tested for DHFR affinity and anti-proliferative against L1210 and L1210/R81. N-bromoacetyl-L-ornithine found to be more potent than other synthesized activity (Table 28).



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 was detrimental. Removal of α- COOH was found to lose the anti-proliferative activity, and reason behind is α-COOH involved in binding activity (Table 29).



In this article synthesis approach were made to derivatives of methotrexate or aminopterin which are basically folic acid antagonists. The synthesis scheme involved protection, de-

Table 26: *In Vitro* anti-proliferative activity against hSC and hPBMC of derivatives of MTX [43].

Comp	A	n	hSC, IC_{50} nM	hPBMC, IC_{50} nM
MTX	-	-	1	1
3a	O	1	0.52	1
3b	O	2	1.4	1.9
3c	S	1	0.3	0.5
3d	S	2	0.77	1.3
MTX-33	C	1	3.8	0.83

Table 27: *Anti-proliferative activity against L1210 of MTX derivatives [44].*

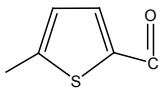
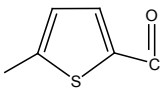
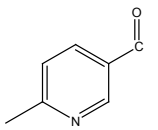
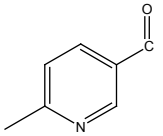
Comp	R_1	Z	L1210 IC_{50} nM	Chang Liver IC_{50} nM
MTX	H	-	20	14
9Aa	H		8.1	3.1
9Ab	Et		3.7	1.6
9Ba	H		88	36
9Bb	Et		268	35

Table 28: *Anti-proliferative activity against L1210 of Halo derivatives of MTX [45].*

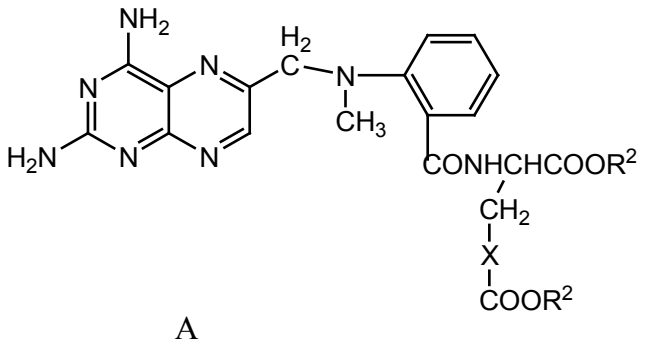
Comp	n	X	DHFR	L1210 Cells IC_{50}, µM	L1210/R81
			IC_{50}, nM		
MTX	-	-	25	0.005	200
1	4	I	-	-	-
2	4	Br	72	0.096	240
3	4	Cl	32	0.033	93
4	3	Br	46	0.126	155
5	3	Cl	32	0.062	105

Table 29: *Inhibitory activity of derivatives of MTX [46].*

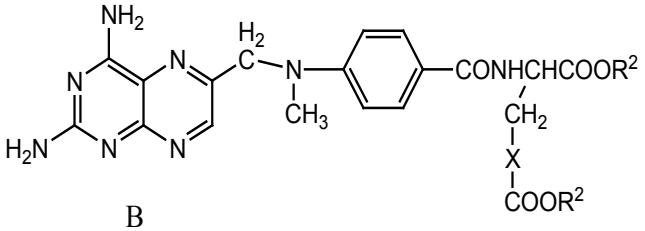
Comp	R	X	n	Y	% inhibition
3	Me	COOH	2	SO_2H	59
4	H	COOH	2	SO_2H	77
5	Me	COOH	2	$PO(OH)_2$	53
6	H	COOH	2	$PO(OH)_2$	100
7	H	COOH	1	$PO(OH)_2$	22
8	H	COOH	3	$PO(OH)_2$	52
9	H	COOH	4	$PO(OH)_2$	44
10	Me	H	0	SO_2H	19
11	Me	H	1	SO_2H	22
12	Me	H	2	SO_2H	18
13	Me	H	3	SO_2H	26
14	Me	H	1	$PO(OH)_2$	22
15	Me	H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

protection and various steps. Synthesis begins from potassium phthalimide and end product is *p*-[(2,4-Diamino-6-pteridinyl) methyl]amino]-benzoic acid which involves a number of intermediate [47].

Ortho and Meta isomers of AMT and MTX have been synthesized and were assayed for biological activities. General structures of these isomers are as follows. Besides these few derivatives were synthesized having $CH_2CH{=}CHCOOH$ instead of saturated side chain. These derivatives were being assayed for binding affinity with DHFR and FPGS from mouse liver. None of the derivative was found to be more potent than the parent drug [48].

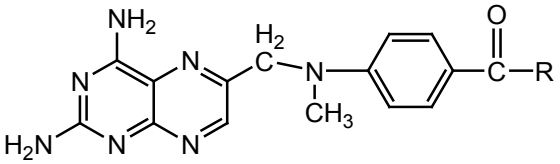


A

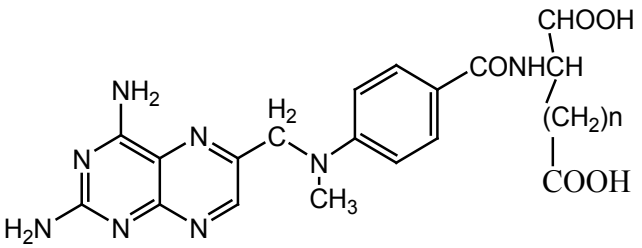


B

Various methotrexate derivatives synthesized by replacement of glutamic acid with amines and amino acid esters. These derivatives were tested for antibacterial and antitumor activity against L1210 in mice. Many derivatives showed significant activity and some show better than MTX. ID_{50} value of MTX is 0.002 (Table 30).



In this research work, glutamate moiety changed to amino acids having longer carbon chain. It was found that cytotoxicity increased by increasing carbon number from 2-5. Anticancer activity was tested on L1210 leukemia and results are comparable to parent drug (Table 31).



γ–Phosphonates and γ–sulphonate derivatives of MTX have been synthesized and tested for DHFR inhibition and L1210 carcinoma. Results showed that γ–sulphonates are more active than γ– phosphonates and both are less active than the parent drug. but γ–phosphonates have more potency in MTX resistant line L1210/R81 (Table 32).

Table 30: *Antitμmor activity against L1210 of amine and ester derivatives of MTX [49].*

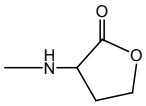
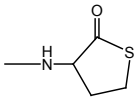
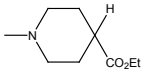
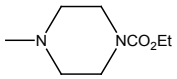
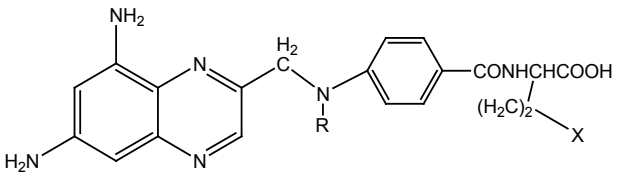
Comp	R	ID_{50}μg/ml
2	-oc (=O)OCH_2 $CH(CH_3)_2$	0.0001
3	Ethyl 4 - amino bμtyrate	0.003
4	Methyl DL-2-amino-bμtyrate	0.02
5	Ethyl L -1eμcinate	0.003
6	Methyl DL-norleμcinate	0.010
7	Ethyl L- methihioninate	0.001
8	Ethyl L-tryptophanate	0.006
9	Methyl L-tyrosinate	0.010
10	Methyl c-carbobenzyloxy-L - lysinate	0.035
11	Dimethyl DL-asparate	0.005
12	Methyl L-prolinate	0.030
13	C yclohexanamine	0.002
14	Morpholine	0.018
15		0.350
16		0.010
17		0.005
18		0.010
19	-N3 (azide)	0.005

Table 31: *Anticancer activity of amino acid derivatives of MTX [50].*

Comp	n	L1210 Binding assay ID_{50}, nM	L1210spectrophoto-metric assay ID_{50}, nM	L1210/R71 ID_{50}, nM
MTX(1)	2	11	9	32
2	3	12	6.7	39
3	4	18	6.9	39
4	5	-	-	35

Table 32: *DHFR inhibition of derivatives of MTX [51].*

Comp	R	X	DHFR inhibition	L1210 IC_{50} μM
			IC_{50} μM	
1 MTX	Me	CO_2H	0.5	0.012
2 AMP	H	CO_2H	0.5	0.0031
3	Me	SO_3H	0.55	0.3
4	H	SO_3H	0.62	0.037
5	Me	$CO_2PO_3H_2$	-	-
6	H	$CO_2PO_3H_2$	-	-
7	Me	PO_3H_2	1.34	0.19
8	H	PO_3H_2	1.26	0.035



A novel drug delivery system was formed using peptide labile spacer attached to MTX and tuftsin which is peptide carrier. These drug delivery systems were tested for cell lines MonoMac 6. It was found that all conjugates trigger toxic effect greater than MTX [52].

In this article 3' Halo, and 3', 5'-dihalo derivatives have been synthesized and synthesis was done in several steps. All derivatives showed a good growth inhibitory effect against lymphosarcoma cell lines (Table 33).

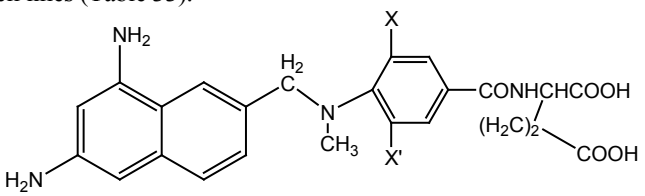


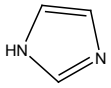
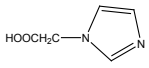
Table 33: *di-Halo derivatives of MTX [53].*

Comp	X	X'
I	H	H
II	F	H
III	F	F
IV	Cl	H
V	Br	H
VI	Cl	Cl
VII	Br	Cl

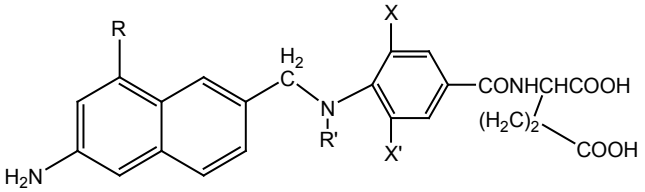
Table 34: *Halo derivatives of MTX [54].*

Comp	R	R'	X	X'
IV	NH_2	CH_3	Cl	H
V	NH_2	CH_3	Cl	Cl
VI	OH	CH_3	Cl	H
VII	NH_2	CH_3	Cl	Cl
VIII	OH	CH_3	Br	H
IX	OH	H	Br	Cl

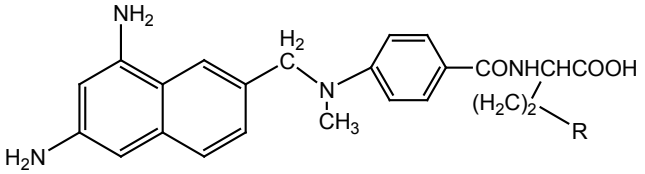
Table 35: *Histidine Derivatives of MTX [55].*

Comp	R	L1210 IC_{50}, μM	L1210DHFR IC_{50}, μM	CEM FPGS IC_{50}, μM
MTX	-COOH	0.007	0.073	3.2
4		0.091	1.3	>200
5		0.15	0.37	>200

In this article halogenated derivatives were synthesized, but after modification in pteridine ring (Table 34).



Derivatives have been synthesized by replacing glutamate moiety with histidine and other related groups. Biological assay data of a few were given yet various derivatives have been synthesized. These derivatives have significant inhibition activity against DHFR but less for FPGS (Table 35).



Conclusion

Changes in Structure of MTX were made to enhance efficacy and find a relation of structural changes and activity. Those derivatives, which are structurally related to MTX show good activity. Increase in carbon chain length between –COOH silibinin conjugate, SO_2H at gamma position, Aza derivatives, aminoalkanedioic acid derivatives, associated with antibodies, Anilides, dihydro-2H,1,4 benzothiazine, thiophene-2-carboxy group instead of benzyl show higher activity and proved to be more potent.

Substitution of alkyl group at 7-position, bisamides, alkylation at 8-position, lysyl derivative of gamma position, methyl group at benzylic carbon, removal of alpha COOH decreases activity as compared to MTX. Gamma substituted binds more effectively than alpha substituted so alpha COOH is more important in activity. Gamma isomers are more actives than alpha isomer. Diesters, monoamides showed varying degree of activity. Less than two CH_2 between COOH proved to be detrimental. So, new researchers can focus on those modifications which show better anticancer activity and can synthesize even better derivatives.

Conflict of Interest

The authors declare that they have no conflict of interests.

References

1. Friedman JM, Weber-Schöndorfer C. Antineoplastic drugs. 3rd edn Elsevier. 2014.
2. Lew CC, Goldman P. The Enzymatic Hydrolysis of Methotrexate and Folic Acid. J Biol Chem. 1967;242:2933–9.
3. Pawlak A, Kutkowska J, Obmińska-Mrukowicz B, et al. Methotrexate induces high level of apoptosis in canine lymphoma/leukemia cell lines. Res Vet Sci. 2017;114:518–23.
4. Khodadadei F, Safarian S, Ghanbari N. Methotrexate-loaded nitrogen-doped graphene quantum dots nanocarriers as an efficient anticancer drug delivery system. Mater Sci Eng C. 2017;79:280–5.

5. Giletti A, Vital M, Lorenzo M, et al. Methotrexate pharmacogenetics in Uruguayan adults with hematological malignant diseases. Eur J Pharm Sci. 2017;109:480–5.
6. Askari BS, Krajinovic M. Dihydrofolate Reductase Gene Variations in Susceptibility to Disease and Treatment Outcomes. Curr Genomics. 2010;11:578–3.
7. Kinder AJ, Hassell AB, Brand J, et al. The treatment of inflammatory arthritis with methotrexate in clinical practice: treatment duration and incidence of adverse drug reactions. Rheumatology. 2005;44:61–6.
8. Haustein UF, Rytter M. Methotrexate in psoriasis: 26 years' experience with low-dose long-term treatment. J Eur Acad Dermatol Venereol. 2000;14:382–8.
9. Calnan J, Davies A. Wound healing : an experimental study. 1965;7:505–12.
10. Stovall TG, Ling FW, Gray LA. Single-dose methotrexate for treatment of ectopic pregnancy. Obs Gynecol. 1991;77:754-7.
11. Espinoza LR, Zakraoui L, Espinoza CG, et al. Psoriatic arthritis: clinical response and side effects to methotrexate therapy. J Rheumatol. 1992;19:872–7.
12. Nuevo PDE, Norma T, Codex DEL, et al. Lysine and Ornithine Analogues of Methotrexate as Inhibitors of Dihydrofolate. 2015;1–13.
13. Rosowsky A, Freisheim JH, Moran HG, et al. Methotrexate Analogues. 26. Inhibition of Dihydrofolate Reductase and Folylpolyglutamate Synthetase Activity and in Vitro Tumor Cell Growth by Methotrexate and Aminopterin Analogues Containing a Basic Amino Acid Side Chain. J Med Chem. 1986;29:655–60.
14. Rosowsky A, Wright JE, Ginty C, et al. Methotrexate Analogues. 15. A Methotrexate Analogue Designed for Active-Site-Directed Irreversible Inactivation of Dihydrofolate Reductase. J Med Chem. 1982;25:960–4.
15. Rosowsky A, Forsch RA, Freisheim JH, et al. Methotrexate Analogues. 23. Synthesis, Dihydrofolate Reductase Affinity, Cytotoxicity, and in Vivo Antitumor Activity of Some Putative Degradation Products of Methotrexate-Poly (L-lysine) Conjugates. J Med Chem. 1984;27:888–93.
16. Kumar AA, Freisheim JH, Kempton RJ. Synthesis and characterization of a fluorescent analogue of Methotrexate. J Med Chem. 1983;26:111-3.
17. Forsch RA, Rosowsky A. Methotrexate Analogues. 22. Selective Cleavage of 2-(Trimethylsily1)ethyl Esters in the Presence of N-tert -Butyloxycarbonyl Groups during the Synthesis of Protected Dilysine and Trilysine Derivatives of Methotrexate. J Med Chem. 1984;1305–9.
18. Nadhum SA, Mohammed MH. Design, Synthesis, Characterization and Preliminary Anticancer Study for Methotrexate Silibinin Conjugates. Iraqi J Pharm Sci. 2015;24:75-84.
19. Rosowsky A, Moran RG, Forsch R, et al. Methotrexate analogues—XVII. Biochem Pharmacol. 1984;33:155–61.
20. Rosowsky A, Forsch R, Uren J, et al. Methotrexate Analogues. Synthesis of New y-Substituted Derivatives as Dihydrofolate Reductase Inhibitors and Potential Anticancer Agents. J Med Chem. 1981;24:1450–5.
21. Suster DC, Tărnăuceanu E, Ionescu D, et al. Potential Anticancer Agents. 16. Methotrexate Analogues with a Modified Peptide Side Chain. J Med Chem. 1978;21:1162–5.
22. Montgomery JA, Piper JR, Elliott RD, et al. Analogues of Methotrexate. J Med Chem. 1979;22:862–8.
23. Rosowsky A, Beardsley GP, Ensminger WD, et al. Methotrexate Analogues. 11. Unambiguous Chemical Synthesis and in Vitro Biological Evaluation ofa- and y-Monoesters as Potential Prodrugs. J Med Chem. 1978;21:380–6.
24. Rosowsky A. Methotrexate Analogs. 2. A Facile Method of Preparation of Lipophilic Derivatives of Methotrexate and 3',5'-Dichloromethotrexateb y Direct Esterification. J Med Chem. 1973;16:1190-973.
25. Rosowsky A, Chen KKN. Methotrexate Analogs. 4.7-Methyl Derivatives of Methotrexate and Dichloromethotrexate. A New Synthesis and Some Biological Studies. J Med Chem. 1974;17:1308–11.
26. Rosowsky A, Ensminger WD, Lazarus H, et al. Methotrexate Analogues. 8. Synthesis and biological Evaluation of Bisamide Derivatives as Potential Prodrugs. J Med Chem. 1977;20:925-30.
27. Chaykovsky M, Hirst M, Lazarus H, et al. Methotrexate Analogues. Synthesis and Biological Properties of Some 8-Alkyl-7, 8-dihydro Analogues. J Med Chem. 1977;20:1323–7.
28. Rosowsky A, Yu CS. Methotrexate Analogues. Direct Coupling of Methotrexate and Diethyl L-Glutamate in the Presence of Peptide Bond-Forming Reagents. J Med Chem. 1978;21:170–5.
29. Martinelli JE, Chaykovsky M, Kisliuk RL, et al. Methotrexate Analogues. 12. Synthesis and Biological Properties of Some Aza Homologues. J Med Chem. 1979;22:869–74.
30. Suster DC, Tarnauceanu E, Botez G, et al. Potential Anticancer.17. Analogues of Methotrextae with a tripeptide side chain. J Med Chem. 1978;21:1165-978.
31. Rosowsky A, Yu CS. Methotrexate Analogues. 18. Enhancement of the Antitumor Effect of Methotrexate and 3',5′-Dichloromethotrexate by the Use of Lipid-Soluble Diesters. J Med Chem. 1983;26:1448–52.
32. Rosowsky A, Bader H, Smith MR, e al. Methotrexate Analogues. 28. Synthesis and Biological Evaluation of Newy-Monoamides of Aminopterin and Methotrexate. J Med Chem. 1986;29:1707–9.
33. Rosowsky A, Bader H, Kohler W, et al. Methotrexate Analogues. 34. Replacement of the Glutamate Moiety in Methotrexate and Aminopterin by Long-Chain 2-Aminoalkanedioic Acids. J Med Chem. 1988;31:1338–44.

34. Rosowsky A, Forsch RA, Bald H, et al. Methotrexate Analogues. 25. Chemical and Biological Studies on the y-tert-Butyl Esters. J Med Chem. 1985;28:660–7.

35. Burstein S, Knapp R. Chemotherapy of Murine Ovarian Carcinoma by Methotrexate-Antibody Conjugates. J Med Chem. 1977;20:950–2.

36. Piper JR, McCaleb GS, Montgomery JA. A synthetic approach to poly(gamma-glutamyl) congugates of methotrexate. J Med Chem. 1983;26:291-4.

37. Horwitz SB, Kisliuk RL. Reduced Derivatives of Methotrexate. J Med Chem. 1968;11:907-8.

38. Rosowsky A, Bader H, Freisheim JH. Synthesis and Biological Activity of Methotrexate Analogues with Two Acid Groups and a Hydrophobic Aromatic Ring in the Side Chain. J Med Chem. 1991;34:574–9.

39. Chaykovsky M, Rosowsky A, Papathanasopoulos N, et al. Methotrexate Analogs. 3. Synthesis and Biological Properties of Some Side-Chain Altered Analogs. J Med Chem. 1974;17:2212–6.

40. Rosowsky A, Yu CS, Uren J, et al. Methotrexate Analogues. 13. Chemical and Pharmacological Studies on Amide, Hydrazide, and Hydroxamic Acid Derivatives of the Glutamate Side Chain. J Med Chem. 1981;24:559–67.

41. Rosowsky A, Forsch RA, Yu CS, et al. Methotrexate analogues. 21. divergent influence of alkyl chain length on the dihydrofolate reductase affinity and cytotoxicity of methotrexate monoesters. J Med Chem. 1984;27:605–9.

42. Rosowsky A, Forsch RA, Wick MM, et al. Methotrexate Analogues. 29. Effect of γ-Aminobutyric Acid Spacers between the Pteroyl and Glutamate Moieties on Enzyme Binding and Cell Growth Inhibition. J Med Chem. 1986;29:1872–6.

43. Matsuoka H, Ohi N, Mihara M, et al. Antirheumatic agents: Novel methotrexate derivatives bearing a benzoxazine or benzothiazine moiety. J Med Chem. 1997;40:105–11.

44. DeGraw JI, Colwell WT, Crase J, et al. Analogues of methotrexate in rheumatoid arthritis. 1. Effects of 10-deazaaminopterin analogues on type II collagen-induced arthritis in mice. J Med Chem. 1997;40:370–6.

45. Rosowsky A, Solan VC, Forsch RA, et al. Methotrexate analogues. 30. Dihydrofolate reductase inhibition and in vitro tumor cell growth inhibition by N epsilon-(haloacetyl)-L-lysine and N delta-(haloacetyl)-L-ornithine analogues and an acivicin analogue of methotrexate. J Med Chem. 1987;30:1463–9.

46. Rosowsky A, Forsch RA, Moran RG, et al. Methotrexate Analogues. 32. Chain Extension, α-Carboxyl Deletion, and γ-Carboxyl Replacement by Sulfonate and Phosphonate: Effect on Enzyme Binding and Cell-Growth Inhibition. J Med Chem. 1988;31:1326–31.

47. Elliottc RD, Temple C, Montgomery JA. Potential Folic Acid Antagonists. IV. Synthetic Approaches to Analogs of Aminopterin and Methotrexate. IV. The Preparation of p-{[(2,4-Diamino-6-pteridinyl)methyl]amino]-benzoic Acids. J Org Chem. 1970;35:1676–80.

48. Rosowsky A, Bader H, Forsch RA, et al. Methotrexate Analogues. 31. Meta and Ortho Isomers of Aminopterin, Compounds with a Double Bond in the Side Chain, and a Novel Analogue Modified at the a-Carbon: Chemical and in Vitro Biological Studies. J Med Chem. 1988;31:763-8.

49. Chaykovsky M, Brown BL, Modest EJ. Methotrexate Analogs. 6. Replacement of Glutamic Acid by Various Amino Acid Esters and Amines. J Med Chem. 1975;18:909–12.

50. Rosowsky A, Forsch R, Uren J, et al. Methotrexate Analogues. 20. Replacement of Glutamate by Longer-Chain Amino Diacids: Effects on Dihydrofolate Reductase Inhibition, Cytotoxicity, and in Vivo Antitumor Activity. J Med Chem. 1983;26:1719–24.

51. Rosowsky A, Moran RC, Forsch RA, et al. Methotrexate analogues-27. Dual inhibition of dihydrofolate reductase and folylpolyglutamate synthetase by methotrexate and aminopterin analogues with a γ-phosphonate group in the side chain. Biochem Pharmacol. 1986;35:3327–33.

52. Bai KB, Láng O, Orbán E, et al. Design, Synthesis, and In Vitro Activity of Novel Drug Delivery Systems Containing Tuftsin Derivatives and Methotrexate. Bioconjug Chem. 2008;19:2260–9.

53. Tomcufcik AS, Seeger DR. The Synthesis of the 3′-Fluoro and 3′,5′-Difluoro Derivatives of 4-Amino-4-deoxy-N10-methy]pteroylglutamic Acid (Methotrexate). J Org Chem. 1961;26:3351–6.

54. Robert BY, Curran V. The Halogenation of 4-Amino-4-deoxy-N10-methylpteroylglutamic Acid (Methotrexate) and Other Pteroylglutamic Acid Derivatives. J Am Chem Soc. 1959;81: 2814-8.

55. Mao Z, Pan J, Kalman TI. Design and synthesis of histidine analogues of folic acid and methotrexate as potential folylpolyglutamate synthetase inhibitors. J Med Chem. 1996;39:4340–4.

***Correspondence to:**

Iqbal M
Department of Chemistry
School of Natural Sciences (SNS)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
Tel: +92 300 6005249
E-mail: mudassir.iqbal@sns.nust.edu.pk