SYNTHESIS AND BIOLOGICAL EVALUATION OF METHOTREXATE ANALOGUES FOR ANTICANCER ACTIVITY

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Summary

Out of the series of compounds synthesized, the most active compounds were M001 and M002. The new entity of methotrexate analogues was confirmed by physiochemical and spectral studies like IR, Mass and ^{1}HNMR spectra. Screening of anticancer activity was carried out in both *in vitro* and *in vivo* in DLA induced Swiss albino female mice. The CTC_{50} values of compounds M001 and M002 was 68µg/ml and 61µg/ml respectively. Both the compounds found to be a potent inhibitor of tumor growth. However, compound M002 posses enhanced anticancer activity as compared to compound M001, by decrease in body weight and prolongation of life span *in vivo* studies. These findings confirm that substitution of methyl quinazoline on pteridine ring of methotrexate, a potent analogue with significant antitumor activity can be synthesized with ease.

Key words: Methotrexate (MTX); Dalton's Lymphoma Ascities (DLA); Pteridine; Quinazoline.

Introduction

Cancer is the most common cause of mortality in the developed countries and accounts for around 12.5% of all deaths worldwide. There are an estimated 2.5 million cases of cancer in India at any given time. Cancer treatments include surgery and radiation therapy followed by chemotherapy. Each has its own limitations and none of these therapies alone or in combination have achieved complete cure in detected cancers. Consequently numerous cancer therapeutic agents of various chemical variants have been synthesized. In recent years, researchers have been exploring various synthetic methods for drug development with no or reduced side effects to target cancer cells. Targeting antifolates have been one of the major focus points for anticancer treatment. Antifolates act by inhibiting two important enzymes, DHFR and thymidylate synthase which are involved in the metabolic pathway of folic acid. [1] The discovery of methotrexate (MTX) had lead to intensive investigations of numerous classical antifolate analogues. MTX is accepted as potent therapeutic agent to treat various types of cancer [2,3] even though it posses high toxic profile with narrow therapeutic index [1-3]. The clinical use of methotrexate causes toxicity to bone marrow, gastrointestinal mucosa and resistance to target enzyme DHFR [1-3].
Currently numerous antifolate drugs are in clinical development with modifications in their functional moiety to minimize problems caused by methotrexate. It was also observed that there was paucity of data on methotrexate analogues targeting pteridine nucleus for antitumor activity. Therefore the present work is aimed to synthesize methotrexate analogues targeting pteridine nucleus with no or reduced side effect profile and enhanced activity as compared to MTX.

Materials and Methods

Chemistry: The chemicals like anthralinic acid, Benzoyl chloride, acetic anhydride, pyridine, and methanol were obtained from department of pharmaceutical chemistry, KMCH College of pharmacy, India. Methotrexate pure sample were procured from SWIZZ pharmaceuticals ltd, Chennai, India. The chemical used were of analytical grade and procured from HIMEDIA chemicals Ltd.
The synthetic schemes for the preparation of test compounds are based on various literatures with some modifications [4,5]. A series of analogues was synthesized and evaluated. In preliminary studies many compounds were unstable with low yield and inactive in inhibiting tumor cells, therefore compounds afforded in moderate to good yield were synthesized and studied. The schematic synthesis of compound M001 and M002 was performed by fusion of functional groups phenyl and methyl substituted benzoxaine with methotrexate molecule**.** The products which precipitated during the course of reaction was filtered, washed with distilled water and allowed to dry. It was finally purified by recrystallization from suitable solvent. The structure of the compounds were determined and confirmed by physiochemical and spectral methods.

Animals: Healthy adult female Swiss mice weighing 20-30 g were obtained from KMCH College of pharmacy animal house, Coimbatore, India and approved by Institutional animal ethics committee ref.no: KMCRET/M.Pharm/01/2007. The experiments were performed as per the recommendations of CPCSEA, Chennai.

Inoculation of DLA cell line: Dalton's Lymphoma Ascites (DLA) tumor cells were procured from the Amala Cancer Research Center, Thrissur, Kerala, India and was propagated in Swiss albino mice by intra peritoneal transplantation. The cells were collected from DLA bearing mice during the log phase of the 11^{th} day by withdrawing the fluid from intraperitoneal cavity. Tumor viability was determined by tryphan blue exclusion test and cells were counted using haemocytometer.
The percentage cytotoxicity was calculated using the formula

$$\text{Percentage cytotoxicity} = \frac{100 - (\text{Total cells} - \text{Dead cells})}{\text{Total cells}} \times 100$$

The cell suspension was suitably diluted with phosphate buffer saline (PBS) to get a concentration of $1x10^6$ cells. 250µl of this fluid is injected into the peritoneal cavity of each mouse by i.p. route except for vehicle control group to obtain DLA tumor.

Acute toxicity studies: Acute oral toxicity were carried out as per the OECD guidelines No. 420 and recorded. Drugs were administered by i.p. route to overnight fasted animals. The animals were observed for any onset of toxic symptoms and gross behavioral changes at 4^{th}, 8^{th}, 12^{th} and 24^{th} hours. Hence all the mice were subjected for *in vivo* anticancer screening.

Biological screening activity - In vivo Dalton's Lymphoma Ascites tumor studies [6]**:**

Study group:

The mice were divided into V groups with five animals in each group. Group I served as the normal vehicle control and received 0.3% carboxymethylcellulose (CMC) suspension, for which inoculation of tumor cells was not done. The remaining group were inoculated with DLA ($1x10^6$ cells) and divided into 4 groups. Group II served as tumor control and treated with 0.3% CMC suspension. Group III, which serve as positive control was treated with MTX at 5 mg/kg body weight. Groups IV and V were treated with test compounds M001 and M002 as a single dose 5 mg/kg body weight. All the treatments were given i.p. route after 24 h of DLA inoculation and continued throughout the study period as a single dose. Tumor growth was assessed by body weight analysis, mean survival time (MST) and percentage (%) increase in life span (%ILS).

Body weight analysis: The decrease in body weight was calculated by the formula

$$\text{Decrease in body weight} = \frac{\text{Gain in body weight of control group} - \text{Gain in body weight of treated group}}{\text{Gain in body weight of control group}} \times 100$$

Mean survival time (MST): The surviving times of DLA tumor bearing mice were observed and MST was calculated

Percentage increase in Life span: Using MST, the % ILS was calculated by the formula

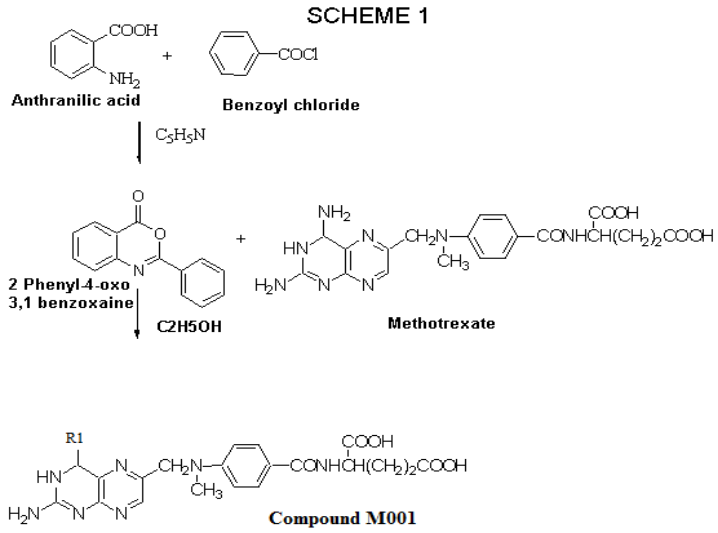
$$\% \text{ ILS} = \frac{\text{MST of treated group} - \text{MST of control group}}{\text{MST of control group}} \times 100$$

Statistical analysis: The *in vivo* data was analyzed by student T test and P< 0.01 and 0.05 was considered as statistically significant.

Results

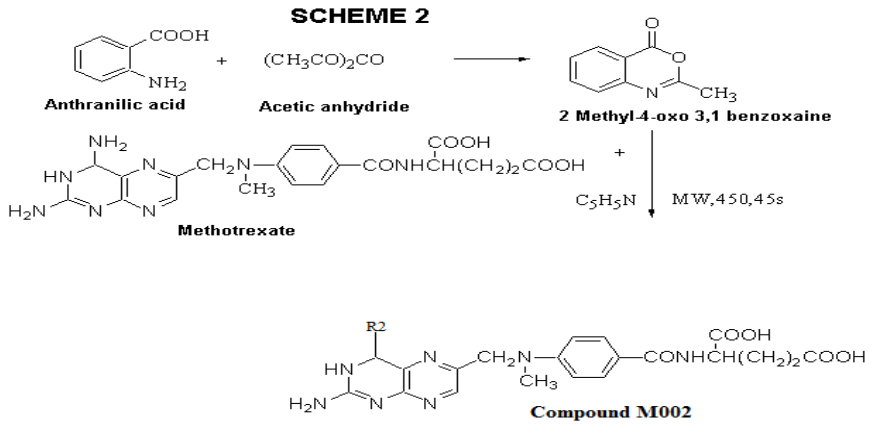
The compounds were synthesized as shown in schemes 1 & 2 (Fig.1 & Fig.1-A).

Figure 1 Synthesis of compound M001



R1- Substitution ($C_{14}H_7NO$)

Figure 1-A Synthesis of compound M002



R2- Substitution ((C_9H_5NO)

The structure of synthesized MTX analogues was confirmed by determining their physiochemical properties like molecular weight, melting point and Rf values (Table 1).

Table 1 Physicochemical properties

Cmpd.	Formula	Molecular Weight	Melting Point [^{0}C]	Yield [%]	R_f Value
Methotrexate	$C_{20}H_{24}N_8O_5$	456.45	123		
Test comp. M001	$C_{34}H_{31}N_9O_6$	661.67	87	78	0.73
Test comp.M002	$C_{29}H_{29}N_9O_6$	599.60	156	89	0.54

Mobile Phase: Benzene:Ethyl acetate[8:2]

The purity of compounds was confirmed by TLC. The structural confirmation was determined by IR and ^{1}HNMR spectroscopy methods. (Table 2) The analysis of the mass spectra reveals that M+ ion at m/z 661.67(M001) and 599.60(M002) characteristically with M+ peak. The parent peak was recorded at 531.5 and 577.40 for compound M001 and M002 respectively.

Table 2 Spectral data of the compounds

Compound	IR Freq. *(KBr, cm^{-1})*	^{1}H NMR (CDCl3, ppm)
Methotrexate	3416 (N-H), 1504 (C=O Carboxylic), 1384 (C=O Aromatic conjugate), 619 (C-H Aromatic)	
M001	3413.39 (N-H), 1613.16 (C=O), 1541.81 (N-H 1^o), 1450.21 (C=O Carboxylic), 902.523 (C-H Aromatic)	2.01 (s, 3H, -NH-), 2.12 (t, 2H, -CH2-), 2.23 (t, 2H, -CH2-COOH), 2.85 (s, 3H, N-CH3), 4.46 (t, 1H, -CH (-CH2)-COOH), 4.61 (s, 2H, -CH2N-), 6.08 (s, 1H, pyrimidinyl), 6.75 (d, 2H, ArH), 7.30-7.78 (m, 11H, ArH), 8.12 (s, 1H, -CONH-), 8.32 (s, 1H, pyrazinyl), 11.10 (s, 2H, -COOH)
M002	3446.17 (N-H), 1637.27 (C=O), 1509.99 (C=O Carboxylic), 1383.68 (C=O Aromatic conjugate), 1100.19 (C-H Aromatic-1,4 disubstitution)	1.03 (s, 3H, -CH3), 2.03 (s, 3H, -NH-), 2.14 (t, 2H, -CH2-), 2.25 (t, 2H, -CH2-COOH), 2.85 (s, 3H, N-CH3), 4.42 (t, 1H, -CH (-CH2)-COOH), 4.68 (s, 2H, -CH2N-), 6.11 (s, 1H, pyrimidinyl), 6.75 (d, 2H, ArH), 7.32-7.66 (m, 7H, ArH), 8.10 (s, 1H, -CONH-), 8.34 (s, 1H, pyrazinyl), 11.06 (s, 2H, -COOH)

Screening of Biological activity: *In vitro* studies revealed the inhibition of tumor cells with CTC_{50} values 68 and 61 µg/ml for compound M001 and M002 respectively. The data clearly suggests that the compound M002 was found to be more potent than compound M001 in inhibiting growth of tumor cells. The safest doses of synthesized compounds were determined by acute toxicity and gross behavioral animal studies as per standard procedures stated in OECD guidelines. The maximum tolerated dose of synthesized compounds was 40mg/kg. So approximately 1/10 dose was selected for the study. The safest doses were selected from acute toxicity and cross behavioral study such that no mortality rate of animals was recorded. Hence all the mice were used for *in vivo* anticancer screening.

In vivo Body weight analysis: After inoculation of the DLA cells, test drugs were administered daily and the change in body weight was observed. The body weight of control and treated group animals were recorded on 0, 11^{th} and 20^{th} day. In control group the body weight was maintained throughout the study period, while there is an increase in body weight was observed in tumor control group till the survival period. But group III – V shows decrease in body weight with the treatment of test compounds. The percentage decrease in body weight in MTX treated group was found to be 16.37 and compound M001 and M002 treated groups shows 14.20 and 11.61 respectively (Table 3).

Table 3 Body weight analysis of test compounds on mice inoculated with DLA $1x10^6$cells

Group	Treatment	Dose (mg/kg)	Body weight			Decrease in body weight from 11^{th} day to 20^{th} day	% Decrease in body weight
			0 day	11^{th} day	20^{th} day		
I	Normal control with CMC	100	24	25	24.4		
II	Tumor control with CMC	100	26	30.11	33.88	------	------
III	Positive control with Methotrexate	5	26.5	30.66	25.64	5.02	16.37
IV	M001	5	27	30.70	26.34	4.36	14.20
V	M002	5	26	33.49	29.60	3.89	11.61

Data expressed as mean $\pm$ SEM of five animals

Mean survival time and % increase in life span: Mean survival time and % increase in life span data was shown in table 4. It reveals control animals were alive till the end of the study period. However MST for tumor control group was found to be 21days and 29, 25 and 26 days for group III to V respectively. The % ILS values were 38.09, 19.04 and 23.80 for groups treated with MTX, M001 and M002 respectively.

Table 4 Mean survival time (MST) and % increase in life span of mice (%ILS)

Group	Treatment	Dose (mg/kg)	MST (in days)	%ILS
I	Normal control with CMC		Alive	-----
II	Tumor control With CMC	100	21 ± 0.4472	------
III	Methotrexate	5	29 ± 0.7071***	38.09
IV	M001	5	25 ± 0.7071**	19.04
V	M002	5	26 ± 0.7071***	23.80

All values are expressed in Mean ± SEM of five animals. Statistical analysis were performed by student 'T' test * * *p<0.001, * *p<0.01

Discussion

The present study was carried out to evaluate the antitumor activity of the synthesized methotrexate analogues for their enhanced potency in DLA tumor cell bearing mice. Though methotrexate cause toxic effects to the rapidly-dividing cells of bone marrow and gastrointestinal mucosa [7,8], still choice of drug in many neoplastic disorders including acute lymphoblastic leukemia [8-11].

During our investigation we have taken into account the antifolate derivatives either classical or non-classical methotrexate as well as the corresponding derivatives. A study by Fry DW, Jackson RC [12] reported that classical and non-classical antifolate has greater activity against murine tumors than MTX [12]. The synthesized compounds were characterized and confirmed for new entity of methotrexate analogues on the basis of physiochemical and spectral studies via IR Mass and ^{1}HNMR. It could be observed through spectral studies compounds M001 and M002 for the confirmation of possible fusion of quinazoline moiety in the pteridine nucleus of methotrexate.

Quinazoline has proven anticancer effect in various types of cancer. More than 300 analogues have been reported with potential anticancer activity [13,14]. Although quinazoline and methotrexate has proven anticancer effect [15], there is possibility for potent activity and reduced toxicity and vice versa of the synthesized analogues.

In vitro studies revealed compound M001 and M002 has potent inhibition against tumor cells with low dose concentration. However, enhanced activity was observed for M002 as compared to M001. It is stated, compounds that is cytotoxic at low dose level are effective as cytotoxic agents. The activity of these analogues might be due to the substitution of quinazoline in the pteridine ring of MTX. Numerous antifolates have been reported with modification in the pteridine and carboxylic ring of MTX to minimize problems caused by MTX. A study by Pazdur R et. al [16] reported enhanced activity of edatrexate, a methotrexate analogue as compared to methotrexate against murine tumors and human xeno grafts [16]. The present study also confirm that MTX analogues possess anticancer activity, furthermore the reliable criteria for judging the anticancer activity of synthesized compounds was *in vivo* DLA induced animal models. The parameters observed where, body weight analysis, mean survival time and percentage increase in life span of animals.

A progressive gain in the body weight was observed in the tumor control group since the inoculation of tumor cells. But, there is decrease in the body weight of animals, treated with test compounds M001 and M002 as compared to the standard positive control, MTX treated group. Hence the synthesized compound was found to be effective in preventing the growth of tumor as indicated by decrease in progressive gain in body weight when compared to control, i.e., mice treated with CMC and positive control, i.e., mice treated with MTX. The compounds M001 and M002 also showed significant increase in the MST and also good % ILS when compared with the control and positive control.

Conclusion

In conclusion, we have synthesized classical antifolate derivatives. The cytotoxicity of the compounds, phenyl and methyl substituted analogue of MTX was evaluated on DLA cells. Among the compounds, the methyl substituted analogue of MTX exhibit potent cytotoxicity, even though it is less cytotoxic than MTX. It is of interest, that pteridine position of MTX represents a site of modification that can yield analogues with improved therapeutic selectivity, when compared to methotrexate. Further, clinical potential of these compounds and its resistance to various cell lines, of course, remain uncertain until additional studies are carried out.

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