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Synthesis of new pyrimidine derivatives via 1,3 dipolar cycloaddition and their *in-silico* molecular docking studies as thymidylate synthase inhibitors

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ABSTRACT

In the present work a new and versatile route for synthesis of fused heterocycles (furo-pyrrolo-pyrido pyrimidine derivatives) using 1, 3 dipolar cycloaddition has been described. The structures of the synthesized compounds were analyzed by IR and ¹H NMR and were purified by recrystallization and column chromatography. *In-silico* molecular docking studies were carried out to analyse the binding affinity of the pyrimidine derivatives towards thymidylate synthase. The synthesized compounds possess good binding interactions with thymidylate synthase showing a docking score of -7.0 for compound 4 and -8.7 for compound 6 which is greater when compared to standards 5-fluoro uracil (-5.0) and Methotrexate (-8.6). The results demonstrate a simple, mild and efficient method for the synthesis of novel compound (furo-pyrrolo-pyrido-pyrimidine derivative 6) and their potential anticancer property as thymidylate synthase inhibitor.

Keywords: Pyrimidine, Cycloaddition, Molecular docking, Thymidylate synthase.

INTRODUCTION

Pyrimidines are the members of the diazine group of heterocyclic compounds and represent the 1, 3-diazine. These units annelate with a variety of ring systems which are available in natural sources like nucleosides and nucleotides etc. Because of their unique biological activity, the derivatives based on this type of heterocyclic system have attracted much attention and many detailed reviews have appeared in the literature. As compounds belonging to this group were known as breakdown products of uric acid at a very early date, the systematic study of the ring system really began with the work of Pinner, who first applied the name pyrimidine to the unsubstituted parent unit. Purines and pyrimidines are heterocyclic compounds which form a significant segment of organic chemistry. Majority of the bioactive molecules are heterocycles. Almost all the biologically active/clinically employed molecules have one way or the other skeletal relationship with natural molecules [1]. They are very widely distributed in nature and are essential to life; they play vital role in the metabolism of all living cells. In the living being very potent molecular unit worth mentioning here is DNA (in the sequence adenine-thymine and guanine-cytosine), vitamins (vitB₁₂ and E families) enzymes and hormones (kinetin, heteroquin, serotonin and histamine etc.).

The importance of uracil and its annelated derivatives are well recognized by synthetic as well as biological chemist. The synthesis of naturally occurring complex molecules containing a uracil ring and its annelated substrates continue to be of great interest due to their wide range of biological activities. Thymine was originally isolated in 1893 from hydrolyzates of bovine thymus. It is one of the four bases in DNA which on hydrolysis give thymine via thymidine [3'-(2'-deoxyD-ribofuranoside)-thymine]. Apart from uridine, other nucleosides derived from uracil are

called pseudouridine and uridine phosphate. Recently uracil moieties were detected in antibiotic tunicamycin [2]. Fluoropyrimidines find diverse use in cancer chemotherapy and other drug applications (**Fig.1**) 5-Fluorouracil has antineoplastic activity and is a valuable drug especially for the treatment of tumors of the colon or rectum, but it has wider application in chemotherapy. Other monopyrimidines of biological interest are 5-fluorocytosine, an antifungal agent; 2-deoxy-5-fluoro-uridine, an antiviral and antineoplastic agent; 5-fluorourotic acid used in yeast molecular genetics and tegafur, an antineoplastic agent which releases 5-FU in vivo [3].

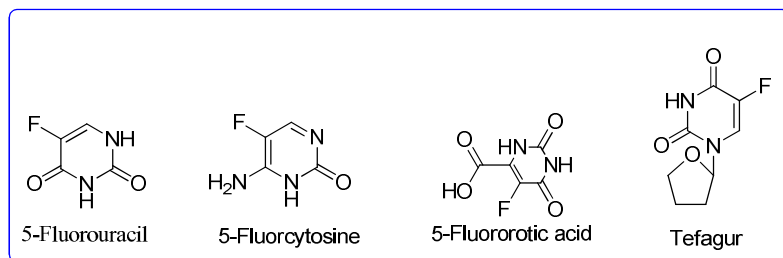


Fig.1- Antitumor drugs

Cytarabine, 4-amino-1-β-D-arabinofuranosylpyrimidin-2(1*H*)-one is a valuable drug in cancer chemotherapy, as in the treatment of acute leukemia of childhood and adult granulocytic leukemia. Certain 5-halo-2-(1*H*)-Pyrimidones arrest the cell cycle of mouse / human cells grown in cultures. The arrest is in the relatively narrow metaphase region [4-6]. Synergistic cell inactivation effects were displayed when human NH1K 3025 cells cultivated in vitro were treated with 5-halo-2-(1*H*)-Pyrimidones in combination with cisplatin. Cis-diaminoplatinum 1-methyl uracil blue $[Pt_4(NH_3)_8(1-meU)_4]^{5+}$ where 1-MeU denotes 1-methyl uracil, referred to as “platinum pyrimidine blues” belongs to a class of platinum complexes which are claimed to have a higher index of Antitumor activity, lower nephrotoxicity than the anticancer drug cisplatin and high aqueous solubility [7-9].

Cycloaddition reactions are the most useful reactions in the armamentarium of synthetic and mechanistic organic chemistry. Conceptually it constitutes one of the simplest reactions in organic chemistry. This involves electron shift, they are ring closure reactions in which the number of σ bonds increase at the expense of π bonds without the loss of any fragment and result in the formation of a cyclic compound (cyclic adduct). Cycloaddition reactions are as old as organic synthesis itself. The reaction of aliphatic diazo compounds with α , β -unsaturated carboxylic esters was discovered by Buchner as early as 1888 [10]. The cycloaddition of diphenyl ketone to olefin and imines were described by Staudinger at the beginning of 19th century [11]. It took more than 50 years before these reactions were recognized as members of two broad classes of cycloaddition i.e. the 1,3 dipolar and [2+2] cycloadditions. Discoveries of the Diels Alder reaction, the Huisgen 1,3-dipolar cycloadditions and the carbene additions of olefins provide the basis for a remarkable flowering of cycloaddition chemistry reflected by outstanding application in synthesis.

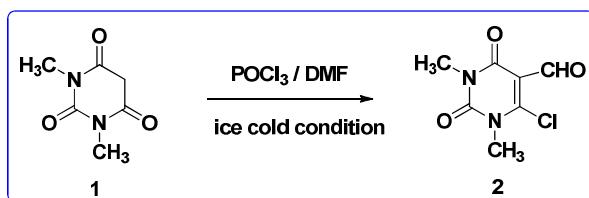
Cycloaddition reactions have figured prominently in both synthetic and mechanistic organic chemistry [12-28]. Current knowledge of underlying principle in this area is the result of a fruitful interplay between theory and experiment. During the past two decades there has been a remarkable interest in the development of (4+2) cycloaddition. These processes offer a powerful solution to many problems in complex and natural product synthesis. Further, ring formation from acyclic precursors account for the popularity of this approach. Classical method for the synthesis of pyrimidine is Bignelli type reactions. However such methods suffer from disadvantages like use of expensive or less available reagents, vigorous reaction conditions, prolonged standing and tedious manipulations in the isolation of the pure products. Nowadays green chemistry, solvent free condition and 1,3 dipolar cycloaddition approach have been used for construction of novel heterocycles.

Reviews on Uracil derivatives have shown that they possess a wide range of biological activity. A large number of reports appeared in literature for their synthesis; however, they usually require harsh conditions, long reaction times and complex synthetic pathways. So, new routes for the synthesis of these have attracted a considerable attention as a rapid entry for the formation of these heterocycles.

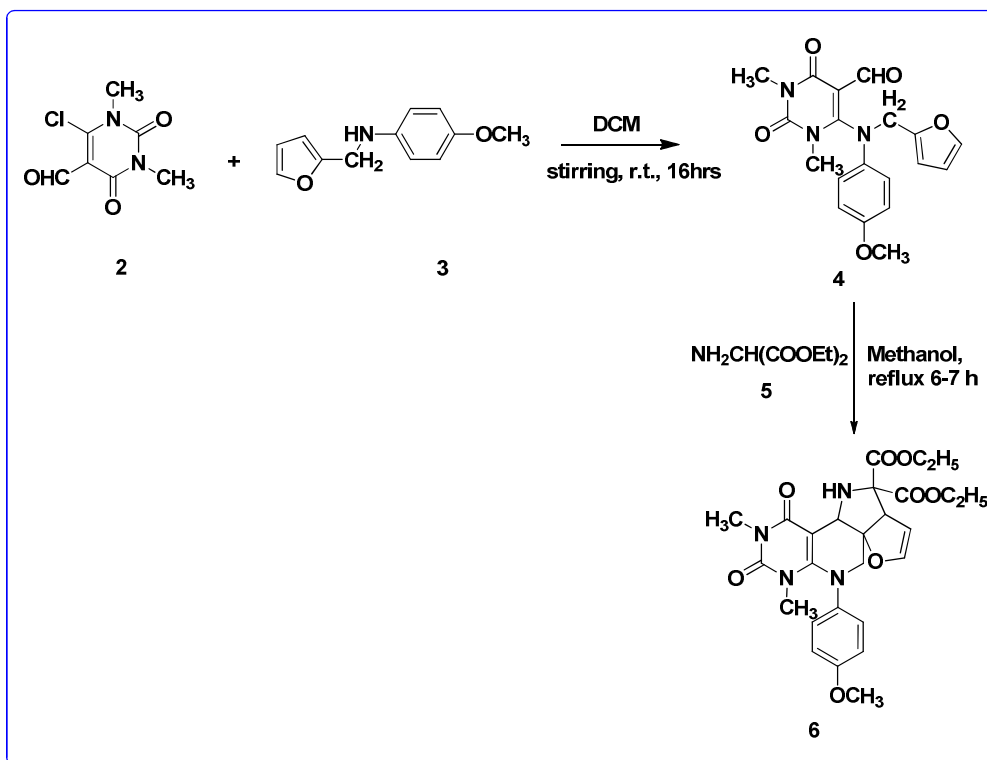
The present work focus mainly on the application of cycloaddition chemistry to the basic skeleton of pyrimidines to construct the newer molecules of biological significance and to characterize them using high resolution spectral techniques IR and ^1H NMR.

MATERIALS AND METHODS

All of the materials were purchased from commercially available sources Sigma-Aldrich, Merck and were utilized without any additional purification. All the reactions were monitored by TLC (Thin layer chromatography). Melting points were recorded on an Elchem digital melting point apparatus in open capillaries and were uncorrected. The IR spectra were recorded on a Perkin Elmer 2000 FTIR spectrophotometer. The ^1H NMR spectra were recorded on the Bruker Advance DPX 300MHZ spectrophotometer using CDCl_3 as solvent and TMS as internal reference. Melting points was determined in open capillary on Buchi B-540 apparatus and are uncorrected. Analytical TLC was performed on precoated silica gel-60 F₅₄ glass plates. Visualization of spots was achieved by exposure to iodine vapour. Column chromatography was performed using silica gel (60-120) and (100-200) and the column was usually eluted using suitable solvents.



Scheme.1 Synthesis of 6-chloro-1, 3-dimethyl -5-formyl uracil



Scheme.2 Synthesis of fused furo-pyrrolo-pyrido pyrimidine derivative 6

Chemistry

The synthetic strategy leading to the key products 6-chloro-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbaldehyde **2**, 6-((furan-2-ylmethyl)(4-methoxyphenyl)amino)-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro pyrimidine-5-carbaldehyde **4**, diethyl 11-(4-methoxyphenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4,4b,5,10,11-octahydrofuro [2",3":3',4']pyrrolo[2',3':4,5]pyrido[2,3-*d*]pyrimidine-6,6(6*aH*)-dicarboxylate **6** were depicted in **Scheme 1&2**. Reaction of 1, 3-dimethyl barbituric acid with POCl₃ and DMF resulted in the formation of formyl uracil **2**. Schiff base was prepared by the reaction of furfural and an amine (para anisidine) which was further reduced using sodium borohydride. The reduced Schiff base underwent condensation reaction with formyl uracil to give 6-(N-furfuryl-N-anisidine)-5-formyl-1, 3-dimethyl-pyrimidinedione **4**. This condensed product when reacted with diethylamino malonate hydrochloride formed the desired final compound **6** under thermal conditions via 1, 3-dipolar cycloaddition.

Molecular docking analysis for anti-cancer efficacy

The binding interactions of compounds, **2**, **4**, **6** and standards 5-fluorouracil, methotrexate with thymidylate synthase were carried out by using Autodock v.1.5.6. The thymidylate synthase was taken from protein data bank (**ID: 1BID**) [29]. Atomic affinity potentials for each ligand were analysed separately and the binding mode of each atom in the ligand with the receptor site was further evaluated. Finally grid maps were calculated for each ligand separately and docking analysis were carried out by using Lamarckian Genetic Algorithm. By using Autodock 4.2 scoring function 5 best poses for each ligand were generated and scored [30]. The binding energy of various docked confirmations of each ligand in complex with the receptor site were analysed separately. Thymidylate synthase (TS) is an enzyme which catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) with 5,10-methylene tetrahydrofolate (CH₂THF) as the methyl donor. Inhibition of thymidylate synthase will block the access of dUMP to the nucleotide-binding site and inhibits dTMP synthesis. This results in deoxynucleotide (dNTP) pool imbalances and increased levels of deoxyuridine triphosphate (dUTP), both of which cause DNA damage.

RESULTS AND DISCUSSION

A cost effective method has been used for the synthesis of 1,3-dimethyl barbituric acid by using following reagents: 1,3 dimethyl urea, malonic acid, acetic acid, acetic anhydride. Here acetic anhydride acts as dehydrating agent and the product obtained was recrystallized from ethanol. The yield of the compound obtained was 75% and the structure of the compound was confirmed by spectroscopic data. The IR spectrum showed band at 1690.7cm⁻¹, 1660.8cm⁻¹ due to carbonyl group of amide. A singlet peak at δ 3.69 and δ 3.3 indicated the presence of CH₂ and CH₃ protons but these were downfield due to attachment of electron withdrawing groups. 1,3 dimethyl barbituric acid reacts with POCl₃ and DMF to form 6-chloro-1, 3-dimethyl-5-formyl Uracil. The addition of POCl₃ to DMF was done by maintaining ice cold condition as the reaction was highly exothermic. The product was purified by recrystallization from petroleum ether. The yield of the compound obtained was 86% and the structure of the compound was confirmed by spectroscopic data. In IR Spectrum two bands appeared at ν_{\max} 1689 cm⁻¹, 1652 cm⁻¹ due to carbonyl group of amide. The peak at 1712 cm⁻¹ was due to conjugation of the carbonyl group of aldehyde with C=C. The peak at 780.8cm⁻¹, indicates the presence of chlorine group.

Schiff base **3** was obtained by simple condensation reaction between furfural and p-anisidine. Firstly aromatic amine was dissolved in methanol to which equimolar amount of furfural was added slowly. As the reaction was exothermic, ice cold condition was maintained to avoid such reaction condition. The yield of the compound obtained was 92% and the structure of the compound was confirmed by spectroscopic data. IR spectrum shows absorption bands at 1626.9 cm⁻¹ and 832.1cm⁻¹ due to presence of C=N of imines and para substituent in the aromatic ring. A peak at 1478.3 and 1246.5 were due to C=C of aromatic ring and C-O of -OCH₃. A peak at δ 3.83 and δ 8.31 were due the presence of -CH₃- proton and -CH- proton of imine. The peaks at δ 6.5-7.6 were due to-CH- protons of aromatic ring.

NaBH₄ is highly soluble in polar solvents like water, alcoholic solution (reacts slowly with polar solvents) while LAH reacts violently. Therefore NaBH₄ was used as reducing agent in the reduction of imine group (C=N) in Schiff base when methanol was used as a solvent. The yield of the compound obtained was 90% and the structure of the compound was confirmed by spectroscopic data. In IR spectrum the peaks at 1463.3cm⁻¹, 820.7 cm⁻¹, was due to C=C stretching in aromatic ring and para substituent in aromatic ring. Formation of compound was confirmed by the peak at 3391.1 cm⁻¹ and 1236.1 cm⁻¹ which indicated the presence of NH stretching group and C-O group. The peak

at δ 4.27 was due to $-\text{CH}_2-$ proton. Peaks at δ 6.21- δ 6.8 were due to (aromatic) $-\text{CH}_2-$ protons. A singlet peak at δ 7.36 and δ 3.8 indicated the presence of $-\text{NH}-$ protons and $-\text{CH}_3-$ protons.

Condensed product (4)

It is a simple reaction between reduced Schiff base and 6-chloro-1, 3 dimethyl -5-formyl uracil where a removal of HCl molecule gives the product. Firstly, equimolar amount of reactants were taken in around bottom flask and CH_3OH was used as solvent. The reactant was stirred in a magnetic stirrer until the reaction was complete. The completion of the reaction was checked by TLC. The above proposed scheme failed as no product was obtained. Therefore an attempt had been made to carry out the same reaction using DCM as solvent. While monitoring the progress of the reaction by TLC, a new spot was obtained which was assumed to be the product. The product formed was purified by column chromatography. The separation of the compound was checked in different solvent system like CHCl_3 : Petroleum ether (7:3), CHCl_3 , ethylacetate: hexane (3:7). Separation of the compound was found to better in ethylacetate: hexane (3:7) solvent system. The yield of the compound obtained was 70% and the structure of the compound was confirmed by spectroscopic data.

IR Spectrum showed absorption bands at 1717cm^{-1} , 1655.2cm^{-1} , 1441.9cm^{-1} , 829.9 , 1244.1cm^{-1} due to $\text{C}=\text{O}$ of aldehyde group, $\text{C}=\text{O}$ of amide, $\text{C}=\text{C}$ of aromatic ring para substituent in the aromatic ring and $\text{C}-\text{O}$ of OCH_3 . The peak at δ 10.13 indicated the presence of aldehyde proton. The peak at δ 6.25- δ 6.88 indicated the presence of aromatic protons. A singlet peak at 4.83 was due to the presence of $-\text{CH}_2-$ protons. A peak at δ 3.77 was due to the presence of $-\text{CH}_3-$ proton in $-\text{OCH}_3-$. The peak at δ 3.40 and δ 2.95 were also due to the presence of $-\text{CH}_3-$ proton but these were in low field due to the attachment of electron withdrawing groups.

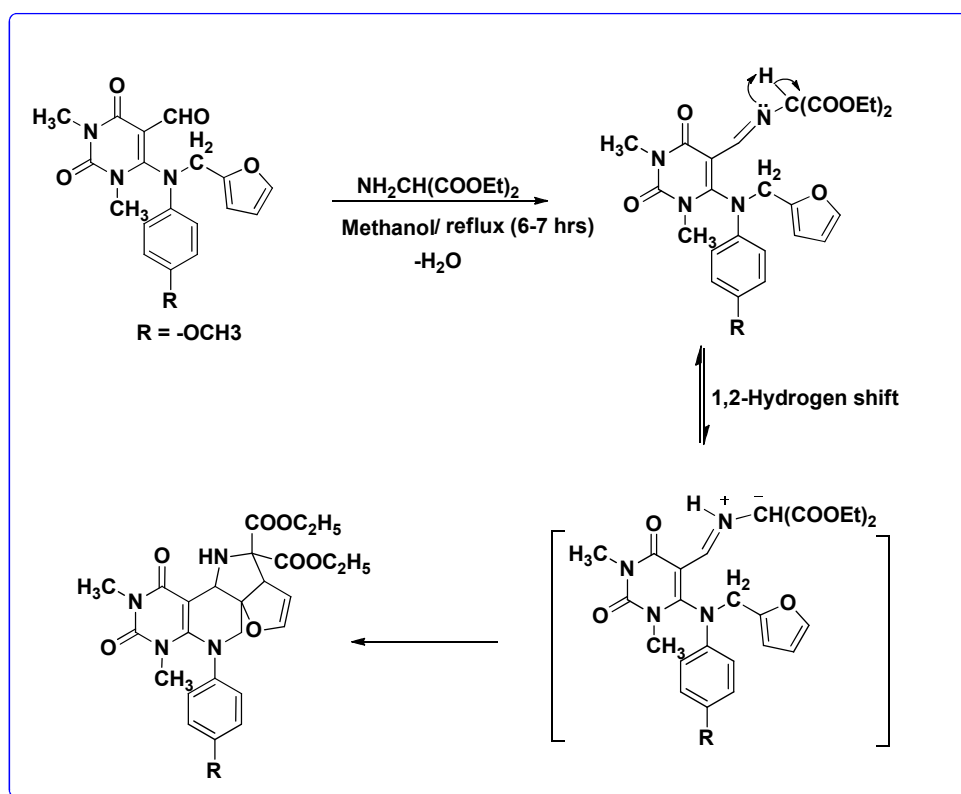


Fig.2 Reaction mechanism for the synthesis of Furo-Pyrrolo-Pyrido-Pyrimidine derivative 6 via 1,3-dipolar cycloaddition reaction

Furo-Pyrrolo-Pyrido-Pyrimidine derivative (6)

Intramolecular 1, 3 dipolar cycloaddition is a powerful concept for the construction of novel heterocycles. In this reaction 6-(N-furfuryl-N-anisidine)-5-formyl-1,3-dimethyl pyrimidinedione and diethylaminomalonnate hydrochloride undergoes intramolecular cycloaddition via azomethine ylide intermediate to form furo-pyrrolo-

pyrido-pyrimidine derivative (**Fig.2**) Various attempts were made to form the desired cyclised product. Equimolar amount of reactants were taken and the reaction was carried out in Synthewave 402 Monomode reactor, toluene was used as the solvent. The reaction failed as diethylaminomalonate hydrochloride was insoluble in toluene. The reaction did not occur when it was carried out in solvent free condition and using methanol as solvent in the domestic microwave. It was observed that the reaction proceeded when it was carried out in a thermal condition using methanol as solvent.

The product obtained was purified by column chromatography. The separation of the compound was checked in different solvent system: ethylacetate: hexane (3:7), CHCl_3 : ethylacetate (2:8), CHCl_3 : ethylacetate (9:1), ethylacetate: hexane (1:1), ethylacetate:hexane (2:1). Separation of the compound was found to better in ethylacetate:hexane (2:1) solvent system. The yield of the compound obtained was 50% and the structure of the compound was confirmed by spectroscopic data. IR spectrum showed bands at 1697.8, 1642.2 due to the presence of carbonyl group. Peaks at 1487.6, 836.8 were due to aromatic C=C stretch. The peaks at 1248.3 and 1225.7 indicated the formation of the compound which was due to C-O stretch in esters and ether. A peak at δ 3.78 was due to $-\text{CH}_3$ -protons in $-\text{OCH}_3$ group and two peaks at δ 3.38 and δ 3.57 were due to $-\text{CH}_3$ - protons in N-CH_3 . Two doublets at δ 6.29 and δ 5.92 were due to $-\text{CH-}$ proton of furfuryl. The peak at 5.59 was due to $-\text{NH-}$ proton. Peaks at δ 6.84 -7.02 were due to $-\text{CH-}$ protons. A triplet at δ 1.26 was due to $-\text{CH}_3$ - protons of methyl group and a quartet peak at δ 4.13 was due to $-\text{CH}_2$ - protons of methylene group.

The results obtained from the molecular docking studies have showed that the compounds **4** and **6** are having good binding interaction towards thymidylate synthase receptor (**Table.1**). The compounds **4** demonstrates a docking score of -7.0 where as compound **6** shows -8.7 which is greater than standards 5-fluoro uracil (-5.0) and methotrexate (-8.6) (**Fig.3**).

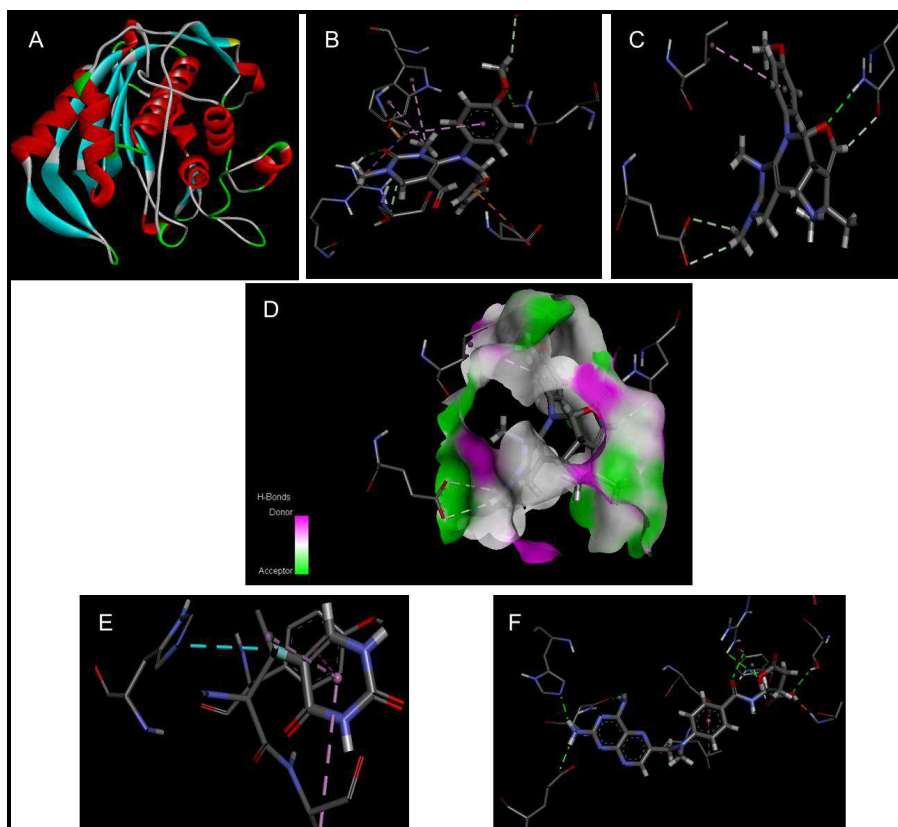


Fig.3 The binding affinity of synthesized pyrimidine derivatives with the PGHS receptor [A] The secondary structure thymidylate synthase (1BD) [B] The interaction of compound 4 [C] The interaction of compound 6 receptor [D] The hydrogen bonding interaction of compound 6 [E] The interaction of 5-fluorouracil [F] The interaction of methotrexate

Table: 1 Molecular docking studies for anticancer efficacy of synthesized pyrimidine derivatives and standards as thymidylate synthase inhibitors

Ligand	AutoDock Score	[grid]H bond	Lipophilicity	Torsion
2	-4.8	-0.9	-0.5	2
4	-7.0	-1.5	-1.2	4
6	-8.7	-2.4	-2.1	5
Std ^a	-5.0	-0.7	-0.8	2
Std ^b	-8.6	-3.1	-1.8	4

^a5-fluorouracil, ^bMethotrexate.*General procedure for the preparation of 1, 3-dimethyl barbituric acid (1)*

A mixture of 1, 3 dimethylurea and malonic acid were stirred for 3 hours. During the procedure acetic anhydride was added dropwise. The mixed solution is then maintained at 70°C and acetic acid was added. The temperature was then raised rapidly to 90°C and kept for 4 h with stirring. The solution was evaporated under reduced pressure and boiled for 10 min with ethanol. On cooling 1, 3-dimethyl barbituric acid crystallised out. It was recrystallised from ethanol as white crystalline solid.

General procedure for the preparation of 6-chloro-1, 3-dimethyl-5-formyl uracil (2)

POCl₃ was added very slowly in 6ml DMF (freshly distilled) so that the sudden raise of temperature could be avoided and then allowed it to come to room temperature. N, N-dimethyl barbituric acid (2gm) was then added into it and refluxed gently for 45 min. After that excess of phosphorus oxychloride was distilled off under reduced pressure and the residual liquid was poured into 100 gm of crushed ice in a 250 ml beaker. It was then allowed to come to room temperature and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulphate and distilled. On evaporation of chloroform under vacuum gave 6-chloro-1,3-dimethyl -5-formyl uracil, (chloroformylated product) which could be recrystallised from petroleum ether.

General procedure for the preparation of Schiff base (3)

Equimolar amount of distilled furfural was weighed and an aromatic amine was weighed. Aromatic amine was dissolved in methanol. To it furfural was added dropwise keeping the solution in ice cold condition. A precipitate was formed which is then filtered. The filtrate obtained was the Schiff base. The product obtained was recrystallized from ethanol.

General procedure for the preparation of 6-(N-furfuryl-N-anisidine)-5-formyl-1, 3-dimethyl-pyrimidinedione 4.

Equimolar amount of reduced Schiff base and chloroformylated product was mixed together and dissolved in dichloromethane. It was then kept for stirring for 16 h. The progress of the reaction was monitored by TLC. After completion of the reaction, it was allowed to cool to room temperature.

General procedure for the preparation of diethyl 11-(4-methoxyphenyl)-1,3-dimethyl-2,4-dioxo-1,2,3,4,4b,5,10,11-octahydrofuro [2'',3'':3',4']pyrrolo[2',3':4,5]pyrido[2,3-d]pyrimidine-6,6(6aH)-dicarboxylate 6.

In a typical case, a mixture of 1mmol of 6-(N-furfuryl-N-anisidine)-5-formyl-1,3-dimethyl pyrimidinedione (condensed product) and 1mmol of Diethyl amino malonate. HCL in methanol was placed in the reaction vessel & refluxed for 6-7 h. The progress of the reaction was monitored by TLC, after completion of the reaction; it was allowed to cool to room temperature. Then the solid thus obtained on evaporation of the solvent was subjected to column chromatography using hexane/ethyl acetate (1:2) as eluent to obtain intramolecularly cyclised product via azomethine ylide intermediate.

CONCLUSION

A simple, mild and efficient method for the synthesis of new pyrimidine derivatives via 1,3-dipolar cycloaddition has been carried out. The results obtained from in-silico molecular docking studies gives a clear idea that the synthesized Furo-Pyrrolo-Pyrido-Pyrimidine derivative (6) possess good binding interaction towards thymidylate synthase enzyme. In future research has to be carried to fully explore the anticancer efficacy of synthesized derivative *in-vitro* and *in-vivo* animal models.

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