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SYNTHESIS, *IN VITRO* BIOLOGICAL EVALUATION AND *IN SILICO* MOLECULAR DOCKING STUDIES OF SOME NOVEL DIHYDROPYRIMIDONES AS POTENTIAL CYTOTOXIC AGENTS

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Abstract:

The major challenge in modern drug discovery has been the design and development of new anticancer drugs with improved efficacy and minimal side effects, especially due to a rapid rise in multidrug resistant tumors. In the recent past, United States Food & Drug Administration (US FDA) newly approved anticancer drugs such as Gefitinib (Iressa), Erlotinib (Tarceva), Lapatinib (Tykerb) and Vandetanib (Caprelsa) possess a pyrimidine nucleus as core moiety exert multiple mechanisms of action. Therefore in the present investigation, we have synthesised a series of pyrimidines (dihydropyrimidones) ***TVD, TVD1-4*** *and evaluated thier potential as anticancer agents by using in vitro brine shrimp (Artemia salina) cytotoxicity bioassay. Among the compounds tested, compound* ***TVD4*** *has showed signifcant cytotoxicity at* ED_{50} *value* ***3.11 ± 0.15 µg/mL****. Consequently, in silico molecular docking studies have also been performed to evaluate the possible underlying mechanism of action of* ***TVD4*** *against DHFR (Dihydrofolate reductase) anticancer drug target. Molecular docking results revealed that the* ***TDV4*** *is less selective towards inhibition of DHFR.*

Keywords: *Cancer, US FDA, Pyrimidine, Dihydropyrimidone, Brine shrimp lethality, Molecular docking, Dihydrofolate reductase (DHFR)*

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QR code

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INTRODUCTION:

Cancer is the name given to a collection of related diseases (Hennessy et al., 2005). In all types of cancer, some of the body's cells begin to divide without stopping and spread into surrounding tissues (Fesik et al., 2005). Cancer can start almost anywhere in the human body, which is made up of trillions of cells (Shea et al., 2016). Normally, human cells grow and divide to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place (Prasad et al., 2016). When cancer develops, however, this orderly process breaks down. As cells become more and more abnormal, old or damaged cells survive when they should die, and new cells form when they are not needed (Inselet al., 2016). These extra cells can divide without stopping and may form growths called tumors (Gottesman et al., 2016). Many cancers form solid tumors, which are masses of tissue. Cancers of the blood, such as leukemias, generally do not form solid tumors. Cancerous tumors are malignant, which means they can spread into, or invade, nearby tissues. In addition, as these tumors grow, some cancer cells can break off and travel to distant places in the body through the blood or the lymph system and form new tumors far from the original tumor. Unlike malignant tumors, benign tumors do not spread into, or invade, nearby tissues. Benign tumors can sometimes be quite large, however. When removed, they usually don't grow back, whereas malignant tumors sometimes do. Unlike most benign tumors elsewhere in the body, benign brain tumors can be life threatening (Cheng et al., 2016).

Everywhere in the world, incredible resources are being devoted in prevention, diagnosis, and treatment of cancer. Cancer is the second leading cause of death worldwide (Nitulescu et al., 2016). Discovery and development of new anticancer agents are the significant effort of several industrial as well as academic organizations, like the National Cancer Institute (NCI) in the United States, the European Organization for Research and Treatment of Cancer (EORTC), and the British Cancer Research Campaign (CRC).Conventional anticancer drug discovery and development have focused on the cytotoxic agents (Mullard et al., 2016). The drug discovery paradigms selected agents that had significant cytostatic or cytotoxic activity on tumor cell lines and caused tumor regression in murine tumor allografts or xenografts. The anticancer agents were discovered mainly by serendipity or inhibiting metabolic pathways crucial to cell division (Hooijberg et al., 1999). Their exact mechanisms of action were often a subject of retrospective investigation. Although this strategy has achieved significant success, the recent developments in molecular biology and an understanding of the pharmacology of cancer at a molecular level have challenged researchers to come up with target-based drugs. These are the agents that are pre-designed to inhibit and/or modify a selected molecular marker deemed important in cancer prognosis, growth, and/or metastasis (Chabner et al., 2005). Several target-based compounds have emerged in recent years (Navarro-Perán et al., 2005).

An antimetabolite is a chemical that inhibits the use of a metabolite, which is another chemical that is part of normal metabolism. Such substances are often similar in structure to the metabolite that they interfere with. Antimetabolites can be used in cancer treatment, as they interfere with DNA production and therefore cell division and the growth of tumors. Because cancer cells spend more time dividing than other cells, inhibiting cell division harms tumor cells more than other cells. We now have a proper understanding and knowledge of enzymes and biosynthesis of purine and pyrimidine nucleotide precursors of RNA and DNA. This intricate metabolic reactions works under a complex web of positive-feedback and negative-feedback controls (Vander Heiden et al., 2011). Most purine or pyrimidine analogs are active only after metabolic activation to the nucleotide form, so these fraudulent nucleotides not only may be incorporated, but also can mimic the natural effector compounds in regulatory pathways. Alternatively, they may deplete critical intermediates, thereby generating enlarged pools of the natural precursors behind a metabolic block, producing effects that can distort the balance of ribonucleoside and deoxyribonucleoside triphosphates. A target of even greater complexity is the incorporation of triphosphates into DNA or RNA and the subsequent modification of these macromolecules (Renslo et al., 2006).

Dihydrofolate reductase (DHFR) is an essential metabolic enzyme that plays critical rolein one-carbon transfer reactions, including the biosynthetic pathways for deoxythymidinemonophosphate (dTMP) (Raoet al., 2000), purines and several amino acids (Banerjee et al., 2002). As such, DHFR has beensuccessfully targeted for both anticancer (eg. methotrexate) and antimicrobial (eg.trimethoprim, pyrimethamine) drug development (Schweitzeret al., 1990). Owing to its essential role in both humanand pathogenic cells, the successful development of anticancer DHFR inhibitors requiresthat the compounds are selective for the carcinogenic cells (Kayeet al., 1998). Hence, it was proposed worthwhile to study the possible protein-ligand interactions using molecular docking studies against cancer targeted DHFR enzyme by using iGemDock software (Hsu et al., 2011).

Pyrimidine is an aromatic heterocyclic organic compound similar to pyridine (Roopanet al., 2016). One of the three diazines, it has the nitrogen atoms

at positions 1 and 3 in the ring (Pullelaet al., 2010). Pyrimidine and its derivatives demonstrate a diverse array of biological and pharmacological activities including anticonvulsant, antibacterial, antifungal, antiviral and anticancer properties (Kappeet al., 2000). This broad spectrum of biochemical targets has been facilitated by the synthetic versatility of pyrimidine, which has allowed the generation of a large number of structurally diverse derivatives including analogues derived from substitution of the aryl ring, and/or derivatization of the pyrimidine nitrogen and C2/C4/C5/C6 carbon positions. Pyrimidines are synthetically versatile substrates, where they can be used for the synthesis of a large variety of heterocyclic compounds and as raw material for drug synthesis(Rafieeet al., 2006). Dihydropyrimidinones are pyrimidine analogs, the products of the Biginelli reaction, are widely used in the pharmaceutical industry as calcium channel blockers, antihypertensive agents, and alpha-1a-antagonists (Padmajaet al., 2013).

Cancer is a primary global cause of mortality, accounting for millions of deaths every year. Though many anticancer agents have been developed to treat different types of cancer effectively, major adverse effects could occur concurrently. Consequently, a huge demand is still there to find some novel molecules to treat this disease in current situation. Hence, we proposed worthwhile to synthesis some novel pyrimidine derivatives (dihydropyrimidones) and studied for their anticancer druggable properties using brine shrimp (Artemia salina) lethality bioassay. The rationale for selecting pyrimidine basic scaffold was, pyrimidine based scaffolds have exerted their cell killing effects through varied mechanisms which indicate their potential to interact with diverse enzymes/targets/receptors. Numerous reviews compiling the results of investigations on the anticancer potential of the pyrimidines based structure in research articles are already present. The promising activity displayed by these pyrimidine based scaffolds clearly places them in forefront as potential future drug candidates. Hence we will have synthesized, characterized and biologically evaluated some novel dihydropyrimidones as potential anticancer agents by using in vitro Artemia salina (Brine Shrimp Lethality bioassay).

MATERIALS:

Instrumentation

- Melting points were taken in open capillary tubes. Purity of the compounds was checked on silica gel G TLC plates of 2 mm thickness using n-hexane and ethyl acetate as solvent system. The visualization of spot was carried out in an UV-chamber. The spectra IR, NMR and Mass have been recorded by sending the pure sample to the Universiti Putra Malaysia (UPM), Jalan Upm, 43400 Serdang, Selangor, Malaysia.

Reagents and chemicals

- Substituted aldehydes and ketones, urea, thiourea, sodium hydroxide, potassium hydroxide, pyridine, triethylamine, Brine Shrimp Eggs, hexane, ethyl acetate, acetone, chloroform, methanol, ethanol, iodine, H_2SO_4 spraying reagent, silica gel (column & TLC) and other regular laboratory chemical of AR grade were procured from the local chemical suppliers via. purchase indent from Laboratory Department, Asia Metropolitan University.

Computational software requirements

- Computer aided drug discovery software along with graphical user interface (GUI) were utilized for molecular modeling, energy minimization, molecular docking and virtual screening protocols.

Table 1: List of software applications used in the present study

S.No	Activity (will be performed)	Software (will be used)	License type (will be obtained)	Source
1)	Molecular modeling (2D-Drawing)	Accelrys Draw	Academic License GPU (General Public User) License	Open Source
2)	Molecular modeling (2D-3D Conversion)	Open Babel	Academic License GPU (General Public User) License	Open Source
3)	Molecular modeling (Molecular Mechanics & Energy Minimization)	ArgusLab v 4.0	Academic License GPU (General Public User) License	Open Source
4)	Molecular Docking & Virtual screening	iGemdock v 2.1	Academic License	Open Source

Table 2: Citations for software applications used in the present investigation

S.No	Software	Citation
1)	**Accelrys Draw**	Draw, A. (2011). Accelrys Software Inc. San Diego.
2)	**Open Babel**	OLBoyle, N. M., Banck, M., James, C. A., Morley, C., Vandermeersch, T., & Hutchison, G. R. (2011). Open Babel: An open chemical toolbox. J Cheminf, 3, 33.
3)	**Argus lab**	Thompson, M. A. (2004). ArgusLab 4.0. 1. Planaria Software LLC, Seattle, WA.
4)	**iGemdock v 2.1**	Hsu, K. C., Chen, Y. F., Lin, S. R., & Yang, J. M. (2011). iGEMDOCK: a graphical environment of enhancing GEMDOCK using pharmacological interactions and post-screening analysis. BMC bioinformatics, 12(1), 1.
5)	**Protein Data Bank (PDB)**	Bank, P. D. (1971). Protein Data Bank. Nature New Biol, 233, 223.

Computational hardware requirements

- The minimum central hardware system configuration include Intel (R) Core (TM) 2Duo Central Processing Unit (CPU), 2.5 GHz, 1 TB hard disk, 2 KV Power Backup, WinXP or higher operating system was used for running all the selected computer aided drug discovery softwares. All softwares were well compatible with the selected system configuration.

X-ray crystallographic structure of dihydrofolate reductase (DHFR)

- X-ray crystallographic data of DHFR Ligand Binding Domain (LBD) was obtained from Brookhaven Protein Data Bank **(http://www.rcsb.org/pdb)**. The protein data bank code (**PCB ID: 4KD7**) deposited by **Lamb, et al., 2013**.

Methods

General procedure for the synthesis of dihydropyrimidone derivatives

- The reaction sequence intended for the preparation of title compounds **(TVD, TVD1-TVD4)** is shown in Scheme 1; we have followed the pre-existing methods for the proposed synthesis.

Scheme 1: Synthesis of Dihydropyrimidones

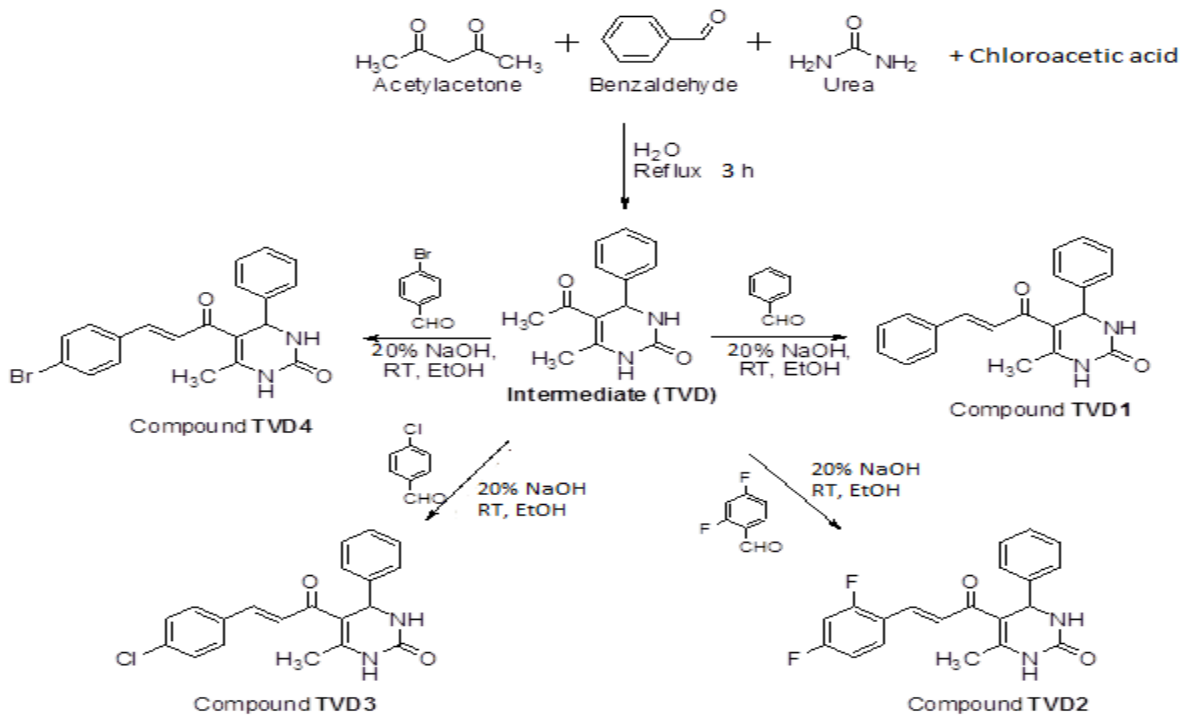
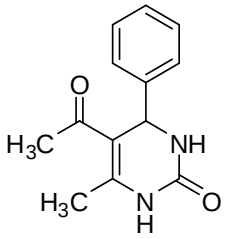
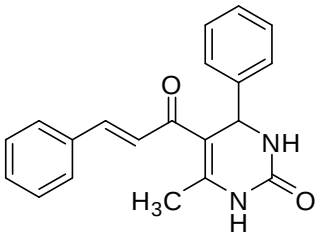


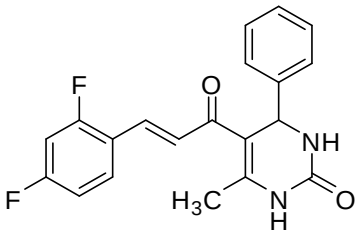
Table 3: Chemical structure of the titled molecules



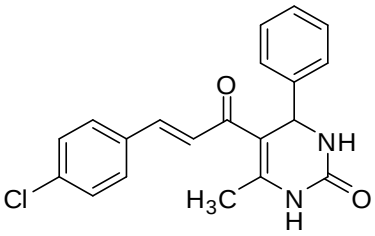
Intermediate (TVD)



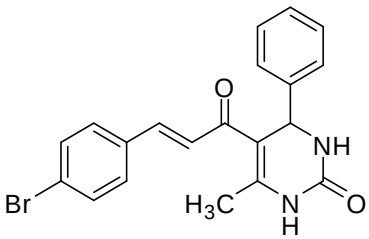
Compound **TVD1**



Compound **TVD2**



Compound **TVD3**



Compound **TVD4**

- The reaction as shown in scheme 1 was started by adding equimolar concentration of substituted acetylacetone, benzaldehyde and urea in q.s. amount of ethanol. To this reaction mixture 10 mole percentage of chloroacetic acid was added and stirred for 10 min, followed by reflux for 3 hrs, reaction mixture turns to yellow. Further the reaction mixture kept aside overnight and examined the TLC profile. After completion of the reaction, the reaction mixture was poured in crushed ice, acidified if necessary with 1:1 dilute hydrochloric acid, and the yellow color solid which separated out was isolated by filtration using Buchner funnel under vacuum filtration setup, dried and purified by recrystallization with ethanol. The crystals of intermediate **TVD** which separated out from the solvent were collected and used for subsequent step of the reaction sequence and also for doing physical characterization and bioassay. The physical properties are depicted in separate tables (Table 4-8).

- The reaction as shown in scheme 1 further proceed by adding substituted benzaldehydes to **TVD** in 1:1 mole concentrations in q.s. amount of ethanol. To this reaction mixture 1.5mL of 20% NaOH solution was added in drop-wise until the reaction mixture turns to yellow. Further the reaction mixture kept aside overnight and examined the TLC profile. After completion of the reaction, the reaction mixture was poured in crushed ice, acidified if necessary with 1:1 dilute hydrochloric acid, and the yellow color solid which separated out was isolated by filtration using Buchner funnel under vacuum filtration setup, dried and purified by recrystallization with ethanol. The crystals of dihydropyrimidones **TVD1-TVD4** which separated out from the solvent were collected and used for doing physical characterization and bioassay. The physical properties are depicted in separate tables for an every individual compound (Table 4-8).

Identification of dihydropyrimidones (TVD, TVD1-TVD4)

- The formation of dihydropyrimidones was identified and analysed by Co-TLC technique with the starting materials. The visualization of the spots was carried out by spraying 5% H_2SO_4 in methanol and heating at 110˚C or under UV light or in Iodine chamber.

Characterization of dihydropyrimidones (TVD, TVD1-TVD4)

- The chemical structures of the synthetic dihydropyrimidones were established on the basis of their physical, chemical and spectral analytical data.
- Melting points were determined in open capillary tube, and expressed in degree Celsius.
- The synthetic chalcones were characterized by UV, IR, NMR & Mass spectral methods.
- NMR & IR spectral data were obtained by sending the samples to Andhra University, Visakhapatnam, India.

In vitro Cytotoxicity Evaluation of Dihydropyrimidones (TVD, TVD1-TVD4)

- **Brine Shrimp Lethality Bioassay (Cytotoxicity Bioassay)**

  The anticancer potential of the synthesized dihydropyrimidones was determined by Brine Shrimp Lethality assay as described by Meyer et al., 1982. Brine Shrimp (Artemia salina) nauplii were hatched in sterile brine solution (prepared using sea water salt 38 g/L and adjusted the pH to 8.5 using 1 N NaOH) under constant aeration for 38 h. After hatching, 10 nauplii were placed in each vial and added various concentrations of drug solutions in a final volume of 5 mL, maintained at 37 ^{o}C for 24 h under light of incandescent lamps and surviving larvae were counted. Each experiment was conducted along with control (vehicle treated) at various concentrations of the test substances. The ED_{50} values (µg/mL) were determined by comparing mean surviving larvae of test and control tubes. The results of cytotoxicity study are given under results section.

General procedure for the ligand preparation

- The chemical structure of the selected ligand **TVD4** was initially modeled as 2D chemical structures using Accelrys Draw software and transformed into 3D chemical structures using Open Babel software and subjected for energy minimization using ArgusLab v 4.0 software. The minimization was executed until the root mean square gradient value reached a value smaller than 0.0001 kcal/mol. Such energy minimized structures were considered for molecular docking studies using iGemdock v 2.1 software. The corresponding docking engine compatible 'MDL MOL' file format has been adapted to ligand by using integral option (save as /MDL MOL).

General procedure for the protein target selection, preparation and validation

- The selection of DHFR Ligand Binding Domain (LBD) for molecular docking studies was carried out based upon several factors such as structure should be determined by X-ray diffraction spectroscopy, and resolution should be between >2.5 A^{o}, it should contain a co-crystallized ligand; the selected protein should not have any protein breaks in their 3D structure. On the other hand, we further consider Ramachandran plot statistics as the important filter for protein selection with none of the residues present in disallowed region. Finally the resultant protein target was prepared for molecular docking simulation in such a way that all heteroatoms (i.e., nonreceptor atoms such as water, ions, etc.) were removed.

General procedure for the software validation

- iGEMDOCK v 2.1 software validation was performed by using X-ray structure (**4KD7)** deposited with co-crystallized ligand was obtained from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb). The Root Mean Square Deviation (RMSD) between the X-ray co-crystallized ligand and docked conformation was 1.84 A^{o} indicated that the parameters for docking simulation was good in reproducing X-ray crystal structure.

General procedure for the Molecular Docking

- Molecular docking technique was employed to dock the bioactive dihydropyrimidone **TVD4** against **4KD7** using iGEMDOCK to locate the interaction between **TAD4** and **4KD7**. iGEMDOCK requires the receptor and ligand coordinates in either Mol2 or PDB format. Non polar hydrogen atoms were removed from the receptor file and their partial charges were added to the corresponding carbon atoms. Molecular docking was performed using standard protein-ligand docking protocol. The binding site was defined by crystallographic ligand of **4KD7**. Default settings were used for all the calculations and docking run was performed.

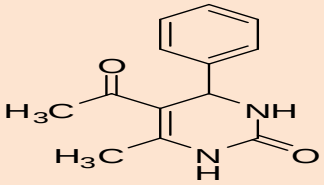
Statistical analysis

- The SPSS 2.0 software was used in data analysis. Data was expressed as mean ± SEM.

RESULTS:

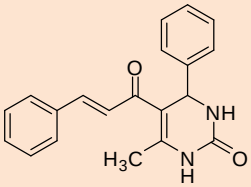
Results of Chemical Synthesis

Table 4: Physical characterization data of dihydropyrimidone TVD.

Dihydropyrimidone (TVD)	
	
(a) Physical state	Solid crystals
(b) Color	Yellowish brown
(c) Nomenclature	5-acetyl-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one
(d) Molecular weight	230.26
(e) Molecular formula	$C_{13}H_{14}N_2O_2$
(f) Melting point (^oC)	213
(g) Yield (%)	85
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography (TLC) • 25% Ethylacetate/Hexane • 0.82 cm • Yellow Fluorescence
(j) UV spectrum data (λ_{max})	300
(k) IR spectrum data (cm^{-1})	1698(C=O), 1743(C=C), 1599(−CONH), 3346(−NH)
(l) ^1H NMR spectrum data (δ ppm)	2.3(s, 3H, CH3), 5.1(s, 1H, C5-Pyrimidine), 7.4(d, J = 15 Hz, 1H, Ar-H), 7.8(d, J = 15 Hz, 1H, Ar-H), 3.3(s, 1H, Ar-NH), 4.0(s, 1H, Ar-NH)
(m) ^{13}C NMR spectrum data (δ ppm)	Not determined
(n) Elemental analysis*	C, 67.81; H, 6.13; N, 12.17; O, 13.90

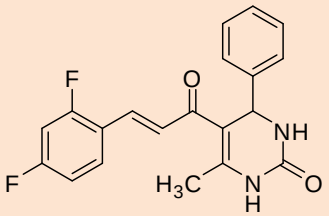
*predicted

Table 5: Physical characterization data of dihydropyrimidone TVD1.

Dihydropyrimidone (TVD1)	
	
(a) Physical state	Solid crystals
(b) Color	Yellow
(c) Nomenclature	5-cinnamoyl-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one
(d) Molecular weight	318.37
(e) Molecular formula	$C_{20}H_{18}N_2O_2$
(f) Melting point (^oC)	135
(g) Yield (%)	87
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography (TLC) • 25% Ethylacetate/Hexane • 0.66 cm • Yellow Fluorescence
(j) UV spectrum data (λ_{max})	284
(k) IR spectrum data (cm^{-1})	1641(C=O), 1617(C=C), 1574(−CONH), 3327(−NH)
(l) ^1H NMR spectrum data (δ ppm)	2.3(s, 3H, CH3), 5.1(s, 1H, C5-Pyrimidine), 7.3(d, J = 15 Hz, 1H, Ar-H), 7.5(d, J = 15 Hz, 1H, Ar-H), 3.3(s, 1H, Ar-NH), 4.1(s, 1H, Ar-NH)
(m) ^{13}C NMR spectrum data (δ ppm)	Not determined
(n) Elemental analysis*	C, 75.45; H, 5.70; N, 8.80; O, 10.05

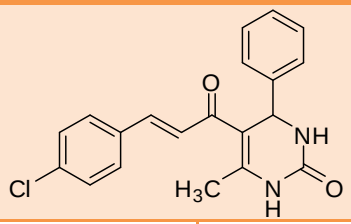
*predicted

Table 6: Physical characterization data of dihydropyrimidone TVD2.

Dihydropyrimidone (TVD2)	
	
(a) Physical state	Solid crystals
(b) Color	Reddish brown
(c) Nomenclature	(E)-5-(3-(2,4-difluorophenyl)acryloyl)-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one
(d) Molecular weight	354.35
(e) Molecular formula	$C_{20}H_{16}F_2N_2O_2$
(f) Melting point (°C)	191
(g) Yield (%)	92
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography (TLC) • 25% Ethylacetate/Hexane • 0.63 cm • Yellow Fluorescence
(j) UV spectrum data (λ_{max})	269
(k) IR spectrum data (cm^{-1})	1645(C=O), 1616(C=C), 1575(−CONH), 3317(−NH)
(l) ^{1}H NMR spectrum data (δ ppm)	2.3(s, 3H, CH3), 5.1(s, 1H, C5-Pyrimidine), 7.4(d, J = 15 Hz, 1H, Ar-H), 7.8(d, J = 15 Hz, 1H, Ar-H), 3.3(s, 1H, Ar-NH), 3.8(s, 1H, Ar-NH)
(m) ^{13}C NMR spectrum data (δ ppm)	Not determined
(n) Elemental analysis*	C, 67.79; H, 4.55; F, 10.72; N, 7.91; O, 9.03

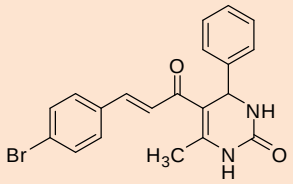
*predicted

Table 7: Physical characterization data of dihydropyrimidone TVD3.

Dihydropyrimidone (TVD3)	
	
(a) Physical state	Solid crystals
(b) Color	Dark yellow
(c) Nomenclature	(E)-5-(3-(4-chlorophenyl)acryloyl)-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one
(d) Molecular weight	352.81
(e) Molecular formula	$C_{20}H_{17}ClN_2O_2$
(f) Melting point (°C)	155
(g) Yield (%)	81
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography (TLC) • 25% Ethylacetate/Hexane • 0.43 cm • Yellow Fluorescence
(j) UV spectrum data (λ_{max})	287
(k) IR spectrum data (cm^{-1})	1630(C=O), 1598(C=C), 1510(−CONH), 3331(−NH)
(l) ^{1}H NMR spectrum data (δ ppm)	2.3(s, 3H, CH3), 5.1(s, 1H, C5-Pyrimidine), 7.4(d, J = 15 Hz, 1H, Ar-H), 7.8(d, J = 15 Hz, 1H, Ar-H), 3.3(s, 1H, Ar-NH), 4.0(s, 1H, Ar-NH)
(m) ^{13}C NMR spectrum data (δ ppm)	Not determined
(n) Elemental analysis*	C, 68.09; H, 4.86; Cl, 10.05; N, 7.94; O, 9.07

*predicted

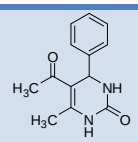
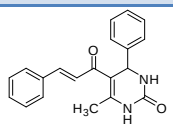
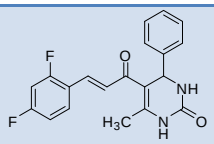
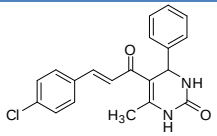
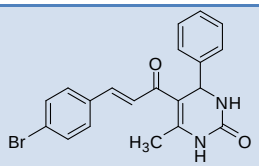
Table 8: Physical characterization data of dihydropyrimidone TVD4.

Dihydropyrimidone (TVD4)	
	
(a) Physical state	Solid crystals
(b) Color	Light yellow
(c) Nomenclature	(E)-5-(3-(4-bromophenyl)acryloyl)-6-methyl-4-phenyl-3,4-dihydropyrimidin-2(1H)-one
(d) Molecular weight	397.27
(e) Molecular formula	$C_{20}H_{17}BrN_2O_2$
(f) Melting point (^oC)	173
(g) Yield (%)	83
(h) Recrystallization solvent	Ethanol
(i) Thin Layer Chromatography (TLC) ❖ **Mobile phase concentration** ❖ **R_f value** ❖ **UV-254nm observation**	Thin Layer Chromatography (TLC) • 25% Ethylacetate/Hexane • 0.38 cm • Yellow Fluorescence
(j) UV spectrum data (λ_{max})	289
(k) IR spectrum data (cm^{-1})	1595(C=O), 1516(C=C), 1471(−CONH), 3305(−NH)
(l) ^{1}H NMR spectrum data (δ ppm)	2.3(s, 3H, CH3), 5.1(s, 1H, C5-Pyrimidine), 7.3(d, J = 15 Hz, 1H, Ar-H), 7.7(d, J = 15 Hz, 1H, Ar-H), 3.3(s, 1H, Ar-NH), 4.0(s, 1H, Ar-NH)
(m) ^{13}C NMR spectrum data (δ ppm)	Not determined
(n) Elemental analysis*	C, 60.47; H, 4.31; Br, 20.11; N, 7.05; O, 8.05

*predicted

Results of Biological Evaluation:

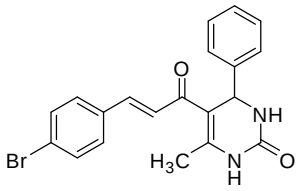
Table 9: Cytotoxicity data of dihydropyrimidones TVD, TVD1-TVD4.

Compound	*Artemia salina lethality* (Brine shrimp) Effective Dose Concentration (ED_{50}, µg/mL)*
 **TVD**	20.15 ± 0.15
 **TVD1**	45.12 ± 0.11
 **TVD2**	6.03 ± 0.12
 **TVD3**	15.87 ± 0.11
 **TVD4**	3.11 ± 0.15

*Mean±SEM, p<0.05

Results of Computational Evaluation

Table 10: Molecular docking information of dihydropyrimidone TVD4 against 4KD7

Compound	iGemdock score Kcal/mol	No. of Hydrogen bonds/Interacting Amino acid residues
 **TVD4**	-106.777	3 / Glu 30, Val 115 and Tyr 121

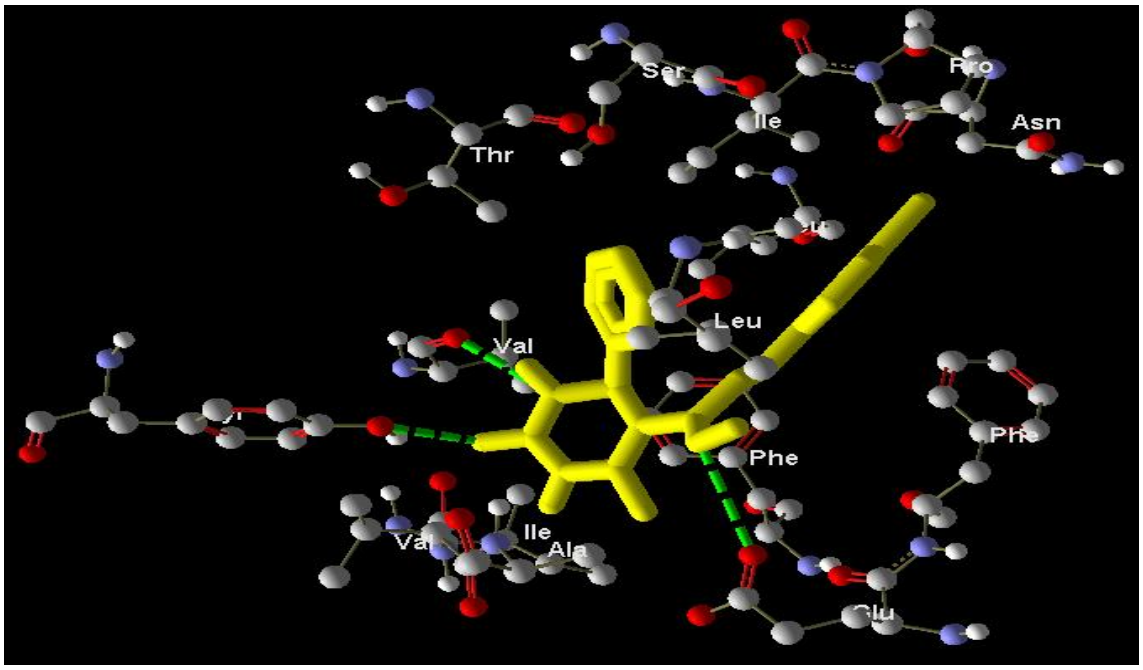


Fig 1: Binding orientation and H-bond interactions (Green) of TVD4 (Yellow) within the active binding site region of 4KD7 (Residues)

DISCUSSION:

Synthesis

The compounds synthesized in the present study were keeping with the expected structures by its spectral data as shown in the Tables **4-8**. Following are the representative UV, IR, ^{1}H-NMR spectra of the compounds **TVD, TVD1-4**, respectively. The results of the spectral data of the compounds **TVD, TVD1-4** were in close agreement with those of the structural features of the dihydropyrimidones and the structures of all the compounds were confirmed by their corresponding spectral data.

Biological Evaluation

Cancer is the uncontrolled growth of cells that interfere with the growth of healthy cells. The usual treatments of Cancer are surgery, chemotherapy (treatment with anticancer drugs), radiation, or some combination of these methods. Anti-Cancer drugs are targeted to control and treat various Cancer like, Breast cancer, Cervical cancer, Small cell lung cancer, Head and Neck cancer, Ovarian cancer, Hodgkin's and Non-Hodgkin's lymphoma, Oesteo-sarcoma, Seminomas of testis, Myeloblastic leukemia, Lymphoblastic leukemia etc. The use and application of drugs synthesized or procured from natural or synthetic sources for cancer inhibition and cure is known as "chemotherapy" and the drugs are more commonly named as chemotherapeutic drugs. As stated earlier, cancer can be defined as a state where cells or tissues of the body start to divide uncontrollably and evade the normal cell cycle as a result of which progression of large tumors occur, and the tumorous cells by the mechanism of metastasis may invade the neighboring normal tissues of the body causing serious implications. (Yang et al., 2006).

Brine shrimp (Artemia salina) was used as a simple bioassay tool for cytotoxicity test on new chemicals. The procedure determined ED50 value

in µg/ mL of active compounds in the brine medium. The activities of known active compounds are manifested as toxicity to shrimps. The advantages of this method are being rapid, reliability, inexpensive and convenient assay (Meyer et al., 1982).

Brine Shrimp Lethality bioassay has an excellent good standard correlation with cytotoxic activity in human solid tumors, and has led to the discovery of new class of natural or synthetic active anticancer agents (McLaughlin et al., 1998).

Brine Shrimp Lethality bioassay has correlation with cytotoxic activity in NCI (National Cancer Institute) cancer cell lines (Mohammed et al., 2009, Mirza et al., 2007, Pimenta et al., 2003).

The investigation of cytotoxicity screening data revealed that the compound **TVD4** demonstrated comparatively the most potent cytotoxicity, with ED_{50} value of 3.11 ± 0.15 µg/mL (Table 9). It is interesting to note that the compound **TVD2** also showed appreciable cytotoxicity with ED_{50} value of 6.03 ± 0.12 µg/mL. The other compounds such as TVD, TVD1 and TVD3 displayed poor level of activity at concentrations 15.87 ± 0.11, 20.15 ± 0.15 and 45.12 ± 0.11 µg/mL, respectively. A close insight into basic chemical structure of the molecules synthesized in the present study clearly displayed the significance of dihydropyrimidone and α,β-unsaturated ketone moieties forming part of the core structure as seen in case of most of the natural products such as pyrimidine analogs and chalcone flavonoids which are currently in clinical use as potential anticancer agents.

The remarkable observation towards the observed cytotoxicity was substitution and unsubstitution of the phenyl ring B of the chalcone bridge which is covalently attached to the dihydropyrimidone nucleus. On the other hand, there was a clear evidence that further substitution of **TDV** by α,β-unsaturated ketone initially reduced the activity as seen in case of **TVD1** but strong enhancement has been noticed on substitution with halogens on ring B as seen in case of the compounds **TVD2-4**. From the observed activity it was clearly suggested that besides dihydropyrimidone the halogen substituent on ring B is equally essential to retain the potency.

Computational Evaluation

Common Mechanism of Action of Anti-Cancer Drugs:

Cancer drugs have been designed to slowly act on the cancerous cells and halt their progression by suppressing them through various molecular mechanisms:

a) They may act by damaging the DNA of cancerous cells. The anticancer drugs cause single strand (SSB) and double strand (DSB) DNA breaks or may lead to manufacture of nonsense DNA or RNA. Examples of drugs in this category include Cisplatin, Mitomycin C, Daunorubicin, Doxorubicin and Etoposide.

b) They inhibit the synthesis of new DNA to stop the cell from replicating because replication of cells leads to growth of tumor. These agents work in a number of different ways. DNA building blocks are folic acid, heterocyclic bases, and nucleotides, which are made naturally within cells. All of these agents work to block some step in the formation of nucleotides or deoxyribonucleotides (necessary for making DNA). When these steps are blocked, the nucleotides, which are the building blocks of DNA and RNA, cannot be synthesized. Thus the cells cannot replicate because they cannot make DNA without the nucleotides. Examples of drugs in this category include methotrexate, fluorouracil, hydroxyurea and mercaptopurine.

c) They stop mitosis or the actual splitting of the original cells into cell into two new cells. Stopping mitosis stops cell division (replication) of the cancer cells and may ultimately halt the progression of the cancer.

In the present investigation we have selected inhibition of synthesis of new DNA as the potential hypothetical mechanism for the observed cytotoxicity potential of bioactive dihydropyrimidone TVD4 (Table 10 and Figure 1). Hence docking simulation has been performed to determine possible protein-ligand interactions within the active binding site region of the 4KD7 (selected anticancer antimetabolite target protein DHFR). In the recent past, the interactions between the selective synthetic DHFR ligands revealed the importance of hydrogen bonding with the amino acids Asn 64, Phe 31 and Phe34 are important for increased affinity to human DHFR (Lambet al., 2013). The compound TVD revealed one hydrogen bond interactions with the amino acids Ala 9; compound TVD1 revealed one hydrogen bond interactions with the amino acids Ser 59; compound TVD2 revealed one hydrogen bond interactions with the amino acids Ser 59; compound TVD3 did not form any hydrogen bond interactions; compound TVD4 revealed one hydrogen bond interactions with the amino acids Tyr 121. Since, the bioactive dihydropyrimidone TVD, TVD1-4 did not displayed the interactions with amino acids which earlier reported to be most vital towards inhibition of DHFR. Therefore, we hypothesized that the cytotoxicity potential of the TDV4 might not be due to DHFR inhibition; further target based screening has to be performed to understand the inherent mechanism of action of the series of compounds synthesized in the present study.

CONCLUSION AND IMPLICATION:

In conclusion, we could synthesis and characterize some novel substituted dihydropyrimidones **TVD, TVD1-TVD4**. These compounds were screened for

in-vitro cytotoxicity study by using Brine Shrimp (*Artemia salina*) lethality bioassay and the results revealed the positive and significant contribution of **TVD4** consisting of 4-bromo substitution at position 4 of ring B of chalcone moiety which is covalently attached to the dihydropyrimidone nucleus towards the observed cytotoxicity (*Artemia salina lethality*) at ED_{50} value **3.11 ± 0.15** µg/mL. Likewise, the compound **TVD2** has also been exhibited significant activity comparatively with **TVD4** at ED_{50} value **6.03 ± 0.12** µg/mL. The observed remarkable activity of **TVD4** and **TVD2** may be due to the pharmacophores such as dihydropyrimidone moiety, α,β-unsaturation moiety, halogen substituents which forming part of basic skeleton of both the molecules. Subsequently, molecular docking studies revealed that the possible underlying mechanism might not be due to the inhibition of DHFR. Further studies have to be processed to understand complete mechanism of cytotoxicity of **TVD4**.

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