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Biological and computational evaluation of some new fully unsaturated 2-substituted-4,6-dichloro symmetric-triazine based chalcone hybrids as potential cytotoxic agents

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ABSTRACT

It has been reported in the literature review that the multifarious biological and pharmacological significance of compounds possess 1,3,5-triazine and chalcone pharmacophores in its basic scaffold as potential cytotoxic agents. In view of its therapeutic potential, 1,3,5-triazine-chalcone hybrid molecules (TCH1-35) were schemed and synthesize , in the present study were screened for their In vitro cytotoxicity potential. Cell proliferation was determined by the MTT assay method. MTT which is a tetrazolium salt is converted into insoluble formazan by mitochondrial dehydrogenases in live cells. Formazan is dissolved in DMSO, and absorbance was measured at dual wavelength of 550 and 630 nm on a Varioskan TM Flash Multimode Reader. A part from this cytotoxic assay to find the best fit lead compounds from the synthesized triazine-chalcone hybrid derivatives, receptor-ligand docking studies were also performed using Schrodinger GLIDE commercial software (Maestro 9.5) version with extra-precision mode and Grids were generated using Glide version 9.5 following the standard procedure recommended by Schrodinger. Glide tools were used to calculate dock score and evaluate conformers. The best fit molecules were identified from the results and were accomplished with good binding efficiency against five selected anticancer targets such as phosphatidyl inositol-4,5-bisphosphate 3 kinase (PI3 K gamma), vascular endothelial growth factor (VEGF), cyclin-dependent kinase-2 (CDK-2), murine sarcoma viral oncogene homologue b1 (BRAF), focal adhesion kinase (FAK).

Keywords: *invitro* Cytotoxicity, MTT assay 1,3,5-triazine-chalcone pharmacophores, Anticancer targets, Schrodinger GLIDE commercial software (Maestro 9.5) version.

INTRODUCTION

Cancer is a class of diseases that cause cells in the body to change and grow out of control [1]. Cancer cell growth is different from normal cell growth. Instead of dying, cancer cells keep on growing and form new cancer cells. These cancer cells can grow into (invade) other tissues, something that normal cells cannot do. In most cases the cancer cells form a tumor. But some cancers, like leukemia, rarely form tumors. Instead, these cancer cells are in the blood and bone marrow [2]. Cancer cell escape from many of the normal homeostatic mechanism that control proliferation. They invade surrounding tissues, gets into the body's circulating system and set up areas of proliferation away from the site of their original appearance [3].

Cancer is the second most important disease leading to death in both the developing and developed countries nowadays. The global burden of cancer continues to increase largely because of the aging and growth of the world population alongside an increasing adoption of cancer-causing behaviors, particularly smoking, in economically developing countries [4]. A substantial proportion of the worldwide burden of cancer could be prevented through the application of existing cancer control knowledge and by implementing programs for tobacco control, vaccination (for liver and cervical cancers), and early detection and treatment, as well as public health campaigns promoting physical activity and a healthier dietary intake. Clinicians, public health professionals, and policy makers can play an active role in accelerating the application of such interventions globally [5].

***Invitro* Cytotoxicity studies**

The invitro cytotoxicity can be evaluated by the simple and economic MTT assay. This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by Mitochondrial succinate dehydrogenase. The MTT enters the cells and passes in to the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with DMSO and the released the solubilized formazan reagent is measured Spectrophotometrically at 570 nm. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of viability of the cells. When the amount of dark purple formazan produced by the cells is treated with a reagent compared with the amount of formazan produced by untreated controlled cells, the effectiveness of the agent in causing death of cells can be deduced through the production of a dose-response curve [6-10].

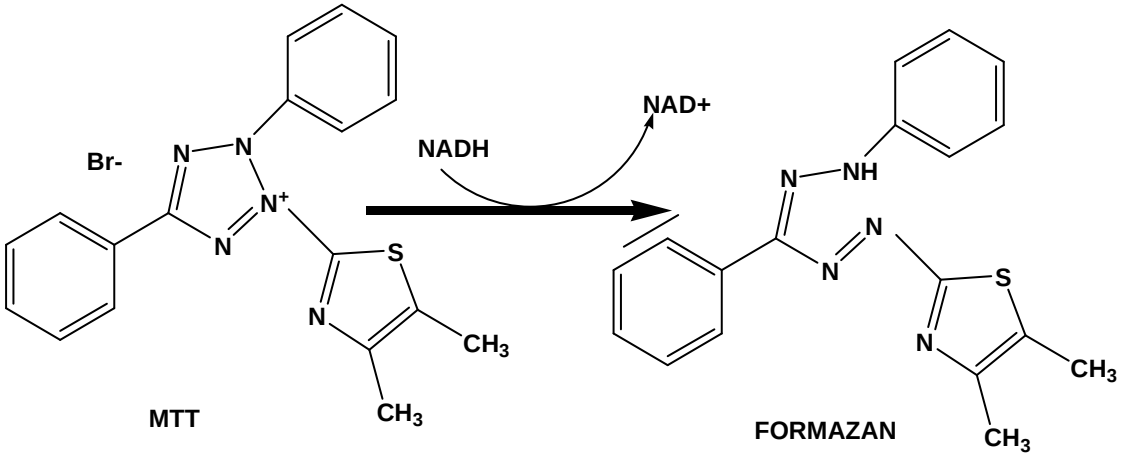


Fig:1 representing the conversion of MTT to Formazan

Experimental section

Cell lines and Reagents

HT-29(Colon cancer),MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines were obtained from national centre for cell science (NCCS) Pune, India. DMEM (Dulbeccos Modified Eagles Medium),MEM (Minimum Essential Media Eagle),MTT [3-(4,5-dimethyl tiazole-2-yl)-2,5-diphenyl tetrazolium bromide], trypsin, EDTA were purchased from sigma chemicals (St.Louis, MO). Fetal bovine serum (FBS) was purchased from arrow labs, 96 well flat bottom tissue culture plates were purchased from Tarson.

Method

A) Maintenance of cell lines

HT-29 and DU-145 cell lines were grown as adherent in DMEM media, whereas MCF-7 was grown in MEM media supplemented with 10% fetal bovine serum. The cultured was maintained in a humidified atmosphere with 5% CO_2.

B) Preparation of samples for Cytotoxicity

Stock solutions of test compounds (TCH 1-35) were prepared (100µg/mL) in DMSO and from them various dilutions were made with sterile water to get the final drug concentrations of 50, 25, 12.5, 6.25, 3.125 µg/mL.

C) Cytotoxicity evaluation

The cells were seeded in 96 well pates at a density of $1x10^4$ (Counted by Tryphan Blue exclusion dye method) per well and were incubated for 24hrs to recover. After incubation the medium was replaced with fresh media

containing different dilutions of the test compounds. Then the plated were incubated for additional 48hrs at 37^0c in DMEM/MEM with 10% FBS medium. Following incubation, the medium was removed and replaced with 90µL of fresh DMEM without FBS. To the above wells,10 µL of MTT reagent (5mg/mL of stock solution in DMEM without FBS) was added and incubated at 37^0C for 3-4hrs,there after the above media was replaced by adding 200 µL of DMSO to each well (to dissolve the blue formazan crystals) and incubated at 37^0C for 10 minutes. The absorbance at 570 nm was measured on a spectrophotometer [11].

Methotrexate was used as a reference drug for comparison. Assay was performed in triplicate for three independent determinations. The cytotoxicity was expressed as IC50(µg/mL) which is the concentration of the compound that inhibited proliferation rate of the tumor cells by 50% as compared to the control untreated cells.IC50 values were determined from the plot: % inhibition versus concentration. All in vitro experiments for cell proliferation/inhibition were performed in triplicates.

$$\% \text{ inhibition at the given concentration} = 1 - \frac{(\text{Mean optical density of Test}) \times 100}{(\text{Mean optical density of control})}$$

IC50=Inv.log (50-c)/m; c and m derived from Y= mx+c , plot of % inhibition Vs log C. The results of the compounds are shown in tables 1 and 2.

***In silico* Molecular docking**

Ligand–protein inverse induced fit docking (LPIIFD) approach has been used as a useful tool in facilitating drug design. In this approach, docking single or multiple small molecules in single or multiple conformations to a receptor site is attempted to find putative ligands. A number of flexible docking algorithms have been introduced. These include multiple-conformer shape matching, genetic algorithm, evolutionary programming, simulated annealing, fragment-based docking, and other novel algorithms [12]. Testing results have shown that these algorithms are capable of finding ligands and binding conformations at a receptor site close to experimentally determined structures. [13-16]. The LPIIFD approach is now applied to the database of compounds synthesized in the present study for finding 'best fit' (hit identification) against selected anticancer protein drug targets. The compound with least binding energy against each individual target protein is considered scope for further study. By these means, it is possible to understand how the compounds interact with the target protein. The results emerging out of these studies can be used to identify new active ligands by using the knowledge obtained from the *in silico* established secondary protein targets [17-21].

In the present research, considerable attention has been focused on identifying some new 1,3,5-triaizine-chalcone hybrids capable of showing significant cytotoxicity [22-28]. The docking simulation was performed using five different anticancer targets involved with cell cycle, cell growth, and DNA replication.

In an attempt to understand the binding mode of our Triazine-chalcone analogues, the ligands (35 compounds) were docked with the crystal structure of the five targets Phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3KS gamma, PDB ID:4FHK), Vascular endothelial growth factor (VEGF,PDB ID:1Y6A), Cyclin-dependent kinase-2 (CDK-2 ,PDB ID: 4BGH), Murine sarcoma viral oncogene homologue b1 (BRAF, PDB ID: 5CSX), Focal adhesion kinase (FAK, PDB ID: 4Q9S) Receptor ligand-binding domain in complex with various substrates, the information was obtained from the Protein Data Bank(www.rcsb.org)

EXPERIMENTAL SECTION

Schrodinger Maestro (9.5) GLIDE commercial version is used as graphical user interface.[29] Protein is prepared using protein preparation wizard and following functions are performed:

a) Automatically imported full PDB files — or any chain within a PDB file — from local databases or the PDB website.
b) Automatically missing hydrogen atoms are added.
c) Metal ionization states are corrected to ensure proper formal charge and force field treatment
d) Bond orders are enumerated to HET groups
e) Co-crystallized water molecules are removed at the user's discretion.
f) Residues with missing atoms or multiple occupancies are highlighted.

g) Quickly and easily determine the most likely ligand protonation state as well as the energy penalties associated with alternate protonation states
h) Optimal protonation states for histidine residues are determined.
i) Potentially transposed heavy atoms in arginine, glutamine, and histidine side chains are corrected.
j) The protein's hydrogen bond network is optimized by means of a systematic, cluster-based approach, which greatly decreases preparation times.
k) A restrained minimization is performed that allows hydrogen atoms to be freely minimized, while allowing for sufficient heavy-atom movement to relax strained bonds, angles, and clashes.

RESULTS

Table: 1 Cytotoxicity Assessment (MTT-dye Reduction Assay) of newly synthesized 1,3,5 Triazine-chalcone hybrids (TCH1-35) IC50 values expressed in µg/mL, for compounds TCH-24,20,10,11,12,9,5,6 and for the standard methotrexate

Compound code	R substituent	**IC50 Values of Cell Lines**		
		HT-29	MCF-7	DU-145
TCH-24	3-FC_6H_4	42±1.986	49±1.356	28±1.654
TCH-20	3-ClC_6H_4	53±2.258	58±2.009	41±2.840
TCH-10	3,5-diOHC_6H_3	38±2.314	46±2.176	24±1.269
TCH-11	4,5-diOHC_6H_3	56±2.986	63±2.986	59±2.0321
TCH-12	2-Me,5-OHC_6H_3	64±2.647	76±1.765	61±2.384
TCH-5	2-$OMeC_6H_4$	81±2.658	89±1.546	74±2.025
TCH-6	3-$OMeC_6H_4$	66±2.458	69±2.582	51±1.036
TCH-9	4-OHC_6H_4	73±2.569	79±2.196	62±1.244
Methotrexate		11±1.463	9±1.002	6±1.005

*Values are expressed as mean ±SEM, *P < 0.05.All the compounds and the standard dissolved in DMSO, diluted with culture medium containing 0.1% DMSO. The control cells were treated with culture medium containing 0.1% DMSO.*

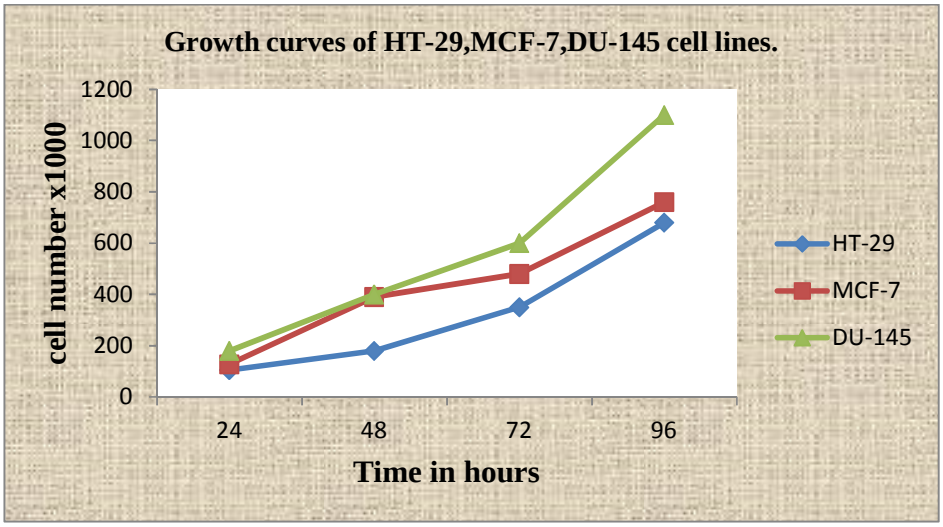


Fig: 2 shows the growth curve of each of the Colon, Breast and prostate cancer cell lines in normal DMEM culture media. The three cell lines maintained exponential growth characteristics until the end of experiments (96 hours)

Cytotoxic MTT assay experiment showing the determination of half maximum inhibitory concentration of synthesized hybrid conjugates treated on HT-29, MCF-7 and DU-145 cells.

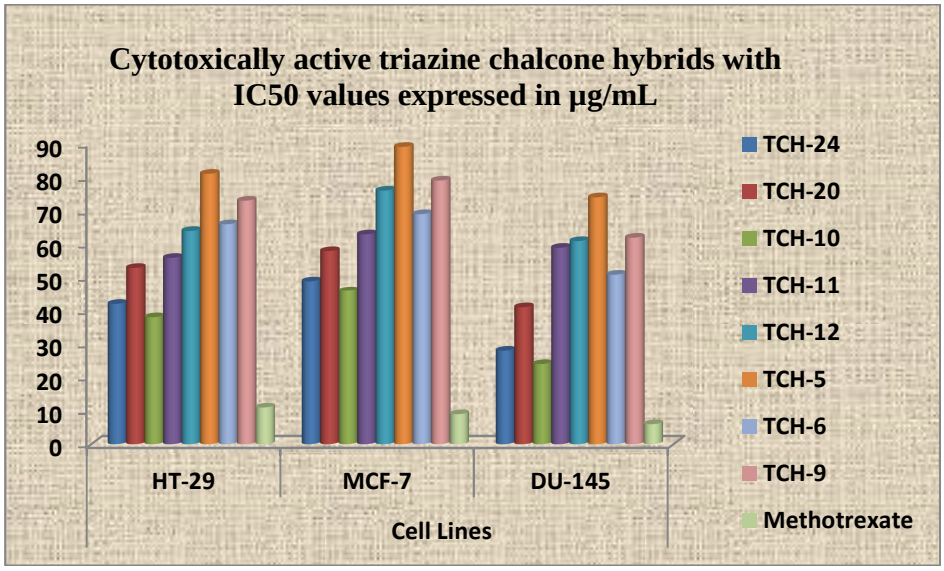


Fig: 3 Indicates cytotoxically potential Triazine chalcone hybrids with excellent IC50 values expressed in µg/mL.
% cell proliferation rate inhibitory activity at half maximun inhibitory concentration (IC50 value) of all the cytotoxically active 6 Triazine chalcone hybrid compounds treated on HT-29, MCF-7 and DU-145 cells in comparision with the standard Methotrexate treated at various concentrations of 50, 25, 12.5, 6.25, 3.125 µg/mL

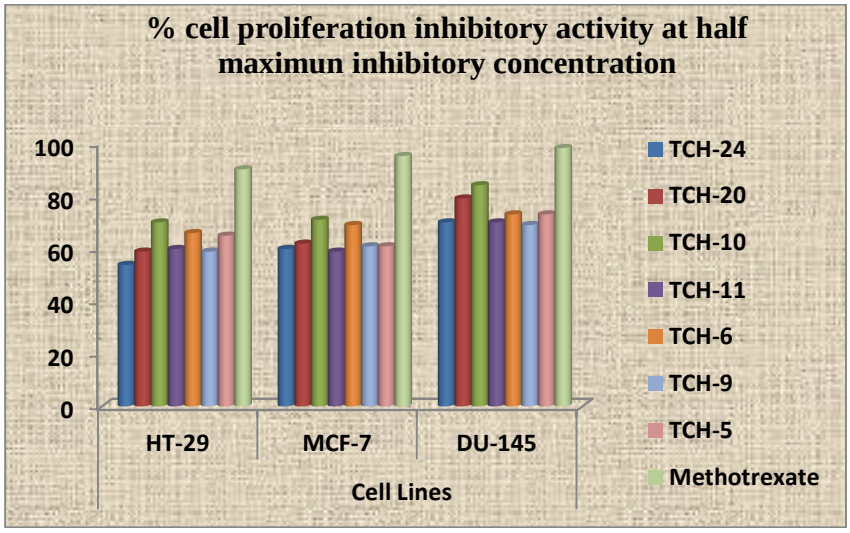
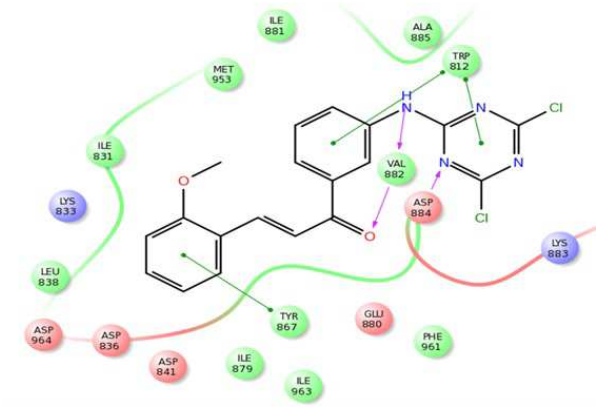


Fig: 4 show the excellent proliferation inhibition of cells at IC50 concentration, calculated and plotted against the Methotrexate as reference standard

Molecular Docking results of 1,3,5-Triazine-chalcone hybrid molecules (TCH 1-35) against selected anticancer drug target PI3KS- Gamma (PDB ID:4FHK) (VEGF,PDB ID:1Y6A), (CDK-2 ,PDB ID: 4BGH), (BRAF, PDB ID: 5CSX), (FAK, PDB ID: 4Q9S).

Fig:5 2D diagram interactions of cytotoxically active antagonists (TCH-5,6, 9, 10, 11, 12, 20, 24,) color figures online are indicative of H-bond interactions of synthesized molecules Docked with anticancer Targets



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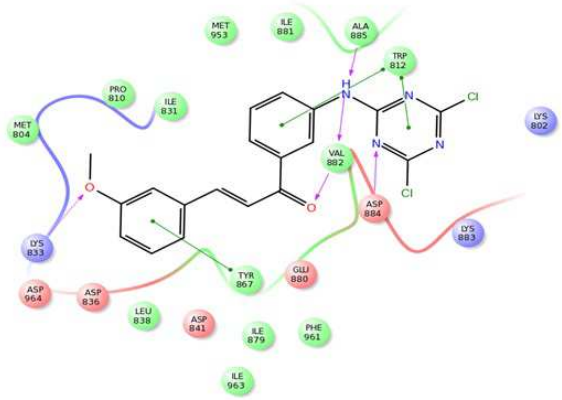

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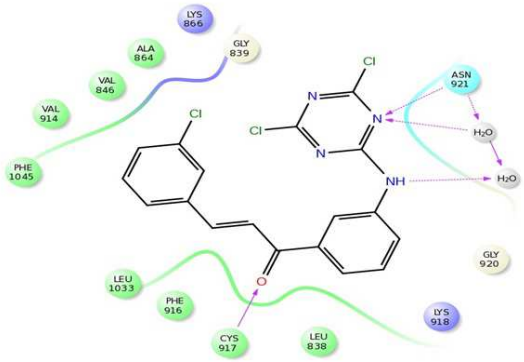

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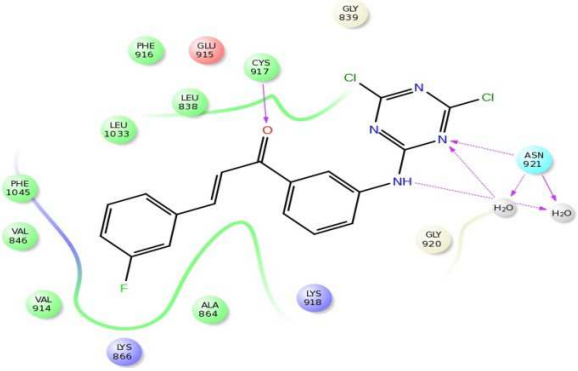

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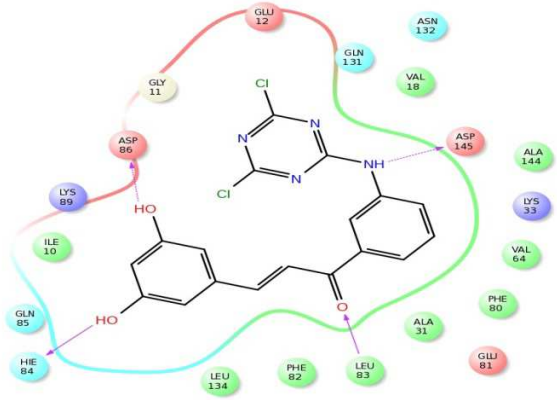

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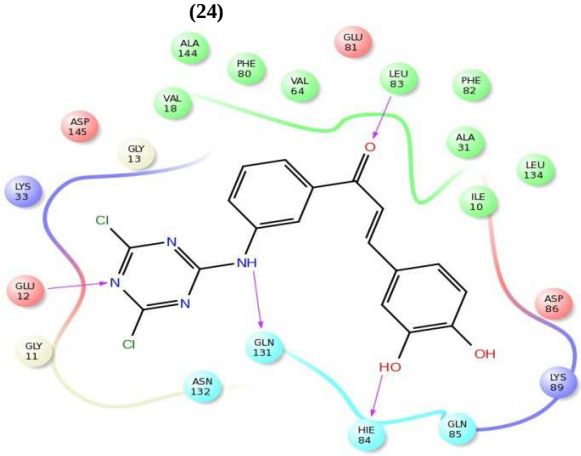

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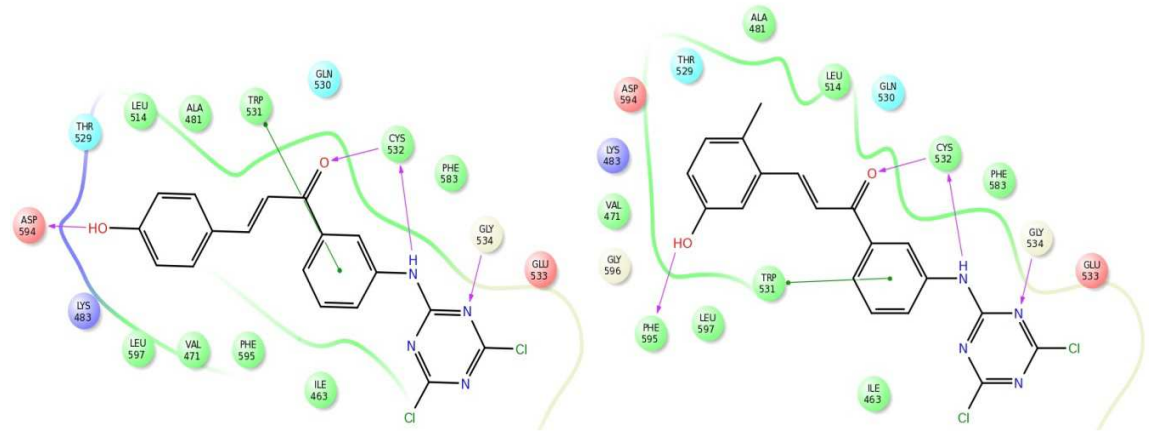
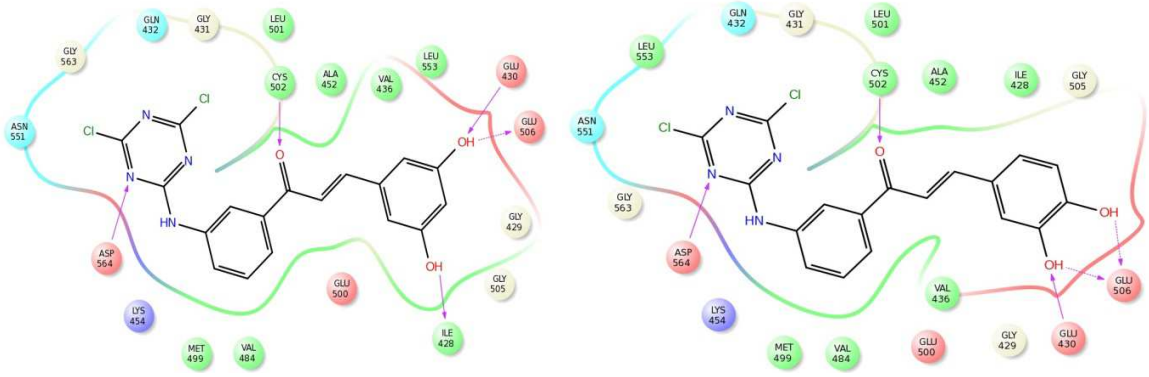


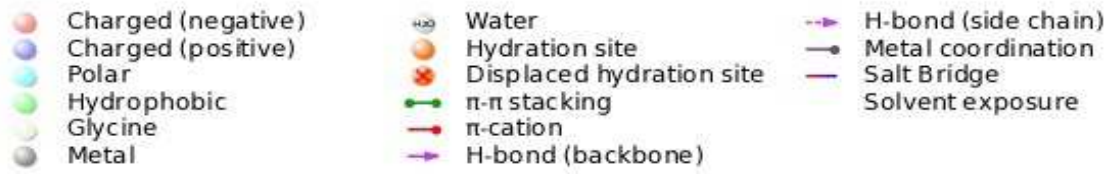
(9) **(12)**



(10) **(11)**

Table: 2 Summarized molecular docking results of 1,3,5-triazine-chacone hybrid molecules (TCH1-35) with their Schrodinger Maestro (9.5) GLIDE / Dock Scores (kcal/mol) and H-bond

Target code	PDB ID	Best Fit Ligand	R Substituent group	Glide score	Glide energy	No.of H Bonds	H-Bond interacting Residues
PI3K γ	4FHK	TCH-20 TCH-6	3-OMe-Phenyl 3-Cl- Phenyl	-11.781 -11.693	-42.141 -40.549	3 5	Val (882) Asp(884) Lys(833) Ala(885) Val(882) Asp(884)
VEGFR	1Y6A	TCH-24 TCH-20	3-F-Phenyl 3-Cl- Phenyl	-8.998 -8.774	-39.318 -40.037	2 2	Cys(917) Asn(921) Cys(917) Asn(921)
CDK2	4BGH	TCH-10 TCH-11	3,5diOH Phenyl 4,5diOH-Phenyl	-10.206 -9.776	-61.168 -55.718	4 4	Asp(86) Hie(84) Asp(145) Leu(83) Glu(12) Gln(131) Hie(84) Leu(83)
BRAF	5CSX	TCH-9 TCH-12	4-OH-Phenyl 2Me,5OHPhenyl	-12.808 -12.304	-44.815 -40.597	4 4	Asp(594) Cyc(532) Gly(534) Phe(595) Cys(532) Gly(534)
FAK	4Q9S	TCH-11 TCH-10	4,5di OHPhenyl 3,5diOH-Phenyl	-8.832 -8.752	-52.373 -47.299	5 5	Asp(564) Cys(502) Glu(430)Glu(506)Ile(428) Asp(564) Cys(502) Glu(430)Glu(506)



interactions against selected five anticancer drug targets.

Statistical analysis

The data were statistically analyzed and all the values are expressed as mean± standard error of mean, the graphs were plotted by using Microsoft excel software. The experimental data obtained was plotted against concentration Vs % inhibition, half maximum reduction in number of metabolically active cells to determine IC50 value.

Discussion

The results of *invitro* Cytotoxic (MTT) assay of the synthesized 1,3,5 triazine-chalcone hybrid molecules is illustrated in Table: 1.The compounds (TCH1-35) have been evaluated for their cytotoxicity against HT-29(Colon cancer), MCF-7(breast cancer) and DU-145 (prostate cancer) cell lines. Methotrexate was used as the reference standard.

The results clearly revealed that most of the synthesized compounds possessed Cytotoxic activity as evidenced by IC50 values. Of all the compounds tested against HT-29 cells lines, The compound TCH-24 having 3fluoro-phenyl moiety in its structure showed appreciable activity with IC50 value of 42µg/mL. This is followed by compounds TCH-20 having 3 chloro-phenyl moiety (IC50 value 53 µg/mL),TCH-12 having 2methyl,5 hydroxy phenyl moiety(IC50 value 64 µg/mL),TCH-11 having 4,5dihydroxy phenyl moiety(IC50 value 56 µg/mL),TCH-10 having 3,5dihydroxy phenyl moiety (IC50 value 38 µg/mL),TCH-9 4-hydroxy phenyl (IC50 value 73 µg/mL),TCH-6 3methoxy phenyl (IC50 value 66 µg/mL),TCH-5 2-methoxy phenyl (IC50 value 81 µg/mL).

Among the compounds tested for cytotoxicity on MCF-7 cell lines again the compound TCH-24 having 3fluoro-phenyl moiety in its structure showed appreciable activity with IC50 value of 49µg/mL. This is followed by compounds TCH-20 having 3 chloro-phenyl moiety (IC50 value 58 µg/mL),TCH-12 having 2methyl,5 hydroxy phenyl moiety(IC50 value 76 µg/mL),TCH-11 having 4,5dihydroxy phenyl moiety(IC50 value 63 µg/mL),TCH-10 having 3,5dihydroxy phenyl moiety (IC50 value 46 µg/mL),TCH-9 4-hydroxy phenyl (IC50 value 79 µg/mL),TCH-6 3methoxy phenyl (IC50 value 69 µg/mL),TCH-5 2-methoxy phenyl (IC50 value 89 µg/mL).

The compounds tested for cytotoxicity on DU-145 cell lines, It is interesting to note that the compound again with 3fluoro-phenyl moiety in its structure showed excellent activity (IC50 value 28µg/mL) and this is much more potent on these cell lines than the other two cell lines tested. This is followed by compounds TCH-20 having 3 chloro-phenyl moiety (IC50 value 41 µg/mL),TCH-12 having 2methyl,5 hydroxy phenyl moiety(IC50 value 61 µg/mL),TCH-11 having 4,5dihydroxy phenyl moiety(IC50 value 59µg/mL),TCH-10 having 3,5dihydroxy phenyl moiety (IC50 value 24 µg/mL),TCH-9 4-hydroxy phenyl (IC50 value 62µg/mL),TCH-6 3methoxy phenyl (IC50 value 51 µg/mL),TCH-5 2-methoxy phenyl (IC50 value 74 µg/mL).

Compounds with different R substituent at different positions were found to exhibit satisfactory results when treated against HT-29,MCF-7,DU-145 cells respectively with TCH-7 (IC50 value 128,132,108 µg/mL).similarly TCH-15(IC50 value 148, 167,130 µg/mL),TCH-18 (IC50 value 123,128,122 µg/mL),TCH-21(IC50 value 118,116, 109 µg/mL) TCH-25 (IC50 value 108,118,102 µg/mL) and the rest of the compounds showed least Cytotoxic activity at higher IC50 values towards all the three types of cell lines.

The molecular docking results of a series of synthesized hybrids with highest glide score and least binding energy scored molecules were selected for target study.TCH-20,6 accomplished the best binding efficiency against target PI3K gamma with Glide score -11.781,-11.693 Kcal/mol and 3,5 hydrogen bonding residues respectively. Similarly TCH-24,20 against VEGFR with Glide score -8.998, -8.774 Kcal/mol and 2,2 hydrogen bonds respectively. Correspondingly compounds TCH-10,11 showed good binding efficiency against target CDK2 with -10.206, -9.776

Kcal/mol and 4,4 hydrogen bonds respectively. Compounds 9,12 with -12.808 ,-12.304 Kcal/mol and formed 4 hydrogen bonds each with BRAF. Compounds 11,10 with -8.832,-8.752 each formed 5 hydrogen bonds with target FAK.

CONCLUSION

The structure-activity relationship study based on the above results clearly indicated the significance of aryl and heteroaryl moieties as a part of the 1,3,5 Triazine chalcone hybrids scaffold and also presence of different electron withdrawing substituents in enhancing the cytotoxicity. When more than one electron withdrawing substituents are present on the aryl and heteroaryl ring, a cumulative effect may be observed. Compounds with more number of electron releasing or electron withdrawing substituents on the aromatic or hetero aromatic ring at different positions can be synthesized to draw meaningful conclusions with respect to the influence of electronic effects on the cytotoxic activity. Molecular docking results of the synthesized triazine-chalcone hybrids were in good keeping with the MTT assay results, that clearly indicating the Cytotoxic activity of these hybrids molecules.

Extensive research is required on 1,3,5-triazine-chalcone hybrid molecules to find novel analogs suitable for clinical applications.

Conflict of interest
The authors confirm that there are no potential conflicts of interest.

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