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Investigating novel leads as anti-cancer agents against carcinoma of cervix by MTT assay

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Abstract: Cyclin Dependent Kinases (CDKs) are known to act as potential therapeutic targets in cancer. In the present research, a novel screening approach has been utilized to screen nearly 1400 drugs available from DrugBank database. After validating the docking protocol, various molecular docking softwares such as Molegro Virtual Docker (MVD), GOLD and AutoDock were utilized to test the underlying efficiency of these programs towards predicting the binding affinities of studied compounds. A consensus scoring and ranking retrieved top 3 drugs viz. Olmesartan, Telmisartan and Candesartan which were examined for anti-proliferative effects against carcinoma of cervix cell line using MTT assay.

Keywords: Cyclin Dependent Kinase, Docking, Cancer, Hela cells, MTT assay

Introduction

Eukaryotic cell cycle progresses by transitions from one stage of the cell cycle to the other and are driven by phosphorylation events mediated by a family of serine/threonine protein kinases termed CDKs (Cyclin Dependent Kinases). Molecules involved in the basic machinery of the cell cycle such as cyclins, cyclin-dependent kinases; and other kinases and phosphates that regulate CDKs were isolated and extensively characterized [1].

CDK4 is a catalytic subunit of the protein kinase complex which is important for cell cycle G1 phase progression [2]. The activity of this kinase is restricted to the G1-S phase, which is controlled by the regulatory D-type cyclin subunits and CDK inhibitor p16 (INK4a). This kinase was found to be responsible for the phosphorylation of retinoblastoma gene product [3].

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It has been hypothesized that the dysregulation of the cell-cycle machinery may contribute to the development of cancer [4]. Hence, inhibitors of cyclin-dependent kinases are anticipated to possess therapeutic utility against a wide variety of proliferative diseases, especially cancer [5].

In the present study, computational approach followed by experimental analysis of few drugs was carried out to test the inhibitory activity against carcinoma of cervix cells. In order to advance the use of computational techniques in studying binding affinities of ligands with receptors, Molegro Virtual Docker (MVD), GOLD and AutoDock softwares are used to screen nearly 1400 drugs available from DrugBank database. Followed by the consensus scoring and ranking of hits, top three drugs are selected to perform cytotoxic activities by MTT assay [6] against HeLa cell lines.

Materials and Methods

Receptor X-ray structure

The X-ray crystal structure of the CDK4 enzyme, 2W9F in complex with 4-[(4-Imidazo[1,2-A]pyridin-3-ylpyrimidin- 2-yl)amino]benzene sulfonamide inhibitor was recovered from Protein Data Bank (PDB: http://www.rcsb.org/pdb) and selected as the receptor model in virtual screening program. We used the DrugBank database (www.drugbank.ca) and the docking software MVD [7] for virtual ligand docking.

Consensus Scoring

Before docking, the 2D structures of 1400 drugs were converted to 3-dimensional formats by using corina make 3D option of Tsar software (www.accelrys.com). The structures were energy minimized using cosmic optimize module and are stored as mol2 formats.

As docking and scoring play important roles in drug design, it has been reported that consensus scoring was generally more effective than single scoring scheme and represented an effective way in improving hit rates in various virtual database screening studies [8-9]. Therefore, in this study, we tested three different scoring functions such as MolDock score of Molegro software, GOLD score score [10] and free energy based scoring scheme of AutoDock [11]. Initially all the drugs were docked using MVD and the resultant best poses of top hits were re-scored using GOLD and AutoDock to generate classes. During ranking, signs of some scoring functions are changed to make certain that a lower score always indicates higher affinity.

Cancer Cell Lines

Carcinoma of cervix (HeLa) cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 4.5 g/L glucose, 2 mM L-glutamine and 5% fetal bovine serum (FBS) (growth medium) at 37°C in 5% CO2 incubator.

In vitro cytotoxicity studies

In vitro cytotoxicity studies were done by MTT assay on top three hits from our screening study. MTT assay relies on the ability of live cells to reduce a water-soluble yellow dye MTT (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a water-insoluble purple formazan product. The MTT assay developed by Mosmann was modified and used to determine the inhibitory effects of test compounds on growth of HeLa cells *in vitro*. Briefly, the trypsinized cells from T-25 flask were seeded in 96-well flat-bottomed tissue culture plate at a density of $5x10^3$ cells/well in growth medium (DMEM supplemented with 10% Fetal calf serum) and cultured at 37°C in 5% CO_2 to adhere. After 48 hrs of incubation, the cells were pretreated with growth medium and mixed with different concentrations of test compounds (2, 4, 8, 16, 32 and 64 µg/ml) in triplicates to achieve a final volume of 100 µl and then cultured for 48 hr. All compounds were prepared as 3mg/ml concentration stock solutions in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the culture was within 0.2%. Culture medium and solvent are used as controls. Each well then received 2 µl of fresh MTT (0.5mg/ml in PBS) followed by incubation for 2hr at 37°C. The growth medium was removed from the wells and replaced with 100 µl of DMSO to solubilise the coloured formazan product. After 30 min incubation, the absorbance of the culture plate was read at a wavelength of 570 nm on an ELISA plate reader, Anthos 2020 spectrophotometer. The mean OD values of each test compound were corrected by subtracting with the mean OD of blanks. Relative percent inhibition activity is expressed as:

% inhibition = 100 – (corrected mean OD of sample x 100 / corrected mean OD of control)

Table 1: Mol dock scores of 2W9F bound co-crystallized ligand

PDB ID	MolDock Score(kcal/mol)			Average MolDock Score (kcal/mol)	Average RMSD Value(A°)
	Run-1	Run-2	Run-3		
2W9F	-145.50	-147.78	-146.30	-146.53	0.678

Table 2: Docking results and rank-sum data of top 14 drugs Vs 2W9F

Drug Name	Binding energies (kcal/mol)			Consensus scoring rank			Rank sum
	MVD	GOLD	Auto Dock	MVD	GOLD	Auto Dock	
Pentagastrin	-158.351	44.53	-5.9	1	2	1	4
Olmesartan	-208.296	60.26	-8.5	3	3	3	9
Teniposide	-174.500	33.31	-7.3	1	2	2	5
Verteporfin	-200.020	13.14	-6.4	3	1	1	5
Montelukast	-175.412	54.34	-7.9	2	3	2	7
candoxatril	-165.536	25.19	-7.8	1	1	2	4
Pemetrexed	-162.825	74.72	-9.0	1	3	3	7
Losartan	-168.644	59.11	-7.4	1	3	2	6
Eprosartan	-170.916	53.51	-7.6	1	2	2	5
Candesartan	-200.317	66.54	-7.3	3	3	2	8
Tiagabine	-169.122	57.11	-7.2	1	3	2	6
Repoglinide	-167.432	11.71	-6.4	1	1	1	3
Telmisartan	-176.844	56.89	-8.9	2	3	3	8
Atorvastatin	-172.108	42.48	-6.6	2	2	1	5

Discussion

From the docking analysis, it has been identified that nearly 14 drugs are found to have binding affinities more than the co-crystallized ligand and hence consensus scoring was applied and a cross-docking routine was carried out with GOLD and AutoDock softwares (Table 2). Ranking was done individually by clustering scores into equally split three classes using Tsar software, of which compounds in class3 represents the highest class or top rank. Classes were generated for all scoring functions and instead of taking an average, rank-sum technique [9] was employed to retrieve best compounds (Table 2). The ranks obtained from each of the scoring functions were added to give the rank-sum. The advantage of a sum over average was that the contribution from the rank for each individual score can more easily be split out for illustrative purposes in the former instance.

Finally, cell based cytotoxic activity of top three drugs against carcinoma of cervix (HeLa) by MTT assay revealed growth inhibitory characteristic of these drugs *in vitro*. Cancer cells were exposed to the selected compounds (Olmesartan, Telmisartan and Candesartan) for 48 h and it was found that cell viability gradually decreased in a dose-dependent manner and the results reported in Table 3 suggest that the proliferation of HeLa cells could be significantly inhibited by candesartan in a concentration dependent manner. The maximum percent inhibition was found to be 52.8% at a tested dose of 64 µg/ml. The morphology of cells after treatment with drugs appeared significantly different than untreated cells, which could probably due to the growth inhibitory and cell death initiating ability of the studied compounds (Figure 1).

Table 3: Inhibitory effects of drugs on the growth of HeLa cells cultured *in vitro*.

S.No	Conc. (µg/ml)	% Inhibition		
		Olmesartan	Telmisartan	Candesartan
1.	0.5	21.9	20.5	30.1
2.	1	26.9	25.3	33.4
3.	2	30.1	28.7	35.8
4.	4	35.6	30.5	38.1
5.	8	38.2	35.8	40.4
6.	16	41.5	38.9	41.5
7.	32	45.1	43.6	42.7
8.	64	48.3	47.3	52.8

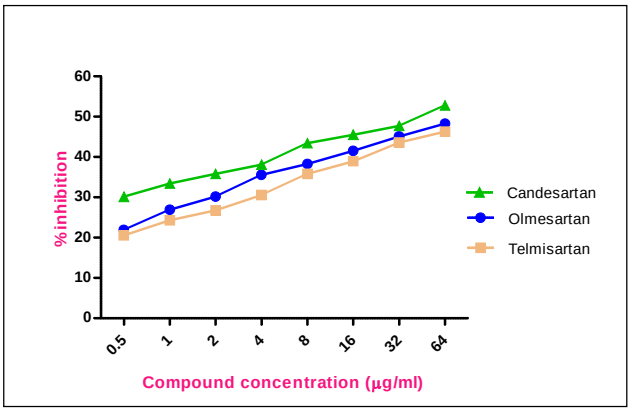

Figure1: Dose-response curves of the inhibitory effects of Olmesartan, Telmisartan and Candesartan on the growth of HeLa cells

Conclusion

Screening studies of 1400 drugs obtained from drug bank database are docked against 2W9F using Molegro Virtual Docker software. Screening procedure followed by cross-docking with GOLD and AutoDock resulted in 14 drugs and the top three drugs viz., Olmesartan, Telmisartan and Candesartan were investigated for their ability to inhibit HeLa cell proliferation using MTT assay. Candesartan was found to be most effective in reducing the growth of HeLa cell lines. Hence, this study employing molecular docking analysis along with experimental observations reveals the importance of various drugs specific to a disease on one hand and may also act as possible anti cancer agents, on the other.

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