JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue
JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)
An International Scholarly Open Access, Peer-reviewed, Refereed Journal

In silico study of BIBR1532 and other TERT inhibitors

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INTRODUCTION

Telomerase is an excellent biomarker for cancer detection as compared to normal cells almost all of the human cancers cells express high levels of telomerase to add to the proliferative effect of these cells. Telomerase activity can be identified by its catalytic protein domain telomerase reverse transcriptase. These are part of an important subgroup of RNA-dependent polymerases. The main function of this molecule is to lengthen telomeres in DNA strands, thereby allowing senescent cells that would otherwise become postmitotic and undergo apoptosis to exceed their lifespan become potentially immortal, as is often the case with cancerous cells. At a molecular level, telomerase is responsible for catalyzing the addition of nucleotides in a TTAGGG sequence to the ends of a chromosome's telomeres, thereby preventing degradation of the chromosomal ends following multiple rounds of replication. There are various regulatory mechanisms that ensure that TERT target shorter telomeres rather than longer ones and in this way do not contribute to aberrant telomere elongation that may lead to Cancer. Having said that, TERT play an important role in cancer cell's rapid proliferation because it directly impacts how many times a cell can divide and thus is capable of inducing immortality in cells.

OBJECTIVES OF RESEARCH

A) To perform molecular docking studies of tcTERT protein to its substrate and known inhibitors and understand the receptor ligand interactions.

B) To investigate whether the BIBR1532 telomerase inhibitor studied in tcTERT system

can be docked in hTERT as well.

C) To discuss previous studies conducted with a focus on telomerase inhibition to treat cancer and the future scope of experimentation in the same field.

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Structure of TERT

One of the most important components of telomerases is the catalytic subunit TERT. The TERT is further composed of 4 main structural domains including -

1) The N-terminal (TEN) which contains the binding site for DNA, for repetitive addition of NTPs and also an RNA domain 1 (RID1) important for RT pseudoknot binding.

2) The telomerase RNA binding domain (TRBD) which contains an RNA domain 2 (RID2) which is important for enzymatic activity through it being bound to TRCR4/5.

3) Reverse transcriptase (RT) which is bound to the RID2 and is shown to be the catalytic core for nucleotide addition.

4) C-Terminal extension (CTE) contains residues that are important for repetitive addition of nucleotides. These are also present in RT.

The expression of TERT is a rate-limiting factor for telomerase activity. (Mitchell, M., Gillis, A., Futahashi, M., Fujiwara, H., & Skordalakes, E. (2010)

TERT's role in cancer and various methods of inhibition

Over two hundred combinations of hTERT polymorphisms and cancer development have been found. There have been strong correlations between the polymorphisms and development of cancer in various individuals. (Andrews, L. G., & Tollefsbol, T. O. (2007)

The regulation of hTERT has also been researched to determine possible mechanisms of telomerase activation in cancer cells : (Daniel, M., Peek, G. W., & Tollefsbol, T. O. (2012)

1) Glycogen synthase kinase 3 (GSK3) seems to be over-expressed in most cancer cells. GSK3 is involved in promoter activation through controlling a network of transcription factors.

2) Leptin is also involved in increasing mRNA expression of hTERT via signal transducer and activation of transcription 3 (STAT3), proposing a mechanism for increased cancer incidence in obese individuals. There are several other regulatory mechanisms that are altered or aberrant in cancer cells, including the Ras signaling pathway and other transcriptional regulators

3) Other important pathways that were studied in relation to TERT activity to determine and inhibit cancer cell proliferation includes the mTOR pathway. The mTOR pathway involves mTOR which is a kinase that is very important in regulating protein synthesis and it interacts with telomerase to increase its expression. Telomerase activity has also been found to be inhibited by phytochemicals such as isoprenoids, genistein, curcumin, etc. These chemicals play a role in inhibiting the mTOR pathway via down-regulation of phosphorylation.

4) N-substituted-dihydropyrazole derivative (13i) inhibits TERT in S180 and HepG2 mice cells.

5) Isothiazolone derivatives are biocides targeting up-regulated hTERT.

6) Gambogic acid is a natural product that inhibits hTERT promoter in BGC-28 human gastric carcinoma cells.

Other chemicals that are in development for clinical treatment to hopefully inhibit cancer include :

1) **Nucleoside Analogues** : Once they are phosphorylated, they work as antimetabolites by being similar enough to nucleotides to be incorporated into growing DNA strands; but they act as chain terminators and stop viral DNA polymerase. However, they have a couple of drawbacks such as resistance to these can be developed very quickly especially considering the high rate to mutation and proliferation in cancer cells. Also, these chemicals are not specific to viral DNA and affect mitochondrial DNA as well which can lead to side effects such as bone marrow suppression. Example - AZT is a thymidine analogue. AZT works by selectively inhibiting HIV's reverse transcriptase, the enzyme that the virus uses to make a DNA copy of its RNA.

2) **Retinoic Acid** : is a metabolite of vitamin A_1 that plays an important role in transcription

mechanisms. It mediates the functions of vitamin A_1 required for growth and development. Hence, if this mechanism is used to prevent the transcription of TERT it can reduce he uncontrolled rate at which cancer cells are defining. However, the issue here is to find a way to specifically target the mutated cells only and not the normally functioning cells as well because if it does then it would lead to premature apoptosis. (Xiao, X., Sidorov, I. A., Gee, J., Lempicki, R. A., & Dimitrov, D. S. (2005)

3) **Quinolone Antibiotics** : such as ofloxacin and levofloxacin have shown to inhibit telomerase activity in transitional cell carcinoma cell lines by some unknown mechanism. This was seen in an MTT assay where the cancer cell lines' absorbance values indicated decreased activity. (Yamakuchi, M., Nakata, M., Kawahara, K.,

Kitajima, I., & Maruyama, I. (1997)

After careful observation and repetitive experimentation it can be seen that many methods used to inhibit telomerase activity focused on two major components of telomerase namely, TERT and TR. Hence, the following sections focus on in silico investigation of drugs that inhibit TERT (the activation of which controls the activity of TR) by known and unknown mechanisms thus far.

Known inhibitors of TERT

Over the years, hTERT has become a major target for cancer therapy due to the simple fact that it is the catalytic subunit of the telomerase primarily responsible for prolonging the life of cells. Rather than a mutant form of hTERT being present in cancer patients it has been noted that the overexpression of hTERT leading to prolonged life cycle of cancer cells is the major cause. Numerous studies have suggested that mutations of different types such as point mutations, duplications etc. in the promoter region of hTERT is the cause of overexpression and hence cancer proliferation due to telomerase. Keeping this in mind drug development and discovery against telomerase has taken place over the years and a comprehensive list of inhibitors is given in Table1. Binding Database, Liu, T. (2019)

Table 1 **IC50 values of various identified TERT inhibitors[14]**

	Molecule	IC50 (nM)
1	9,10-Phenanthrenequinone	5.00E+3
2	Acridine Yellow	2.17E+4
3	Tanshinone IIA	500
4	Acridine orange	1.22E+4
5	Ethacridine	8.20E+3
6	Camptothecin	>3.00E+5
7	7,8-Dihydroxyflavone	3.60E+4
8	3',4',7,8-Tetramethoxyflavone	>4.00E+4
9	7,8,4'-Trihydroxyflavone	3.00E+3
10	7,3',4'-Trihydroxyflavone	>4.00E+4
11	Staurosporine	8.32E+3
12	1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-me thylpent-3-enyl acetate	3.73E+4
13	(-)-Epigallocatechin gallate	1.00E+3
14	BIBR1532	93

BIBR1532 as a novel telomerase inhibitor

BIBR1532 (IC50 = 93 nM) was first studied in the biological system of *Tribolium castaneum* (Red flour beetle) and was discovered to be a potent telomerase inhibitor that specifically targets the TERT in this case tcTERT. The molecular process by which it inhibited TERT is still unknown however the structural basis has been elucidated to a certain extent. It involves the BIBR1532 binding to a hydrophobic pocket near the TRBD residues which are in turn responsible for binding to the activation domain (CR4/5). The failure to activate the CR4/5 domain has an impact on the catalytic ability of the molecule as the RT is not able to function. Thus, BIBR1532 has defined a new category of telomerase inhibitors that function along similar principles as non-nucleoside reverse transcriptase inhibitors (NNRTIs) that were used to counter HIV1 by binding and blocking HIV RT and not allow it to convert RNA into DNA. (Hoffman, H., Harkisheimer, M., Sweeney, M., & Skordalakes, E. (2015)

METHODOLOGY

Materials

Docking protocols were performed in *BioVIA Discovery Studio v19* in a computer with specifications of 4GB of RAM, 3.20 GHz Processor.

Various steps followed in Drug development

In silico drug development is based on a sequence of rational steps. First step is to select a protein structure (or pdb) file from the existing ones. In this investigation two TERT were selected - firstly, a hTERT without any inhibitor bound to it (pdb id: 5UGW) and secondly, a tcTERT bound to BIBR1532 (pdb id: 5CQG) was selected. The tcTERT structure was a homodimer with two chains - A and B and in order to simplify the preparation and minimization procedures only the A chain was used, the B chain was deleted. Next step is to identify the active binding site of our primary TERT which in this case is tcTERT as it already has the BIBR1532 inhibitor bound to it. This also tells us the location where the new inhibitors are meant to be docked. After the completion of initial steps the protein should be isolated and docking simulations with various inhibitors should be performed. RMSD calculations should be used to justify the docking procedure and binding energies of the various inhibitors displayed by the software should be used to judge the affinity ligand has to the protein and the extent to which the ligand can bind properly to the protein. (Hoffman, H., Harkisheimer, M., Sweeney, M., & Skordalakes, E. (2015)

Experimental techniques to analyze Protein-Ligand complexes

There are three major techniques used to determine the structure of Protein-Ligand complexes in most experiments and these include, X-ray crystallography, Nuclear magnetic resonance spectroscopy (NMR), Electron microscopy. In this investigation however we only used PDB structures that were obtained from X-ray crystallography experiments.

Protein and Ligand Preparation and Energy minimization

Preparation of proteins and ligands is an essential step when using molecular docking techniques this is because the X-ray crystallography method that was used to generate these structures are not 100% accurate and might miss some residues which are in the first place very hard to model. Hence, the prepare step solely focuses on completing the protein structure by adding missing residues, hydrogens and balances the valency of the molecule as a whole.

Generally when studying proteins in a biological system they have optimized geometry and exist in the least possible energy state hence, minimization may not be such a crucial step while trying to study proteins in silico. However, when dealing with ligands it is important to minimize them first as unlike proteins they are much smaller and ligands already have conformation which would be influenced by the velocity CDOCKER applies to molecules during the docking procedure. Hence, it is important to perform minimization on ligands and bring them to the least possible energy state before docking.

The TERT protein was prepared using *Prepare protein protocol* in Discovery Studio v19 with parameters set as *Force Field = CHARMm | Keep Ligands = True | Keep Water = None* and the rest all parameters set to default conditions. A file was created by consisting of the following inhibitors. They were prepared using the *Prepare ligands protocol* with all the parameters set to default. Minimization of ligands was done using the *minimize protocol* in the *Small molecules option* with all other parameter set default except *Max Steps = 4000* using Smart Minimizer.

Molecular Docking

Docking Protocol was used with *Input Site Sphere = 22.583420, 6.294565, -32.145367* and a radius of 20 Å with all parameters set default. With active site specified by PDB site data CDocker was used. The *active site was expanded* before docking was performed. In the CDocker protocol used no changes were made all options were set to default. The RMSD calculations were done to the initial bound ligand which in this case was BIBR1532 to validate the docking procedure.

RESULTS AND DISCUSSION

Docking Studies

The RMSD calculated for the closest binding structure having most of the interactions of the original pdb structure was ***0.4510 Å*** *(Pose no.4)*. CDocker energies calculated by the program from the set of inhibitors to the protein prepared. A few of them having high resemblance to the original interactions are given in Table2. Then, the CDocker energy and CDocker Interaction energies are calculated for each pose of all the other inhibitors and compared to the values of BIBR1532 as this docking was present in the original pdb structure of 5CQG already and implied successful inhibition by BIBR1532 of the tcTERT. Hence, the closer the CDocker energy values of the docked inhibitors to that of BIBR1532 the better the inhibitory effect on tcTERT. Binding energy of all the docked ligands with the

proteinstructurewasalsocalculatedtodeduce theextentto whichthetwo moleculeswerebindingtoeachother.Apartfromthis,scoring functions such as PLP1, PLP2, PMF, PMF04, Jain, LigScore1, LigScore2, Ludi1, Ludi2 and Ludi 3 were also used. These were to understandtheinteractionsbetweentheligandsand amino acid residues present in the protein at the binding site.

Results

Table 2 **Results of docking all known listed inhibitors in tcTERT protein (pdb id:5CQG)**

	Molecule	(-)CDocker Energy	(-)CDocker Interaction Energy
1	9,10-Phenanthrenequinone	10.425	23.924
2	Acridine Yellow	15.754	27.222
3	Tanshinone IIA	13.403	32.623
4	Acridine orange	-11.182	25.435
5	Ethacridine	7.947	31.518
6	Camptothecin	-24.426	38.008
7	7,8-Dihydroxyflavone	29.618	31.622
8	3',4',7,8-Tetramethoxyflavone	20.227	42.407
9	7,8,4'-Trihydroxyflavone	31.379	33.97

10	7,3',4'-Trihydroxyflavone	31.966	35.5
11	Staurosporine	-119.885	31.104
12	1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl acetate	20.536	43.52
13	(-)-Epigallocatechin gallate	39.046	39.12
14	BIBR1532	14.012	46.711

Table 2 **Binding Energy values of all listed inhibitors with tcTERT protein (pdb id:5CQG)**

	Molecule	**Binding Energy**
1	9,10-Phenanthrenequinone(6763)	-46.5189
2	Acridine Yellow (7081)	-58.3852
3	Tanshinone IIA(164676)	-56.7242
4	Acridine orange(62344)	-18.7551
5	Ethacridine (2017)	-21.59
6	Camptothecin (24360)	-46.7625
7	7,8-Dihydroxyflavone (1880)	-62.999
8	3',4',7,8-Tetramethoxyflavone (4033898)	-67.2015
9	7,8,4'-Trihydroxyflavone (688853)	-152.672

10	7,3',4'-Trihydroxyflavone (5322065)	-167.174
11	Staurosporine (44299148)	-23.7057
12	1-(5,8-dihydroxy-1,4-dioxo-1,4-dihyd ronaphthalen-2-yl)-4-methylpent-3- enyl acetate(91929176)	-121.21
13	(-)-Epigallocatechin gallate (65064)	-160.647
14	BIBR1532	-90.1034

Figure 1 **Heat map highlighting the most active amino acid residues interacting with the list of inhibitors**

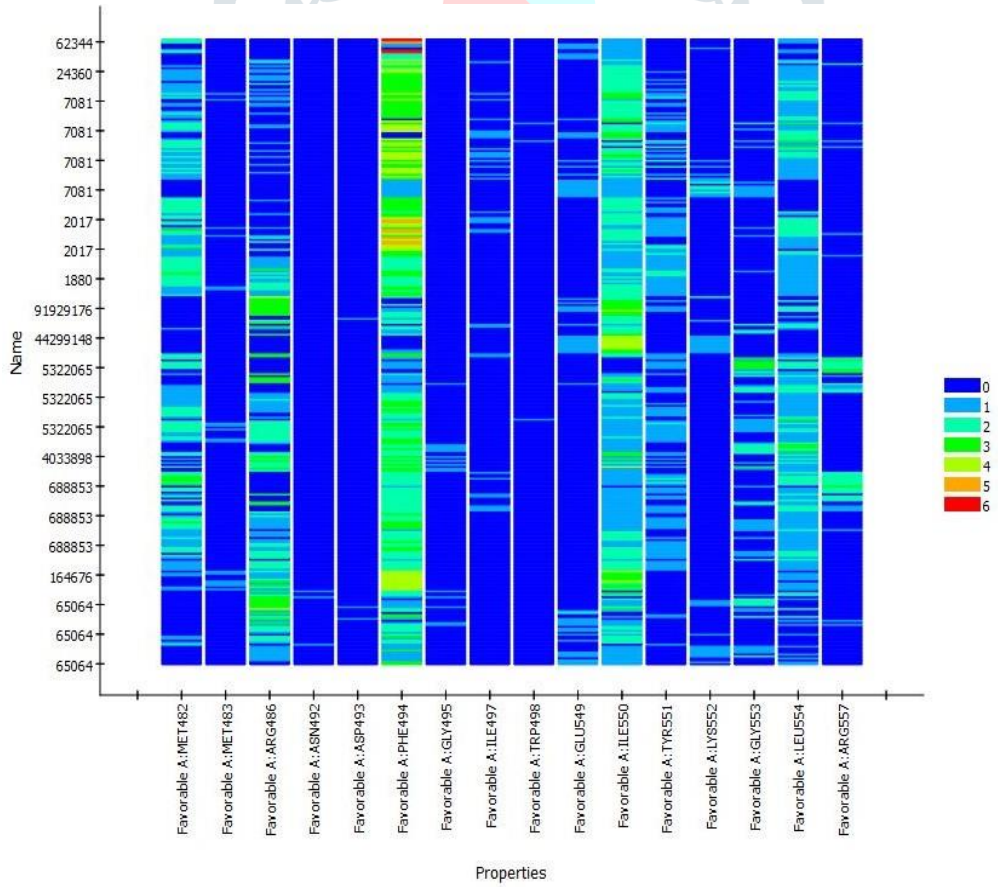


Table 3 **Amino acid residues ranked in order of number of interactions taking place between them and inhibitors at the binding site**

Statistical Residue Analysis

Total Interaction Count						
Favorable	Unfavorable	HydrogenBond	Charge	Hydrophobic	Halogen	Other
1626	17	510	103	1158	0	189

Top 5 Residues with Favorable Interactions (5)							
Residue	Favorable	Unfavorable	HydrogenBond	Charge	Hydrophobic	Halogen	Other
A:ILE550	298	5	84	0	272	0	11
A:PHE494	293	0	78	42	272	0	0
A:LEU554	242	0	12	0	240	0	0
A:MET482	192	0	57	0	10	0	168
A:ARG486	178	6	116	28	113	0	0

Top 5 Residues with Unfavorable Interactions (3)							
Residue	Unfavorable	Favorable	HydrogenBond	Charge	Hydrophobic	Halogen	Other
A:ARG486	6	178	116	28	113	0	0
A:ARG557	6	40	27	33	6	0	0
A:ILE550	5	298	84	0	272	0	11

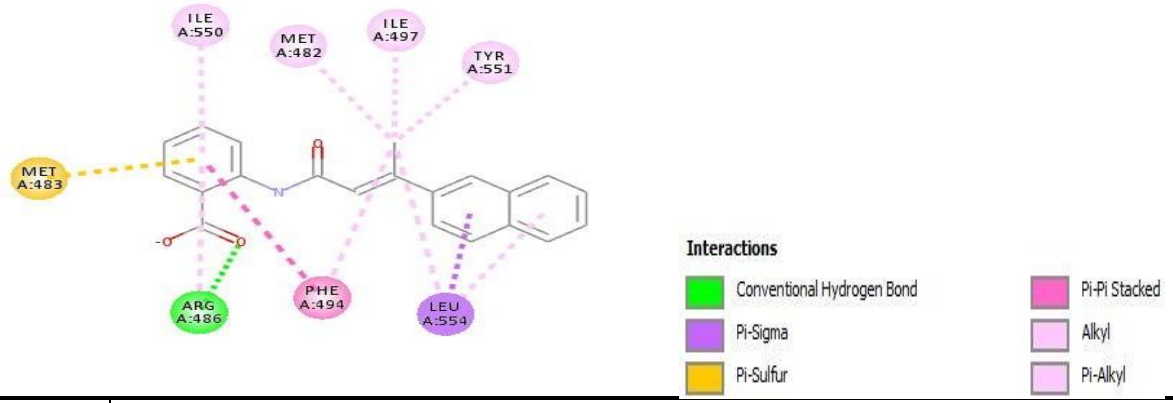
Top 5 Residues with HydrogenBond Interactions (5)							
Residue	HydrogenBond	Unfavorable	Favorable	Charge	Hydrophobic	Halogen	Other
A:ARG486	116	6	178	28	113	0	0
A:ILE550	84	5	298	0	272	0	11
A:PHE494	78	0	293	42	272	0	0
A:GLY553	63	0	76	0	21	0	0
A:MET482	57	0	192	0	10	0	168

Top 5 Residues with Charge Interactions (3)							
Residue	Charge	Unfavorable	HydrogenBond	Favorable	Hydrophobic	Halogen	Other
A:PHE494	42	0	78	293	272	0	0
A:ARG557	33	6	27	40	6	0	0
A:ARG486	28	6	116	178	113	0	0

Top 5 Residues with Hydrophobic Interactions (5)							
Residue	Hydrophobic	Unfavorable	HydrogenBond	Charge	Favorable	Halogen	Other
A:PHE494	272	0	78	42	293	0	0
A:ILE550	272	5	84	0	298	0	11
A:LEU554	240	0	12	0	242	0	0
A:TYR551	143	0	1	0	144	0	0
A:ARG486	113	6	116	28	178	0	0

Top 5 Residues with Other Interactions (5)							
Residue	Other	Unfavorable	HydrogenBond	Charge	Hydrophobic	Halogen	Favorable
A:MET482	168	0	57	0	10	0	192
A:ILE550	11	5	84	0	272	0	298
A:MET483	7	0	0	0	10	0	17
A:GLU549	2	0	52	0	0	0	54
A:ASN492	1	0	2	0	0	0	3

Figure 2 **BIBR1532 interaction with important amino acid residues in the binding site of the protein**



Discussion

CDocker Energy value of a ligand is used to compare the prepared, minimized and docked structure of that ligand to the original pdb structure of the same ligand that was downloaded. The more negative the CDocker energy value the more closely the docked ligand resembles the original structure and hence the docking that was carried out in silico has a better chance of being successfully repeated in-vitro and/or in-vivo systems.

Note : there are more negative CDocker energy values for the inhibitors but the assumption is BIBR1532 showcases ideal inhibitory conditions and hence values closest to BIBR1532's values are desirable.

Since, BIBR1532 is an inhibitor that is shown to already be successful in in-vitro and/or in-vivo systems, it is used as a reference point for what the desired CDocker energy value for all the listed inhibitors should be. This CDocker energy value also includes the solvent conditions of the protein and hence, values close to this may have a similar inhibitory effect on the protein. BIBR1532 has a CDocker energy value of **-14.012.** The inhibitors closest to this value are 9,10-Phenanthrenequinone (**CDocker energy =10.425**), Acridine Yellow (**CDocker energy =15.754**), Tanshinone IIA (**CDocker energy =13.403**).

Binding energies indicate how well the ligand and protein are binding to each other. The energy state after binding has to be lower than the initial energy state of both ligands and proteins in order to become more stable. Both the compounds exist in a higher energy state before binding to each other. Binding energies are calculated by subtracting the initial energy states of the compounds from the final energy state of the complex which must give a negative value if the compounds are indeed binding. Hence, the more negative the binding energy, the more stable the resulting complex indicating that the ligand and the protein have bound very well to each other. BIBR1532 has a binding energy of **-90.1034** which is higher than most of the inhibitors except a few including 1-(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-4-methylpent-3-enyl acetate(**-121.21**),(-)-Epigallocatechin gallate(**-160.647**),7,8,4'-Trihydroxyflavone (**-152.672**), 7,3',4'-Trihydroxyflavone (**-167.174**). It could be hypothesized using this data that these inhibitors show similar/better inhibitory effects than BIBR1532 on tcTERT.

Figure 2 and Table 3 clearly highlight the amino acid residues in the protein with which the ligands are interacting the most. Hence, a valid conclusion being that these residues must be essential for proper binding of the ligand to the protein. Further, some of the most important residues that were present in the binding site of tcTERT after docking all the known inhibitors of TERT include **M482, F494, I550, L554, R486**. Upon comparing this data with the residues present in the binding site of tcTERT where BIBR1532 binds (Figure 4), there is a clear similarity as all of the residues that were important for binding of BIBR1532, are essential for the binding of the other listed inhibitors as well. It can be hypothesized that these inhibitors may showcase a similar inhibitory effect to that of the BIBR1532.

CONCLUSION ANDRECOMMENDATIONS

Limitations of theExperiment

The major limitation of this study was the use of tcTERT as the primary protein of interest rather than hTERT. This is because whenever studying diseases in humans such as cancer it is important to understand the ligand-receptor reactions that are taking place within humans itself. When using other organisms, in this case red flour beetles, there are a number of challenges and differences that need to be accounted for when applying the information learned to human systems such as difference in genome or proteome size or even difference in sequence length of the same protein of interest.

In order to make up for this difference I had initially planned to dock the same list of inhibitors along with the BIBR1532 inhibitor in a hTERT (pdb id: 5UGW) however, I wasn't successfully able to define a binding site. The protocol followed was to superimpose the tcTERT structure on to hTERT structure and by locating where the BIBR1532 inhibitor in the tcTERT, I tried to locate the active binding site of hTERT however was unsuccessful and hence could not present the data of inhibitors bound to hTERT. Another limitation in this investigation was time. Provided enough time I could have further analysed my results through pharmacophore generation and come up with a proper docking protocol for the same list of inhibitors with hTERT.

Conclusion

In conclusion, finding new telomerase inhibitors has become an important part of cancer therapeutics in modern day. Various methods of telomerase inhibition were discussed in this paper such as retinoic acids, nucleoside analogues and antibiotics targeting various aspects of telomerase function but primarily the catalytic unit TERT which is a leading factor in cancer cells proliferative effect. In-silico research is leading the way as the most efficient process through which new telomerase inhibiting drugs are discovered and thus should be at the forefront of cancer research. (Andrews, L. G., & Tollefsbol, T. O. (2007)

In this paper, already known inhibitors of telomerase have been primarily used to justify the docking protocol. All listed inhibitors were docked using this protocol and they showcased similar attributes to the original inhibitor - BIBR1532 that was already docked in the pdb structure (pdb id: 5CQG). These inhibitors and their effects were further studied using functions such as CDocker energy, Binding energy and Amino acid residue interactions. The information gained is enough to hypothesize a few compounds that may (when further analyzed) show similar inhibitory effects on the protein of interest - tcTERT. Further analyses of this investigation would include finding novel pharmacophores, comparing them, creating a database and possibly finding a new drug. (Bryan, C., Rice, C., Hoffman, H., Harkisheimer, M., Sweeney, M., & Skordalakes,

E. (2015)

In the future, strides are being made to develop approaches that inhibit the target proteins linked with telomerase activity rather than directly inhibiting components of telomerase itself such as TERT or TR as although these are present in lower frequency in non-cancer cells they can still be wrongly targeted in normal functioning cells by drugs aimed specifically to target these.

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ACKNOWLEDGEMENTS

The support from Department of Biotechnology, Government of India for Bioinformatics Facility (BIF), Dr. Madhu Chopra, Dr. B R Ambedkar Centre for Biomedical Research, University of Delhi, New Delhi - 110 007, India is highly acknowledged.