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Natural Compounds Targeting Transforming Growth Factor-β: *In Silico* and *In Vitro* Study

Pushpendra Singh[1,*], Felix Bast[1], Ravi Shankar Singh[2]

1 Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda, Punjab, India;

2 Department of Molecular Medicine & Biotechnology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

*Corresponding author. Tel: 9569330844; Email: pushsingh02@gmail.com

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Research Article

Abstract

Inhibition of the tumor-promoting effects of transforming growth factor beta receptor (TGFβR) in carcinogenesis provides a better therapeutic intervention. Various natural compounds, inhibitors of TGFβR have been used for *in vitro* and *in vivo* anticancer study. Although very few TGFβR inhibitors are now intensifying in preclinical studies. In this study our aim to investigate TGFβR1, TGFβR2 and TAK1 inhibitor by using molecular docking and *in vitro* study. Our result revealed that some compounds have better docking energy. Moreover, the effect of two lead molecules epigallocatechin gallate (EGCG) and myricetin on the mRNA expression of TGFβR1 was reported after the 48 hrs treatments in HepG2 and PC3 cancer cell lines. The RT-PCR showed that compound EGCG and myricetin reduced the mRNA expression of TGFβR1 at 80 μM concentration. This molecular docking study provides a better understanding of binding of compounds to the active site of proteins and to summarize the various binding energy, hydrophobic, hydrogen, an electrostatic bond that are decisive for the protein-ligand interactions. Further experimental work will be required for validation of our results.

Keywords: Natural compounds; Cancer; Maestro 9.6; Molecular docking; *In vitro*

1. Introduction

The Transforming Growth Factor β (TGFβ) family have a group of multifunctional regulatory proteins that balance a vast variety of physiological functions including cell proliferation, cell cycle arrest and apoptosis. TGFβR1 53kDa protein is also known as activin encoded by TGFβR1 human gene. TGFβ activated protein kinase 1 (TAK1) was acknowledged for protein kinase action that invigorating by TGFβ signaling transduction [1]. Furthermore, it is also demonstrated that TAK1 cytokine play a significant role in inflammation. TGFβ initiates signals by binding to the TGFβR2 and stabilizes the heteromeric complex with the TGFβR1. As a result, TGFβR1 is trans-phosphorylated and activated by TGFβR2 [2,3]. The activated TGFβ1R then propagates the signals through interaction with receptor-associated Smads [4]. Deregulations of TGFβ signaling pathway responsible for cancer initiation and progression and interrupting the tumor promoter properties of TGFβ signaling would be an attractive therapeutic strategy [5-7]. Monoclonal antibodies and small molecule inhibitors that target TGFβ signaling are the most notable strategy have been used in preclinical studies, but they did not prove much promise as anticancer drugs owing to multifaceted roles of TGFβ signaling [8,9].

In recent years, natural compounds have been investigated intensely in the clinical trial of ovarian, breast, cervical, pancreatic and prostate cancers [10]. A number of propitious agents are in clinical development based on discriminatory activity against anti-cancer molecular targets currently being developed. In this context, we studied TGFβ signaling inhibitors (TGFβR1, TGFβR2, and TAK1) from natural sources using *in silico* and *in vitro* approaches.

2. Material and Methods

2.1 Selection of ligands and protein molecules

In silico, molecular docking protocol and standard setting of parameter adapted from our published literature [11-16]. Marine and natural compounds selected as ligand molecules that had been reported as an anticancer activity in the published literature [17-29]. The X-ray crystal structure of TGFβR1 (PDB; 2X7O), TGFβR2 (PDB; 1KS6), and TAK1 (PDB; 4L3P) kinase protein retrieved from the Protein Data Bank (PDB) (Figure 1).

2.2 Preparation of ligands molecules

Input ligand molecules were prepared using ligprep wizard applications where the addition of hydrogen atoms, 2D to 3D conversion followed by optimized

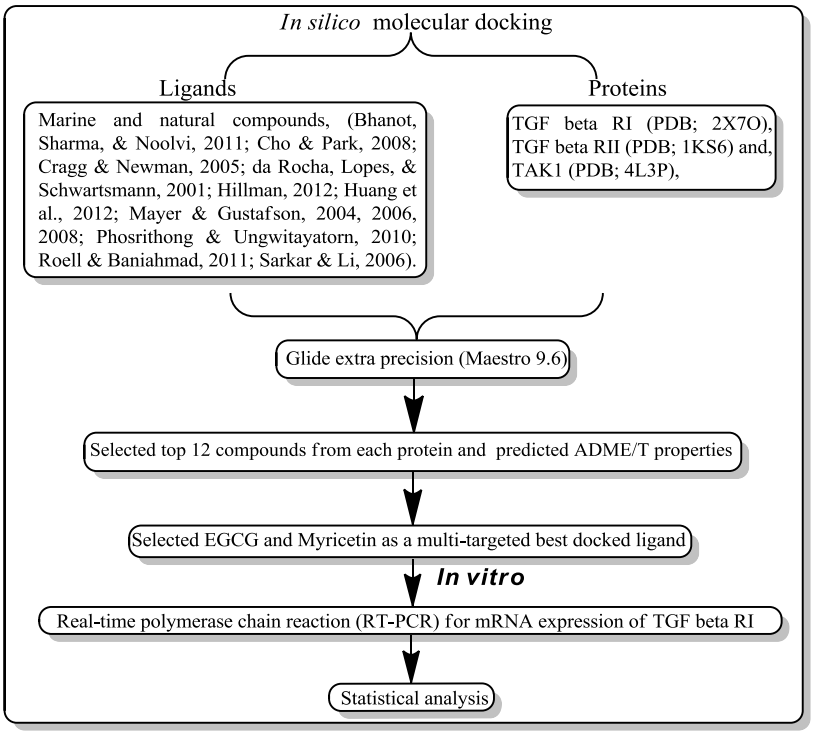

Figure 1. Workflow of study design.

potential for liquid simulations (OPLS_2005) force field was applied [30-32]. Finally, ten conformations for each ligand were generated and performed molecular docking.

2.3 Preparation of protein molecules

Protein molecules were prepared using respective wizard applications (standard methods) where changes such as the addition of hydrogen atoms, fixing of the charges and orientation of groups, assigning bond orders, creation of disulphide bonds, was done into the PDB structure. After the completion of ligands and protein preparation, a receptor-grid file was generated.

2.4 GLIDE (Grid-based Ligand Docking with Energetics) molecular docking

The active site (binding pocket) and functional residues of protein were identified and characterized by GLIDE from Schrodinger package. GLIDE-XP Molecular docking studies using the selected ligand molecules were conducted using Maestro 9.6 [33-35]. Each of these selected compounds was docked into target protein molecules and a compound possessing the lowest energy was selected. After the configuration of receptor-grid file, flexible ligands with rigid receptor molecular docking were performed. The concluding energy evaluation was done on the basis of G-score.

2.5 Absorption, distribution, metabolism, excretion and toxicity (ADME/T) studies

ADME properties such as poor compound solubility, gastric emptying time and lack of ability to permeate the intestinal wall can all diminish the extent to which a drug is absorbed after oral administration. Therefore, *in silico* ADME/T predictive tools are accommodating approach that could abolish unfortunate compounds, before invested valuable time and money in primary testing of compounds. QikProp application of Maestro 9.6 predicts properties such as logBB, overall CNS activity, Caco-2, and MDCK cell permeability and logKhsa for human serum albumin binding, etc. [30-36].

2.6 Reagents and cell lines culture

DMEM media, 1% penicillin, streptomycin and Fetal Bovine Serum (FBS) procured from Invitrogen, and Ham's F-12 media from HiMedia. EGCG and myricetin purchased from MP Biomedicals Pvt. Ltd. and dissolved in Dimethyl Sulfoxide (DMSO) as a 20 mM stock solution. We used final DMSO concentration in culture medium were 0.25% (vol/vol). Hepatocellular carcinoma (HepG2) and Prostate Cancer (PC3), cell lines were procured from NCCS, Pune, India. Cells were grown in phenol red media containing 10% FBS and 1% penicillin and streptomycin and have to grow in a 37°C incubator with 5% CO_2.

2.7 Total RNA isolation, cDNA synthesis and quantitative RT-PCR

One million cells/well were plated into the six-well culture plate media supplemented with 10% FBS and 1% penicillin/streptomycin then incubated at 37°C overnight with 5% CO_2. Cells were exposed to 80 µM EGCG and Myricetin for 48 h. After 48 h total RNA was extracted using Tri reagent (Life Technologies, Gaithersburg, MD) according to manufacturer's

instruction. RNA was quantified at 260/280 nm absorbance and agarose gel electrophoresis. Total RNA (2 µg) was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) using random hexamers in a final volume of 20 µl as described by the manufacturer. RT-PCRs using SYBR green reagent with gene-specific primers (listed in Table 1) and GAPDH gene primers (internal control) were performed in duplicate in a final volume of 20 µl in an ABI 7900 real-time PCR thermal cycler (Applied Biosystems). Relative gene expression levels were calculated after normalization with internal control GAPDH gene using the $2^{-\delta\delta Ct}$ method, where Ct is the threshold value. Results were expressed as fold over control.

3. Statistical Analysis

Statistical values are expressed as the mean ± SEM (SigmaPlot). The value of $p<0.05$ was considered statistically significant.

4. Results and Discussion

4.1 Molecular docking of compounds with TGFβR1, TGFβR2 and TAK1

Molecular docking result of TGFβR1 (PDB; 2X7O) against natural compounds revealed compound CID65064 (EGCG), CID72277, CID5281672 (myricetin), CID9892144, CID72276, CID72281, CID5280343, CID9064, CID6445533, CID5280445, CID44259, CID5281614, CID65064 have better Gscore (as compared to control) -13.05, -11.6, -11.36, -11.14, -11.09, -10.94, -10.93, -10.58, -10.47, -10.42, -10.3, -9.98 Kcal/mol, respectively. Moreover, amino acids Ile211, Val219, Val231, Ala230, Tyr249, Leu260, Val279, Phe262, Ile339, Leu340, and Ala360 involved in the hydrophobic interaction at the active site of the protein. Furthermore, it has been shown that amino acids Leu278, Hid183 and Lys337 form back-bone hydrogen bond at the active site of protein with EGCG. Additionally, it has shown that amino acids Lys232 and Glu245 involved in side-chain hydrogen bonding, in addition, to back-bone hydrogen bonding (Hid283) at the active site of TGFβR1 with myricetin (Figure 2 and Table 2).

Molecular docking result of TGFβR2 against natural compounds revealed compound CID72276, CID5280343, CID9064, CID5281614, CID65064 (EGCG), CID42607750, CID6400741, CID5281672 (myricetin) have better G-score -8.03, -7.81, -7.71, -7.57, -7.3, -6.98, -6.42, -6.36 Kcal/mol, respectively. Further, it was observed that amino acids Gln41, Lys42, Trp65, Met100 and Phe126 form hydrophobic, side chain hydrogen, backbone hydrogen, π-cation and π-π interactions during protein-ligand interactions of TGFβR2 with EGCG. Consequently, it was shown that amino acids Gln41, Trp65, Met100, Glu128 and Phe126 form hydrophobic, side chain hydrogen, backbone hydrogen, π-cation, and π-π interactions during protein-ligand interactions of TGFβR2 with myricetin. Moreover, molecular docking result of TAK1 against natural compounds revealed compound CID42607750, 5281672, 6445533, 72277, 5280445, 5280343, 9892144, 5281614, 442793, 5281670, 9064 have better G-score (as compared to control) -12.01, -11.67, -11.07, -10.66, -10.2, -10.12, -9.96, -9.42, -9.3, -9.08, -8.87 Kcal/mol, respectively. Moreover, protein-ligand interactions showed that amino acids Phe47, Ala61, Leu81, Val90, Met104, Tyr106, Ala107, Cys174 and Phe176

Table 1. Primer sets used for amplification.

S. No.	Name of gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
1.	TGFβRI	5'-TGCACATCGTCCTGTGGAC-3'	5'-GTCTCAAACTGCTCTGAAGTGTTC-3'
2.	GAPDH	ACGGATTTGGTCGTATTGGGCG	CTCCTGGAAGATGGTGATGG

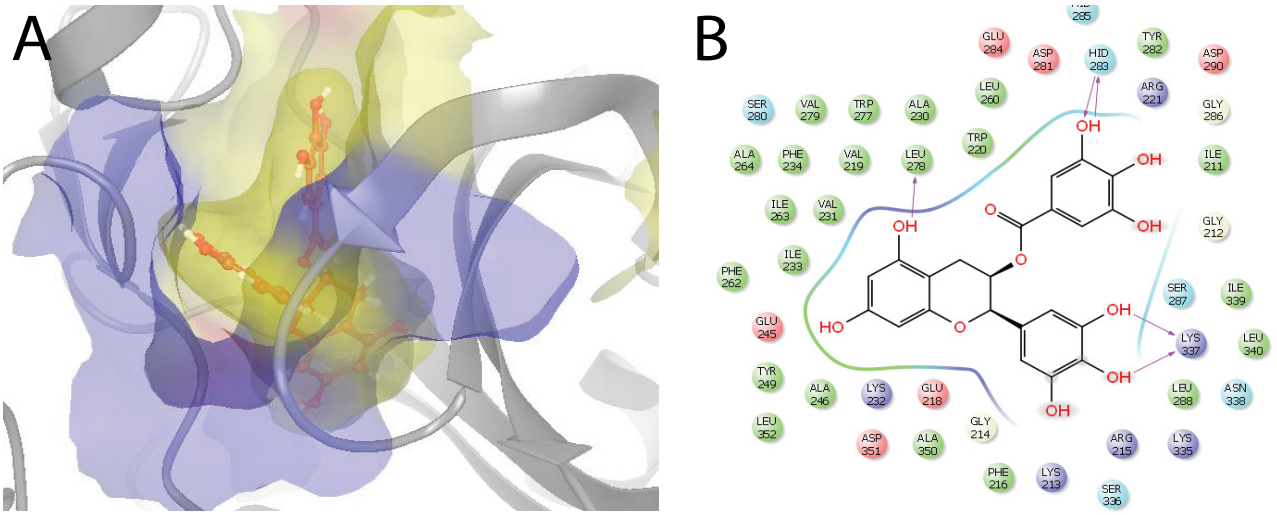

Figure 2. (a) Ribbon presentation of TGFβRI (PDB; 2X7O) protein molecule with CID 65064; (b) Protein-ligand interactions profile of 2X70 with CID 65064.

Table 2: Lowest binding energy for the ligand- TGFβR1 (PDB; 2X7O) and TGFβR2 (PDB; 4XJJ) protein interactions.

Ligand type	Compounds ID	G-Score	Lipophilic Evdw	HBond	Electro	Protein-ligands interactions
	SD 208	-9.95	-5.15	-0.7	-0.35	Hid283
	R 268712	-8.04	-5.5	-1.2	-0.9	Lys232, Ser287, Asp290
	CID4521392	-7.87	-5.16	-0.81	-0.25	Hid283, Lys335
TGFβR	A 83-01	-7.33	-6.89	-0.05	-0.58	Lys337
inhibitors	GW 788388	-7.29	-6.3	-0.35	-0.42	Lys232
(Controls)	449054	-7.23	-4.73	-0.32	-0.36	Lys232, Hid283
	LY 364947	-7.07	-5.09	-0.17	-0.32	Lys232
	D 4476	-7.05	-5.66	-0.88	-0.29	Lys232, Glu245, Hid183
	CID11655119	-7.03	-7.29	0	-0.34	
	SB 525334	-6.46	-4.8	-0.47	-0.54	Lys232, Hid183
	CID65064	-13.05	-6.2	-5.13	-1.36	Leu278, Hid183, Lys337
	CID72277	-11.6	-4.67	-4.12	-1.22	Lys232, Glu245, Hid183, Asp351
	CID5281672	-11.36	-5.02	-3.96	-0.99	Lys232, Glu245, Hid283
TGFβR1	CID9892144	-11.14	-6.83	-2.8	-0.54	Lys213, Lys232, Hid183, Ser287
	CID72276	-11.09	-4.72	-3.46	-1.12	Lys232, Glu245, Hid183, Asp351
	CID72281	-10.94	-4.97	-2.94	-0.98	Lys232, Glu245, Hid183, Asp351
	CID5280343	-10.93	-4.98	-3.53	-0.99	Lys232, Glu245, Hid183
	CID9064	-10.58	-4.69	-2.96	-1.13	Lys232, Glu245, Hid183, Asp351
	CID6445533	-10.47	-5.29	-3.06	-1.18	Arg215, Leu278, Asp281, Asp351
	CID5280445	-10.42	-4.94	-3.04	-1.01	Lys232, Glu245, Hid183
	CID44259	-10.3	-5.71	-1.33	-0.52	Asp281, Hid283
	CID5281614	-9.98	-4.88	-2.51	-0.82	Asp281, Glu245, Asp351
	CID72276	-8.03	-3.69	-3.51	-0.77	Gln41, Trp65, Met100, Phe111, Phe126
	CID5280343	-7.81	-3.83	-3.03	-0.82	Gln41, Trp65, Met100, Phe111, Phe126
	CID9064	-7.71	-3.71	-3.03	-0.8	Gln41, Trp65, Met100, Phe111, Phe126
TGFβR2	CID5281614	-7.57	-3.81	-3.03	-0.76	Trp65, Met100, Phe126
	CID65064	-7.3	-3.29	-3.3	-1.15	Gln41, Lys42, Trp65, Met100, Phe126
	CID42607750	-6.98	-2.87	-3.07	-1.3	Met100, Lys101, Phe126
	CID6400741	-6.42	-3.32	-2.23	-1.42	Gln41, Trp65, Met100, Phe111, Phe126
	CID5281672	-6.36	-2.57	-2.94	-0.92	Gln41, Trp65, Met100, Glu128, Phe126
	CID636888	-6.27	-4.12	-2.29	-0.6	Gln41, Glu119, Phe126
	CID72277	-6.25	-1.99	-3.87	-0.9	Gln41, Trp65, Phe126
	CID5281605	-5.87	-2.2	-2.4	-0.74	Gln41, Trp65, Phe126
	CID439260	-5.78	-1.93	-2.4	-0.85	Gln41, Phe126

involved in hydrophobic interaction at the active site of the protein. Moreover, Additionally, it have shown that amino acids Val42, Ala 46, Glu105 and Ala107, involved in side-chain hydrogen bonding whereas amino acid Lys63 and Asp175 involved in back-bone hydrogen bonding at the active site of TAK1 with myricetin. Additionally amino acid Arg44 and Ala107 of TAK1 form backbone hydrogen bonding with EGCG (Figure 3 and Table 3).

Previously, it was reported that EGCG can inhibit TGFβ induced epithelial-mesenchymal transitions via down-regulation of phosphorylated Smad2 and Erk1/2 in non-small cell lung cancer cells [37]. Moreover, EGCG significantly subdues the HSP27 induction prompted by TGFβ in a dose-dependent manner. EGCG significantly suppressed the TGFβ induced phosphorylation of stress-activated kinases and c-Jun N-terminal kinases [38]. Furthermore, it was noticed that EGCG interacts with TGFβR2 and inhibits the expression of α-smooth muscle actin via the TGFβ-Smad2/3 pathway in human lung fibroblast cells [39,40]. *In vivo* study showed that myricitrin was noticeably ameliorated the expression of TGFβ1 and alpha-smooth muscle actin that led to antioxidant, anti-inflammatory and antifibrotic activity [41]. Furthermore, *in vivo* study in streptozotocin with cadmium-induced nephrotoxic diabetic rats unveil that myricetin up regulates the TGFβ1 and downregulate the peroxisome proliferator-activated receptor alpha proteins expression that led to restraints dyslipidemia and renal mesangial cell proliferation [42].

4.2 ADME/T studies

ADME/T (absorption, distribution, metabolism, elimination and toxicity,) properties of lead molecules were assessed through the Qikprop application of Maestro 9.6. EGCG and myricetin have the best Gscore against TGFβR1, TGFβR2, and TAK1.

Furthermore, EGCG and myricetin have their excellent QPlogPo/w, QPlogHerg K^+ channels, QPlogBB, QPlogKP, QPlogK HSA values that accomplish the Lipinski's rule of five. However, these compounds do not have excellent QPP Caco, QPP MDCK values and percentage of human oral absorption. Therefore, EGCG and myricetin structural alteration and optimization are required to predict structures that have better QPP Caco, QPP MDCK and rate of human oral absorption activity (Table 4).

4.3 Effect of EGCG and myricetin on TGFβR1 mRNA expression in HepG2 and PC3 cancer cell lines

We investigated whether EGCG and myricetin could decrease the TGFβR1 expression in HepG2 and PC3 cells. After 48 h of treatment with EGCG and myricetin, determine the mRNA expression of TGFβR1 by using RT-PCR. As shown in Figure 4, RT-PCR demonstrates that EGCG have a prospective for forceful inhibition of TGFβR1. The RT-PCR densitometric bands examination showed that EGCG reduced the mRNA expression of TGFβR1 by 15% in HepG2 (*P<0.05) and 70% (**p<0.001) in PC3 cells at 80 μM concentration. Whereas, RT-PCR densitometric bands investigation showed that myricetin reduced the mRNA expression of TGFβR1 by 20% in HepG2 (*P<0.05) and 80% (**p<0.001) in PC3 cells at 80 μM concentrations. TGFβ signaling plays a leading role in the initiation, promotion, and progression of various types of human cancer including prostate, breast lung, and liver cancer. Several lines of evidence suggest that carcinoma cells frequently lose anti-proliferative response to TGFβ. High levels of TGFβ can promote tumor growth in an autocrine and paracrine manner through the stimulation of angiogenesis and metastasis. Blocking the tumor-promoting effects of TGFβ by small molecule inhibitors provides an excellent therapeutic opportunity to improve the treatment of cancer.

5. Conclusion

TGFβ increases the tumor motility, invasion, and metastasis. Inhibition of TGFβ signaling offers a novel approach for the treatment of cancer. Monoclonal

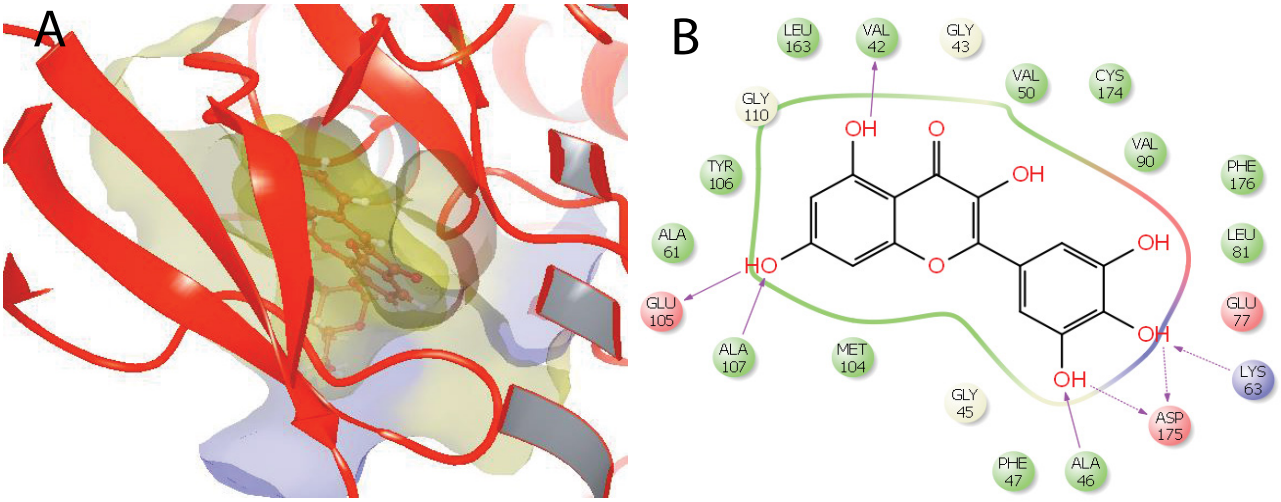

Figure 3. (a) Ribbon presentation of TAK1 (PDB; 4L3P) protein molecule with CID5281672; (b) Protein- ligand interactions profile of 4L3P with CID5281672.

Table 3: Lowest binding energy for the ligand- TAK1 (PDB; 4L3P) protein interactions.

Ligand type	Compounds CID	G-Score	Lipophilic Evdw	H-Bond	Electro	Protein-ligands interactions
TAK1 Inhibitors	CID9863776	-6.82	-3.29	-2.88	-0.44	Ser111, Asn114
	CID57369807	-6.04	-1.97	-3.02	-0.78	Ser111, Asn114, Asn161
Natural Compounds	CID42607750	-12.01	-5.02	-4.48	-1.24	Arg44, Glu105, Ala107, Ser111, Asn114
	CID5281672	-11.67	-3.84	-4.75	-1.69	Val42, Ala 46, Lys63, Glu105, Asp175
	CID6445533	-11.07	-6.55	-2.36	-0.79	Arg44, Glu105, Ala107, Ser111, Asn114
	CID72277	-10.66	-4.62	-3.83	-0.82	Arg44, Glu105, Ala107, Ser111, Asn114
	CID5280445	-10.2	-4.25	-2.95	-1.57	Val42, Ala 46, Lys63, Glu105, Asp175
	CID5280343	-10.12	-3.81	-3.5	-1.53	Val42, Gly43, Lys63, Glu105, Asp175
	CID9892144	-9.96	-6.97	-2.45	-0.58	Ala107, Ser111
	CID5281614	-9.42	-4.35	-3.3	-1.34	Val42, Ala 46, Glu105, Ala107, Asp175
	CID442793	-9.3	-5.04	-2.42	-1.03	Lys63, Glu105, Ala107, Asp175
	CID5281670	-9.08	-5.16	-2.71	-0.78	Lys63, Ala107, Asp175
	CID9064	-8.87	-5.11	-2.46	-0.89	Val42, Arg44, Ala107, Asp175
	CID65064	-8.87	-4.14	-4.41	-0.96	Arg44, Asp175

Table 4: Evaluation of drug-like properties of the lead molecules by Qikprop Maestro.

Compounds	QPlog $P_{o/w}$ (-2.0 to 6.5)	QPlog HERG (acceptable range: above -5.0)	QPP Caco (nm/s) <25-poor >500- great	QPlog BB (-3-1.2)	QPP MDCK (nm/s) <25-poor >500- great	QPlog Kp (-8.0 to -0.1)	QPlog Khsa (Acceptable range: -1.5 to 1.5).	Percentage of human oral absorption; (<25% is poor and >80% is high)
CID65064 (EGCG)	-0.233	-5.558	1.14	-4.177	0.326	-7.415	-0.437	0.684
CID72277	-0.167	-4.684	22.042	-2.338	8.009	-5.482	-0.562	37.049
CID5281672 (myricetin)	-0.276	-4.894	7.673	-2.827	2.56	-6.301	-0.493	28.209
CID9892144	3.838	-5.92	123.998	-1.804	51.813	-3.591	0.723	86.885
CID72276	0.479	-4.852	60.119	-1.858	23.692	-4.599	-0.43	61.589
CID72281	1.782	-5.025	133.02	-1.525	55.9	-4.057	0.007	75.395
CID5280343	0.384	-5.011	20.522	-2.331	7.414	-5.442	-0.348	52.678
CID9064	0.439	-4.845	52.516	-1.913	20.471	-4.716	-0.429	60.305
CID6445533	1.744	-6.907	35.048	-1.764	14.628	-4.913	-0.194	64.802
CID5280445	0.973	-5.14	41.633	-1.975	15.927	-4.849	-0.187	61.625
CID44259	3.765	-6.13	304.782	-0.05	151.525	-4.131	0.787	93.45
CID5281614	0.505	-5.178	45.418	-1.943	17.497	-4.758	-0.364	59.565
CID42607750	-0.607	-5.487	12.878	-3.215	4.48	-5.266	-0.883	17.34
CID5281614	0.458	-4.924	53.31	-1.808	20.806	-4.65	-0.388	60.532
CID442793	3.271	-4.52	506.992	-1.443	237.406	-2.569	0.154	94.512
CID9571127	2.291	-4.098	0.079	-4.908	0.03	-6.406	-0.439	0
CID23663412	3.425	-4.65	8.604	-4.303	3.837	-3.649	-0.49	37.809

QPlog $P_{o/w}$ (-2.0 to 6.5) Predicted octanol/water partition coefficient; QPlog Herg (acceptable range: above -5.0) Predicted IC50 value for blockage of HERG K+ channels; QPPCaco (nm/sec) <25-poor >500- great Predicted apparent Caco-2 cell permeability in nm/s. Caco-2 cells is a model for the gut blood barrier; QPlogBB (-3-1.2) Predicted brain/blood partition coefficient; QPPMDCK (nm/s) <25-poor >500- great Predicted apparent MDCK cell permeability in nm/s. MDCK cells are considered to be a good mimic for the blood-brain barrier. QPlogKP- Predicted skin permeability; Q P log Khsa; Prediction of binding to human serum albumin; (acceptable range: -1.5 to 1.5); Percentage of human oral absorption; (<25% is poor and >80% is high).

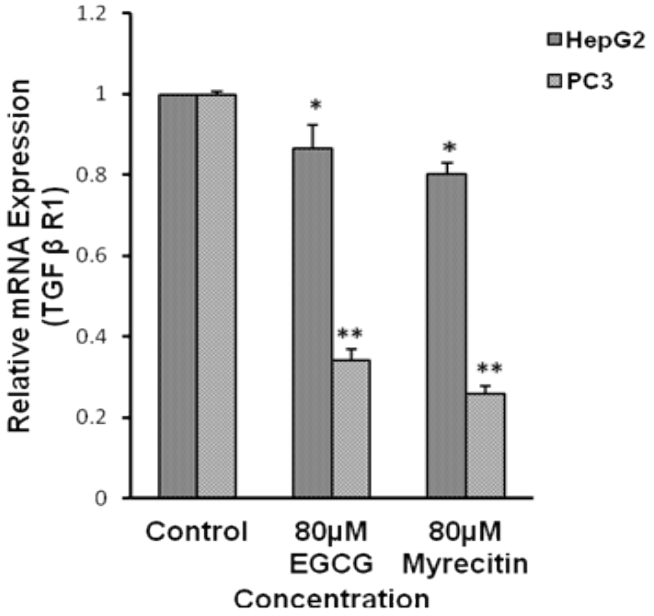

Figure 4. Represented effect of EGCG on TGFβR1 mRNA turnover in HepG2 and PC3.

Note: Cells were treated with 80 μM EGCG and myrecitin for 48 h of treatment. TGFβR1 mRNA were determined by SYBR® Green based RTPCR using GAPDH as internal control. EGCG and myrecitin down-regulated the expression of TGFβR1 mRNA in HepG2 and PC3. Data are s.e.m. (*p<0.05 control vs. EGCG and Myrecitin, **p<0.001 control vs. EGCG and myrecitin)

antibodies and small compounds used as a TGFβ inhibitor but due to cross activity these inhibitors fail in the preclinical trial. Our study showed some compounds has better docking energy such as EGCG and myricetin. Moreover, the RT-PCR showed that compound EGCG and myricetin reduced the mRNA expression of TGFβR1 at 80 μM concentration. This molecular docking study enhanced understanding of binding of compounds to the active site of proteins and to recapitulate the various binding energy, hydrophobic, hydrogen, an electrostatic bond that are important for the protein-ligand interactions. Further experimental work will be required for validation of our results.

6. Acknowledgement

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