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6-Gingerol is the most Potent Anticancerous Compound in Ginger (*Zingiber officinale* Rosc.)

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Abstract

Cancer is one of the most deadly diseases in the world, which is caused due to uncontrolled growth of cells or malfunction of genes that control normal cell growth and division. Because of high death rate associated with cancer and because of serious side effects of chemotherapy and radiation therapy, many cancer patients seek alternative complementary methods of treatment. Ginger (*Zingiber officinale* Rosc.) is an important spice crop with immense medicinal properties and health beneficial effects. All the ginger ligands showed good interaction with the selected targets but based on ADME/Toxicity analysis 6-gingerol was superior with respect to absorption, solubility, and less neurotoxic effect as compared to other ginger ligands. 6-gingerol was also found cytotoxic to all the three cancer cells lines were studied. The cytotoxicity increased with increase in concentration of 6-gingerol. The IC50 values recorded for different cancer cell lines, 24 h. after treatment (100 µM for HCT15, 102 µM for L929 and 102 µM for Raw 264.7) showed uniform cytotoxicity in the three cell lines studied. The study highlights the potential of 6-gingerol for drug development against cancer.

Keywords: 6-gingerol; Biomarkers; Molecular docking; ADME/Toxicity; Cell culture

Introduction

Cancer is a leading cause of death worldwide, with 8.2 million deaths in 2012. More than half of all cancer deaths each year are due to lung, stomach, liver, colorectal and female breast cancers [1]. There are several biomarkers like, Epidermal Growth Factor Receptor (EGFR), c-Met, Phosphoinositide 3-kinase (PI3k), Cyclooxygenase-2 (COX-2), Nuclear factor –kappa β (NF-k β) and Activator protein (AP-1) which express in different types of cancer. EGFR overexpression is thought to play important role in the activation of various malignant tumors [2]. c-Met has been shown to be deregulated and associated with high tumor grade and poor prognosis in a number of human cancers. Deregulation of the receptor tyrosine kinase c-Met has been implicated in several human cancers and is considered as an attractive target for small molecule drug discovery [3]. PI3k is a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking which in turn are involved in cancer. The rapid progress made in developing novel PI3k inhibitors in recent years promises bright prospects for finding a PI3k-targeted anticancer drug in the near future [4]. COX-2 an inducible prostaglandin is considered as a promising target for the treatment of various human cancers [5]. NF-kβ transcription factors play an important role in the inducible regulation of a variety of genes involved in the inflammatory and proliferative responses of cells [6]. Disregulation of NF-κβ has been linked to cancer, inflammation and autoimmune diseases, septic shock, viral infection, and improper immune development. AP-1 activation is linked to growth regulation, cell transformation, inflammation, and innate immune response. AP-1 has been implicated in regulation of genes involved in apoptosis and proliferation. Targeting AP-1 or its activating kinases could be promising agents for the treatment of several cancers [7].

Because of high death rate associated with cancer and because of serious side effects of chemotherapy and radiation therapy, many cancer patients seek alternative complementary methods of treatment. Plants have been used for treating diseases since time immemorial. More than 50 per cent of modern drugs in clinical use are of natural products [8]. Ginger is valued for its spicy and medicinal properties and it has been used as medicine from Vedic period and is called "maha aushadh", means the great medicine. The importance of ginger has been increased recently because of its low toxicity and its broad spectrum of biological and pharmacological applications, viz. antitumor, antioxidant, anti-inflammatory, antiapoptotic, cytotoxic, anti-proliferative and anti-platelet activities [8-13]. Of the various compounds present in ginger, gingerols are the most potent and pharmacologically active compounds and possess anti-inflammatory, analgesic, antipyretic, gastro protective, cardiotonic and antihepatotoxic activities. Of the gingerols, the most potent and pharmacologically active compound is 6-gingerol and is now a target for drug development. Gingerols are thermally labile due to the presence of a β-hydroxy keto group in the structure and undergo dehydration readily to form the corresponding shogaols.

Pharmacological investigations have revealed that ginger and its major pungent ingredients have chemo preventive and chemotherapeutic effects on a variety of cancer cell lines and on animal models [14]. Considering the importance of gingerol, the present study focuses to identify cancer targets for gingerols and shogoal using in silico tools and to validate anti cancerous properties of gingerol.

Materials and Methods

The study is based on in silico screening of potential cancer targets for gingerols and shogaols, molecular docking is used to identify the interaction between ginger ligands and cancer targets, comparison of efficacy of ginger ligands and approved drugs against cancer targets and to validate the anticancerous properties of gingerol using different tumour cell lines.

Retrieval of structure of ginger ligands and approved drugs

3D Structure of four ginger ligands viz. 6-gingerol, 8-gingerol, 10-gingerol and 6-shogaol and two approved drugs viz. quercitrin and disulfiram were retrieved from Pubchem online database.

Preparation of ginger ligands and approved drugs and filtration: Preparation of retrieved ginger ligands and approved drugs was performed using ligand preparation wizard of Discovery Studio 4.0 (DS 4.0), which removed duplicates, enumerated tautomers/isomers, added hydrogen bonds and minimized energy using CHARMm (Chemistry at Harvard Macromolecular Mechanics) force field [15]. Filtration of prepared ligands and approved drugs were done by Lipinski"s and Veber rules [16] that sets the criteria for drug like properties. To pass Lipinsk's and Veber rules, a compound should have molecular weight <500 daltons, number of hydrogen bond donors <5, number of hydrogen bond acceptors <10 and partition coefficient (LogP) <5. After filtration of ginger ligands and approved drugs, molecular docking was performed with selected cancer targets [17].

Preparation of target protein molecules and active site prediction: Preparation of the retrieved protein was performed by using protein Preparation Wizard of DS 4.0, which correct the protein structures by removing extra chain of target protein, internal ligand, crystallographic water molecules and hetero atoms. Hydrogen atoms were added to correct the chemistry of protein and energy minimization was performed to avail a stable conformation by employing CHARMm force field [18]. The energy minimized structure was used as the template for molecular docking. The Receptor cavity and current selection tools of DS 4.0 were used to analyse the binding mode of ligands in the selected region. A grid receptor sphere was generated, including the selected binding active site and incorporating all the critical functional residues.

Molecular docking: Molecular docking was performed between prepared target proteins of cancer with four ginger ligands, NF-kβ and AP-1 were also docked with approved drugs like Disulfiram and Quercitrin by 'C-DOCKER' docking protocol of DS 4.0 [19]. The pose contained minimum difference between –C-DOCKER and –C-DOCKER interaction energy was considered as the best interaction, along with the lowest binding energy calculated as the scoring function [20]. Number of hydrogen bonds between the targets and the ligands were also recorded. The optimal distance between two atoms connected by a hydrogen bond is set to 1.9 Å with a tolerance of 0.5 Å [21].

ADME/Toxicity evaluation: In silico tool 'ADME/Toxicity descriptors' provided by DS 4.0 presented in Table 1 was used for the evaluation of pharmacokinetic parameters and assess the quality of the molecules in terms of absorption, distribution, metabolism, excretion and toxicity after human ingestion. This technique reduces the cost and chance of clinical failures of new drugs. The parameters calculated by this descriptor included aqueous solubility, Human Intestinal Absorption, Blood-Brain-Barrier (BBB) penetration, cytochrome P450 inhibition and Hepatotoxicity levels.

Human Intestinal Absorption level		BBB Level		Aq. Solubility Level		Hepatotoxicity prediction		CPY2D6 prediction	
Level	Intensity	Level	Intensity	Level	Drug-likeness	Level	Value	Level	Value
0	Good	0	Very high penetration	0	Extremly low	0	Nontoxic (False)	0	Non-inhibitor (False)
1	Moderate	1	High	1	No, very low, but possible	1	Toxic (True)	1	Inhibitor (True)
2	Poor	2	Medium	2	Yes, low				
3	Very Poor	3	Low	3	Yes, good				
		4	Undefined	4	Yes, optimal				
				5	No, too soluble				

Table 1: Standard level of ADMET descriptors from DS 4.0.

Maintenance of cell lines and *in vitro* cytotoxicity assay

Based on docking score and ADME/Toxicity analysis 6-gingerol was carried forward for *in vitro* cytotoxicity assay using three different cancer cell lines which included, Human colon cancer (HCT15), mouse leukaemic monocyte macrophage (Raw 264.7) and murine fibro sarcoma (L929) cells which were received from Amala Cancer Research Centre, Thrissur (Kerala). 6-gingerol standard (HPLC grade, 98% pure) was procured from Sigma- Aldrich Company. The cells were cultured in RPMI-1640 medium supplemented with 10 per cent fetal bovine serum (FBS), 4.5 g glucose, 1 per cent each HEPES buffer, sodium pyruvate and antibiotic (penicillin and streptomycin) at 37°C.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was done to evaluate the proliferative capacity of cells. A 96 well plate was used with 100 µl medium containing cells. After 48 h of incubation, the cells were treated with gradient concentration (17, 34, 68, 102, 136 and 170 µM) of 6-gingerol which was dissolved in ethanol. The experiment was replicated thrice for HCT15, Raw 264.7 and L929. Observations were recorded at 24 h intervals. The spent medium was removed and 100 µl of fresh medium and 10 µl of MTT

(5 mg/ml in PBS) were added to the wells and cells were incubated at 37°C in dark for 4 h. The formazan product was dissolved by adding 100 µl of DMSO. The absorbance was measured at 570 nm using monochromatic ELISA reader (VERSA max microplate reader).

Results and Discussion

Retrieval of ginger ligands, approved drugs and identified cancer targets

3D structure of four ginger ligands viz. 6-gingerol (CID: 442793), 8-gingerol (CID: 168114), 10-gingerol (CID: 168115), 6-shogaol (CID: 5281794) and quercitrin (CID: 5280459) and disulfiram (CID: 3117) downloaded in sdf. format from pubchem online database. All the ligands having benzene ring except disulfiram.

3D structure of identified cancer targets were retrieved from PDB online database and saved in PDB File (Text). Structure of cancer target proteins retrieved based on X-ray diffraction method and resolution power.

Preparation of ginger ligands and approved drugs and identified cancer targets

Preparation of the ligands was done by using ligand Preparation wizard of DS 4.0 to change ionization and generate tautomer, isomers and 3D coordinates. After preparation all ligands were filtered with "Lipinski's and Veber rules' [16], the details are presented in Table 2. Of the four ligands from ginger filtered using lipinski's and veber rules, two viz. 6-gingerol and 6-shogaol passed lipinski's and veber rules while 8-gingerol and 10-gingerol failed. In the case of approved drugs, disulfiram passed lipinski's and veber rules while quercitrin failed.

Compound name	Partition coefficient (XLogP3)	Hydrogen Bond Donar (No.)	Hydrogen Bond Acceptor (No.)	No. of rotation bonds (No.)	Lipinski's and Veber rules
6-gingerol	<5	<5	<10	10	Pass
8-gingerol	<5	<5	<10	12	Fail
10-gingerol	>5	<5	<10	14	Fail
6-shogaol	<5	<5	<10	09	Pass
Disulfiram	<5	<5	<10	07	Pass
Quercitrin	<5	>5	>10	03	Fail

Table 2: Filtration of ginger ligands and approved drugs using Lipinki's and Veber rules.

All the non-standard residues were removed from the target proteins during the protein preparation. Energy minimization of protein structure reduced the effect of potential energy, vander-waals energy and electrostatic energy. The maximum numbers of active sites from prepared proteins were found nine in 3LN1 (COX-2) and minimum two in 4HMY (AP-1). Only one active site was selected from each target for docking which had maximum number of amino acid residues in their active site.

Molecular docking analysis

Four ginger ligands and two approved drugs were docked with identified cancer targets by DS 4.0. The docking scores are listed in the Table 3. The minimum difference between -CDOCKER and -CDOCKER interaction energy was found in ginger ligands and targets as 2.287 Kcal/mol (6-gingerol with c-Met), 1.9043 kcal/mol (8-gingerol with c-Met), 4.2519 kcal/mol (10-gingerol with PI3k), 5.8666 kcal/mol (6-shohaol with PI3k). In the case of approved drugs the difference was around 6 kcal/mol. The binding energy of identified targets with 6-gingerol ranged from -61.3134 to -138.2092, 8-gingerol from -21.9807 to -140.5949, 10-gingerol from -87.531 to -131.1699 and 6-shogaol from -38.7325 to -117.683. Among the ginger ligands, 6-gingerol showed the lowest scores of -107.9914 Kcal/mol, -66.7825 kcal/mol and -76.0004 kcal/mol while interacting with EGFR, c-Met and NF-kβ respectively at the residues LYS745, MET789, MET1160, PRO1158 and ASn20 of active site 3, 1 and Receptor cavity with hydrogen bond lengths 1.8 Å, 2.0 Å, 1.9 Å, 2.3 Å and 2.2 Å respectively (Figure 1a, 1b and 1c). In the case of PI3k and COX-2 lowest score (-87.5317 and -89.9435) was showed while interacting with ginger ligand 10-gingerol at the residues SER806, LYS833, LYS890, ASP964, HIS200 and ASN368 with hydrogen bond lengths 2.2 Å, 1.8 Å, 2.4 Å, 2.2 Å, 2.0 Å, 1.9 Å and 2.1 Å respectively (Figure 1d and 1e). In case of AP-1, 8-gingerol showed lowest score (-140.5949) while interacting at the residues LYS127, THR45 and ILE46 with hydrogen bond lengths 1.8 Å, 2.8 Å and 2.2 Å respectively (Figure 1f).

Targets name	Compounds name	(-) CDOCKER energy (Kcal/mol)	(-) CDOCKER Interaction energy (Kcal/mol)	Binding energy (Kcal/mol)	Amino acids bound to H-bond	No. H-bonds
EGFR	6-gingerol	40.8626	45.0524	-107.9914	Lys745; Met793	2
	8-gingerol	42.2719	47.0784	-55.2001	Lys745; Asn842; Asp855	3
	10-gingerol	47.6207	51.7268	-131.1699	Lys745; Asp855	2
	6-shagaol	28.2694	42.1514	-107.9644	Lys745; Asp855	4
C-Met	6-gingerol	36.7507	39.0377	-66.7825	Met1160; Pro1158	2
	8-gingerol	39.1134	41.0177	-21.9807	Met1160	1
	10-gingerol	40.0831	38.3251	-	-	-

	6-shagaol	26.9714	37.2637	-46.9104	Met1160	1
PI3K	6-gingerol	39.8259	43.7252	-83.9303	Lys833; Asp964; Asp836	3
	8-gingerol	46.1988	49.6355	-77.5847	Lys833; Lys890	2
	10-gingerol	51.8771	56.129	-87.5317	Ser806; Lys833; Lys890; Asp964	5
	6-shagaol	33.5313	39.3979	-42.7721	Ser806	1
COX-2	6-gingerol	35.1914	40.4706	-61.3134	His200; Thr369	4
	8-gingerol	40.9833	48.2271	-48.4629	Thr198; Asn368; Gln440	5
	10-gingerol	39.1249	44.6087	-89.9435	His200; Asn368	3
	6-shagaol	30.9843	42.787	-38.7325	Thr198; Asn368	4
NF–kβ	6-gingerol	30.7019	35.3497	-76.0004	Asn202	1
	8-gingerol	23.7329	31.2246	-37.3553	Asn202	1
	10-gingerol	-	-	-	-	-
	6-shagaol	19.2346	33.0365	-40.7234	Ile205	2
	Disulfiram	17.5971	24.1089	-23.2973	Ser126	1
AP-1	6-gingerol	42.6345	51.0834	-138.2092	Thr32; Thr45; Ile46	3
	8-gingerol	47.2985	53.279	-140.5949	Lys127; Thr45; Ile46	3
	10-gingerol	42.8941	41.2684	-95.172	Thr45	1
	6-shagaol	30.7921	47.6857	-117.683	Thr45	2
	Quercitrin	62.7811	69.1273	-478.0884	Gly29; Thr45; Lys127; Thr32	4

Table 3: Docking of ginger ligands with selected cancer targets.

EGFR was seen overexpressed in a variety of cancer like NSCLC [22], prostate cancer [23]. c-Met is found overexpressed in variety of cancers like, breast cancer [24] and ovarian cancer [25]. NF-kβ activated in different types of solid tumors like prostate, breast, cervical, pancreatic, gastric, ovarian and lung cancer [26,27]. PI3k is a signaling molecule that plays a critical role in regulating apoptosis. Mutated phosphoinositide 3-kinase causes cancer development, is highly activated in variety of cancer like, gastric, colon, breast, pancreatic, prostate, cervical, ovarian, skin and lung cancer [27,28]. COX-2 is overexpressed in every premalignant and malignant condition colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck and esophagus [29].

Drug likeliness analysis

The ADME/Toxicity analysis of ginger ligands and approved drugs are listed in Table 4. Adsorption, Distribution, Metabolism, Excretion and Toxicity (ADME/T) descriptor levels of the analogs were obtained from the ADME Descriptors protocol of DS 4.0 which is presented in Table 1. Among the ginger ligands 6-gingerol showed good solubility and adsorption with medium BBB level, nontoxic and non-inhibitor of the enzyme CYP2D6 in metabolism of xenobiotic in the body. In case of approved drugs likes, disulfiram showed low solubility, good absorption, very high penetration, toxic and non-inhibitor while quercitrin showed good solubility, very poor absorption, very low penetration, toxic and non-inhibitor of the enzyme CYP2D6 in metabolism of xenobiotic in the body.

Based on docking result and ADME/Toxicity analysis, 6-gingerol was found superior among ginger ligands and approved drugs. Hence 6-gingerol was carried forward to validate the anticancerous properties through cell culture studies.

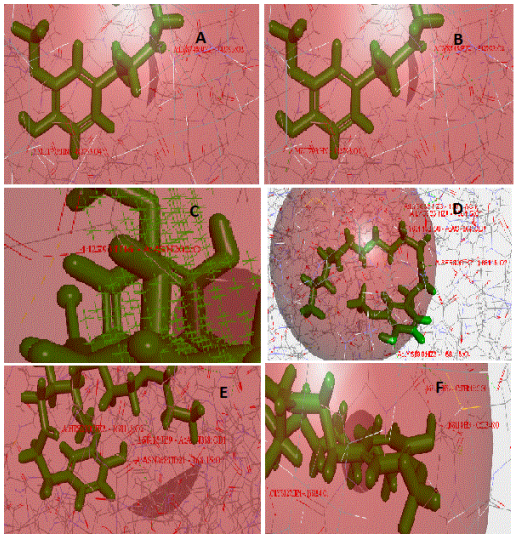

Figure 1: Docking of 6-gingerol with 1XKK, 4GG7 and 2V2T (a, b and c), 10-gingerol with 1E8W and 3LN1 (d and e) and 8-gingerol with 4HMY (f).

Compounds name	ADMET Solubility level	ADMET Absorption level	ADMET BBB Level	Hepatotoxic prediction	CYP2D6 Prediction
6-gingerol	3	0	2	False	False
8-gingerol	3	0	1	False	False
10-gingerol	3	0	1	False	False
6-shogaol	2	0	1	False	False
Disulfiram	2	0	0	True	False
Quercitrin	3	3	4	True	False

Table 4: ADME/Toxicity properties of ginger ligands and approved drugs.

In vitro cytotoxicity of 6-gingerol

MTT assay was performed to determine the cytotoxicity of 6-gingerol on HCT15, L929 and Raw 264.7 cells with 17, 34, 68, 102, 136 and 170 M concentrations. 6-gingerol was found to inhibit the cell growth in all the cells studied. The viability of the cells decreased significantly by 6-gingerol in a dose dependent manner. Cytotoxicity of 6-gingerol on different cancer cell lines at different concentrations 24 h. after treatment is shown in Figure 2. The IC50 value of 6-gingerol on different cancer cell lines viz. HCT15, L929 and Raw 264.7 was observed at 100 µM, 102 µM and 102 µM respectively 24 h. after treatment (Table 5).

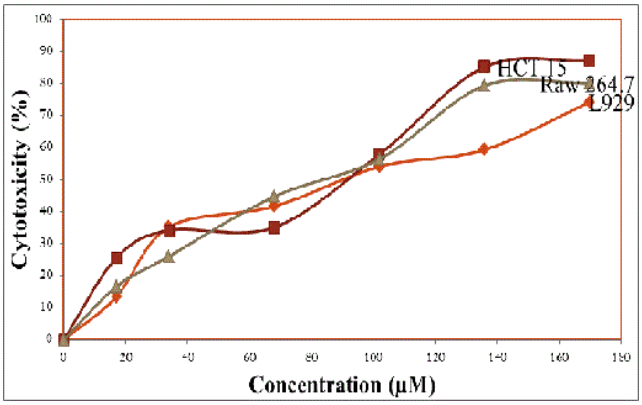

Figure 2: Cytotoxicity of 6-gingerol in different cell lines 24 hours after treatment.

Sixteen percent reduction was observed in cell viability at 10 µM concentration of 6-gingerol and 6-paradol [29] when anticancerous effect was studied in MDA-MB-231 cells (breast cancer). In this investigations, 13 per cent reduction in cell viability was observed in L929 (murine fibro sarcoma cell), 25 per cent in HCT15 (colon cancer cell) and 26 per cent in Raw 264.7 (mouse leukaemic monocyte macrophage cell) at 17 µM concentration of 6-gingerol (Figure 2 and Table 5).

Concentration of 6-gingerol (µM)	Percentage of dead cells*		
	L929	HCT15	Raw 264.7
17	13.35	25.47	26.30
34	35.16	34.1	49.09
68	41.61	35.03	63.64
102	54.04	57.87	65.33
136	59.32	85.17	79.64
170	74.22	87.11	87.15

Table 5: Effect of 6-ginerol on cytotoxicity in cancer cell line 24 hour after treatment. *Percentage of dead cells calculated over control. Percentage of dead cells in control=0 Control is the cell line without 6-ginerol and percentage of dead cells observed in control is zero.

Conclusion

Cancer is a leading cause of death worldwide and more than half of cancer deaths are due to lung, stomach, liver, colorectal and female breast [1]. The present investigations paved way to prove the effectiveness of 6-gingerol as an anticancerous phytocompound through molecular docking and cell culture studies and to highlight the potential of 6-gingerol for drug development against cancer. Pharmacological investigations have revealed that ginger and its major pungent ingredients have chemopreventive and chemotherapeutic effects on a variety of cancer cell lines [19]. As 6-gingerol is identified as a very good phytocompound compared to other ginger ligands like

8-gingerol, 10- gingerol and 6-shogaol and approved drugs like, Disulfiram and Quercitrin, research thrust may be focused on drug development using 6-gingerol against cancer.

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