Screening of phytochemicals against KEAP1- NRF2 interaction to reactivate NRF2 Functioning: Pharmacoinformatics based approach

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Abstract: The transcription factor NF- E2 p45-related factor 2 (NRF2; encoded by *NFE2L2*) and its principal negative regulator, the E3 ligase adaptor Kelch- like ECH-associated protein 1 (KEAP1), significantly contribute to regulation of redox, metabolic and protein homeostasis, as well as inflammation. Therefore, activation of NRF2 imparts cytoprotective effect in numerous pathophysiological conditions including chronic diseases of the lung and liver; autoimmune, neurodegenerative and metabolic disorders and cancer initiation. The objective of this study was to screen library of phytochemicals to identify phytochemicals for direct inhibition of the Keap1–Nrf2 interaction using molecular docking approach. As a result, natural compounds such as 3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide, Phlorizin, Diffutin, Liquiritin and Dihydrogenistin were identified as direct inhibitors of the Keap1–Nrf2 interaction, as evident from its high binding affinity and occupancy of specific binding sites of Klech domain. Thus these phytochemical could be proposed to improve cell resistance to oxidative stress, suggesting their potential as antioxidants. Moreover, the selected compounds fulfilled the Lipinksi rule and appropriate ADMET properties potentiating its efficacy. However, these need to be validated through experimental approaches to ensure its safety profile and efficacy.

Keywords: Medicinal plants, phytochemicals, molecular docking, KEAP1, NRF2

INTRODUCTION

The constant exposure of human body to oxidative and reactive chemicals can lead to oxidative stress causing inflammation that is a type of protective response of body against certain physiological conditions. Chronic inflammation caused by various regulatory mechanisms is a major contributor of cancer (Leon-Cabrera *et al*., 2019). The protein-protein interaction between KEAP1 and NRF2 exhibit one such mechanism that is responsible for cytoprotective response to exogenous or endogenous oxidative and electrophonic stresses (Xing *et al*., 2015). The involvement of Nrf2 in anti-oxidant responses intensified its importance in various physiological and pathological processes. Therefore targeting the interaction between these two proteins presents an excellent opportunity for the prevention and treatment of various oxidative, inflammatory and degenerative diseases. The oxidative homeostasis of cell is maintained by cellular level of Nrf2 that is regulated by its inhibitor KEAP1 (Abed *et al*., 2015).

KEAP1 acts as a sensor for oxidative stresses and a repressor for Nrf2 (Tu *et al*., 2019). The KEAP1 harbors cysteine residues specific for its activity such as i.e. Cys 273 and Cys 288 (Yamamoto *et al*., 2008). The later has low expression in cell under normal conditions because of 26S proteosomal degradation by Keap1 (Saito *et al*., 2016) KEAP1 is an adaptor component of Cullin 3- based ubiquitin E3 ligase complex. In the absence of stress KEAP1 promotes proteosomal degradation of cytoplasmic Nrf2 as it supports ubiquitination followed by proteosomal degradation of Nrf2 in basal state of cell. (Furukawa *et al*., 2005, Kobayashi *et al*., 2004, Staurengo-Ferrari *et al*., 2018a).

One of the theory proposes that when cell is exposed to oxidative stress cysteine residues in the BTB and IVR domain is modified causing change in conformation of KEAP1 protein leading to dissociation of Nrf2 (Wei *et al*., 2019). It is reported that Nrf2 plays important role in cellular redox homeostasis of lung for acute to chronic respiratory tract conditions such as respiratory infections, asthma, (ARDS) and lung cancer etc. (Korytina *et al*., 2019). Recent research also suggest that oxidative stress is the cause of different kidney diseases and Nrf2 has protective role in diseases like renal inflammation, hypertension and other pathological conditions related to kidney (Staurengo-Ferrari *et al*., 2018b). Being master regulator for anti-inflammatory and redox responses Nrf2 has also significant role in liver diseases (Chambel *et al*., 2015). Moreover, the Keap1 binds to Nrf2 through DLG and ETGE motifs of Neh2 domain of Nrf2 and upon oxidative stress specific cysteine residues in KEAP1 undergoes covalent modification which intern liberate Nrf2 to transcribe various genes involved in relieving electrophilic or oxidative stress (Dikovskaya *et al*., 2019, Wei *et al*., 2019).

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In current study, phytochemicals are used to predict a potential inhibitor against KEAP1, which blocks the Nfr2 binding site of this protein and helps to alleviate the level of Nrf2. The use of phytochemical based medicines is increasing due to their structural diversity, availability, exhibition of multiple target activities and negligible side effects (Velu *et al*., 2018). With the help of molecular docking software MOE (Molecular Operating Environment) a library of about 5000 phytochemicals has been screened against KEAP1 to identify potential inhibitors of KEAP1 protein.

MATERIALS AND METHODS

Structure retrieval and optimization

The 3D structure of KEAP1 protein was retrieved from protein data bank (PDB) using PDB ID: 2FLU. The retrieved structure was optimized removing ligand and solvent residues, 3D protonation and energy minimization using Molecular Operating Environment (MOE) (Wen *et al*., 2019).

Ligand library preparation

About 5,000 phytochemicals were retrieved from PubChem (Kim *et al*., 2018), MPD3 (Mumtaz *et al*., 2017) and MAPS database (Emerson *et al*., 2014) and a ready-to-dock library was prepared. To scan the inhibitors, docking have been performed against KEAP1 via MOE software package.

Molecular docking

Ready-to-dock library of phytochemicals was docked with interacting residues of KEAP1 by using docking algorithm of MOE software. The MOE program validates accurate conformation of ligand to get minimum energy structure. After docking, phytochemicals with best and top conformation were determined on the basis of S-score and RMSD value.

In silico analysis of drug likeness and ADMET properties

The best docking scoring phytochemical was then subjected to further selection on the basis of Lipinski's rule of five (Ro5) (Benet *et al*., 2016). This was simply done by Molinspiration server (Jarrahpour *et al*., 2012) for calculation of their physicochemical properties. In order to evaluate drug like characteristics the candidates were subjected to SwissADME software (Daina *et al*., 2017). The calculation of ADMET properties i.e. Absorption, Distribution, Metabolism, Excretion, and Toxicity are an important indication of drug candidate's behavior, fate and level of toxicity in human body.

RESULTS

Database screening and docking study

The 3D structure of KEAP1 was retrieved from Protein Data Bank by PDB ID: 2FLU. Among other structures available for activity of KEAP1 this was the only structure with low RMSD value and a resolution of 1.50. Moreover it was frequently used in previous studies (Canning *et al*., 2015). This structure was optimized to find the antagonist that could interfere with the interaction and does not allow KEAP1 to bind with NRF2. Docking was performed by using ready-to-dock library of phytochemicals. All the residues participating in the reaction of KEAP1 with other proteins or ligands were found with the help of MOE site finder tool. The library of Phytochemical was docked against the KEAP1 protein and docked compounds were ranked on the basis of stringent filter that accounts four factors, maximum occupancy of binding pocket with minimum Gibbs free energy, strength of hydrogen bonding +and other potential non-covalent interaction. Out of 5000 docked molecules, six top ranking docking poses were selected based on maximum binding sites occupied by ligand, minimum S-score and lower RMSD values. These candidate compounds exhibited their minimum binding energy in the range of - 18 Kcal/mol to - 26 Kcal/mol.

However, for all the selected binding sites 3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide was ranked at the top as it exhibited maximum binding score and binding affinity. Most of the phytochemicals i-e four out of top five tend to exhibit strong binding affinity toward amino acid Arg415 and Ser602 suggesting that these are most active residues. These are followed by Ser508, which exhibited interaction in selected two compounds respectively.

ADMET/Drug scan results

The drug likeness of the proposed KEAP1 inhibitors were predicted by ADMET based drug scan tool at Molinspiration server. Among selected candidate Four shows 0 violations while one shows 1 violation to the Lipinski's rule of five and acceptable drug-like properties, i.e. molecular weight (table 2). All the candidate compounds were subjected to Swiss ADME to assess them for their drug like properties, server in order to further validate the potential of drug likeliness (table 3).

DISCUSSION

Keap1-Nrf2 regulatory mechanism is one of the most important pathways against oxidative stress in human body and Insilco analysis of their interaction provides an excellent opportunity to design a potent drug for diseases caused by irregularities in this pathway (Lu *et al*., 2016). There are various ways such as insilico and in-vitro analysis to study the effect of small inhibitory molecules on the partnership of KEAP1 and Nrf2 proteins (Aakre *et al*., 2015). But molecular docking is a simple and cost-efficient method to predict molecular interaction therefore we use this approach to predict potential inhibitors of KEAP1 that can effectively block its binding to Nrf2. The

Table 1: Interaction detail of top seven bioactive phytochemicals in the proposed site of KEAP1 protein

S. No.	PubChem ID	Chemical name	Docking score	RMSD value	Receptor	Interaction
1	574839	3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide	-26	1.6	Ser602 Ser363 Asn382 Arg380 Arg415 Arg483 Ser508 Asn414 Gly354	H-acceptor H-donor H-donor H-donor pi-cation H-donor H-donor H-acceptor H-acceptor
2	6072	Phlorizin	-21	1.15	Ser602 Gln530 Arg415	H-acceptor H-donor H-acceptor
3	442352	Diffutin	-20	1.33	Arg415 Ser602	H-donor H-acceptor
4	503737	Liquiritin	-18	1.70	Arg415 Ser602 Val 465	H-donor H-acceptor H-donor
5	11070108	Dihydrogenistin	-18	0.75	Ser602 Arg415	H-acceptor H- donor

Table 2: Results of compounds examined for Lipinski rule

Compound	Molecular weight (g/mol)	Number of HBA(nON)	Number of HBD nOHNH	MLogP
Lipinski's rule of five	<500	<10	<5	<5
3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide	219.29	4	2	1.54
Phlorizin	436.41	10	7	0.40
Diffutin	464.47	10	5	0.89
Liquiritin	418.40	9	5	0.41
Dihydrogenistin	434.40	10	6	0.55

structure of KEAP1 (PDB ID: 2FLU) was subjected to molecular docking and screened against a ligand library of 5000 phytochemicals. The potential of these compounds to interfere with KEAP1-Nrf2 binding was checked. Which lead towards five potent phytochemicals as follows: 3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide, Phlorizin, Diffutin, Liquiritin and Dihydrogenistin. These phytochemical were screened based on their binding affinity and docking score. Several studies aimed to discover potent inhibitor of KEAP1 protein. In one study small egg derived peptides were used as inhibitor of KEAP1 and Nrf2 interaction by using both Insilco and in vivo approaches. Their result shows that egg derived DKK and DDW has inhibitory activity against KEAP1 by occupying its key residues that are involved in its binding with Nrf2. Particularly DDW which has binding affinity for most of the residues in Kelch domain of KEAP1 (Li *et al*., 2017). In another study compounds from traditional Chinese poly herbal drug were screed for Keap1 inhibition to study the effect of oxidative stress on psychological disorders. Their results demonstrated that among the 10 compounds 3 shows higher binding affinity then control as follows: baicalin with binding affinity (−7.45 kcal/mol), oroxyloside with binding affinity (−7.89 kcal/mol) and liquiritin with binding affinity (−6.86 kcal/mol). These active compounds significantly enhance Nrf2 activity by acting as KEAP1 inhibitor by covalently modifying cysteine residues of KEAP1 which release Nrf2 from its inhibition by KEAP1. As a result these compounds reduced the cellular level of reactive oxygen species (ROS) by acting as KEAP1 inhibitor (Satoh *et al*., 2015). In a similar study by Jiang *et al.* (2014) a synthetic compound named compound 2 was reported to be an excellent inhibitor of protein-protein interaction between KEAP1 and Nrf2 as well as it significantly enhance Nrf2-ARE interaction. Their docking results shows that compound 2 has strong polar interaction with Arg 415 and Arg 483 while occupying all five sub pockets of KEAP1 (Jiang *et al*., 2014).

An *in-vitro* analysis by (Cosimelli *et al*., 2019) reported three high throughput synthetic inhibitors for Nrf2/KEAP1 inactivation. Nine indole derivates were

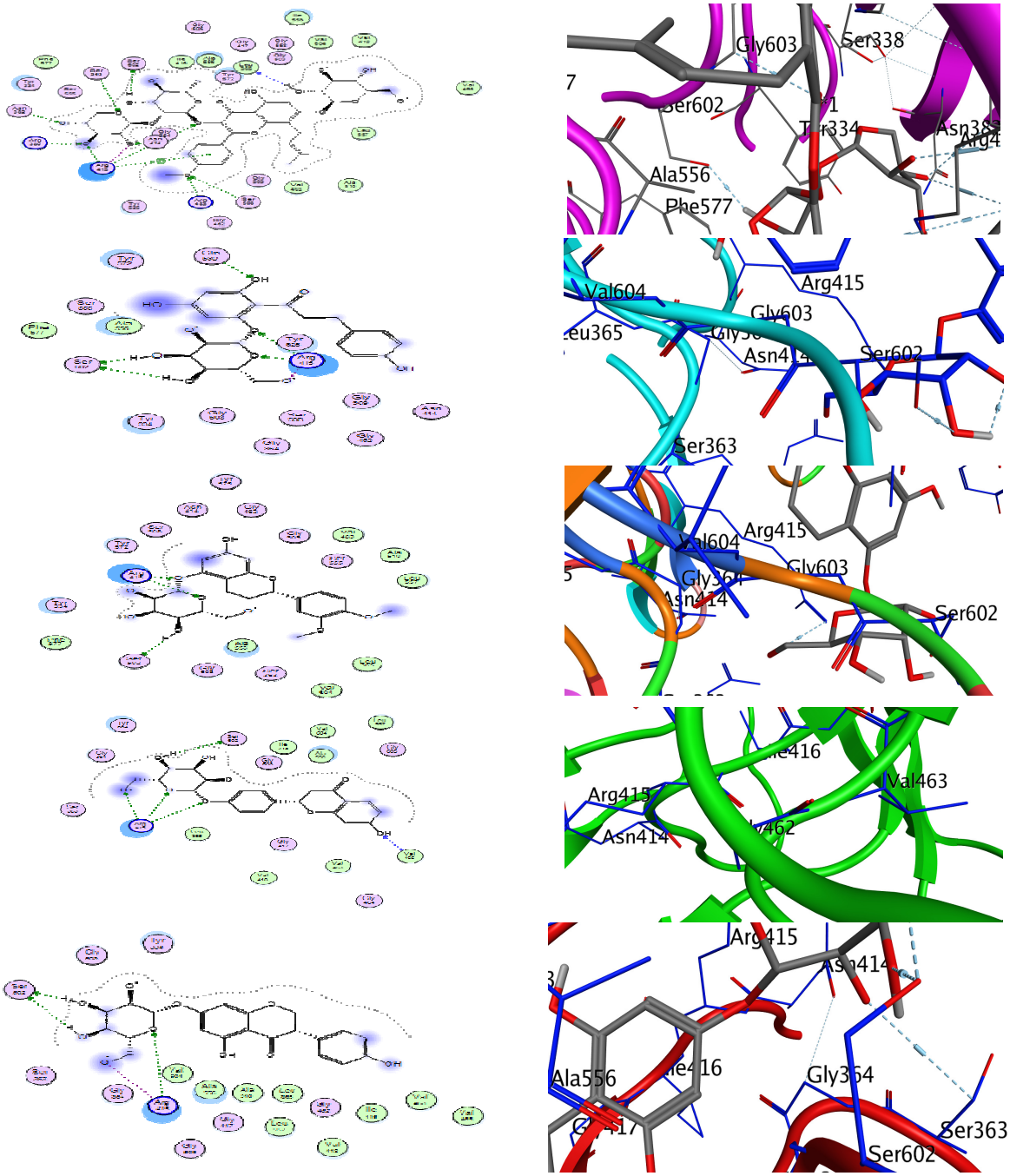

Fig. 2D and 3D illustration of selected docked complex

tested for their inhibitory activity out of which 3 compounds give good results and based upon experimental results and molecular docking analysis one compound, name 9g was particularly active against this pathway. 9g binds to active residues arginine 483 and serine 508 in pockets of KEAP1. The ampholytic nature of this proposed inhibitor is first time reported for KEAP1/Nrf2 regulatory pathway.

Furthermore recently a potential KEAP1 inhibitor has been reported by (Jiang *et al*., 2018) that has the potential to be used as an effective drug against LPS- induced Cardiomyopathy. They used an in house library of 569 synthetic chemicals to dock against KEAP1, out of which one synthetic compound named 'ZJO1' shows 81.25% inhibition of KEAP1-Nrf2 protein-protein interaction. In contrast to previously reported synthetic inhibitors, our study shows five potential phytochemical based ligands of KEAP1 that can acts as an inhibitor for interaction between KEAP1 and Nrf2 by blocking the Nrf2 binding site of KEAP1 protein.

Table 3: ADMET profiling enlisting absorption, metabolism and toxicity related drug like parameters of candidate compounds

Compounds	3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide	Phlorizin	Diffutin	Liquiritin	Dihydrogenistin
A. Absorption					
Blood-Brain Barrier	Yes	No	No	No	No
Gastro-Intestinal Absorption	High	Low	Low	Low	Low
P-glycoprotein substrate	No	Yes	Yes	Yes	Yes
B. Metabolism					
CYP450 1A2 Inhibitor	No	No	No	No	No
CYP450 2C9 Inhibitor	No	No	No	No	No
CYP450 2D6 Inhibitor	No	No	No	No	No
CYP450 2C19 Inhibitor	No	No	No	No	No
CYP450 3A4 Inhibitor	No	No	No	No	No

CONCLUSION

In the current study phytochemicals including 3-(Dimethylamino)-3-imino-N-(4-methylphenyl) propanamide, Phlorizin, Diffutin, Liquiritin and Dihydrogenistin are identified as the potential phytochemicals that shows strong binding capability with KEAP1 protein, also demonstrating all of the drug-like properties. The results of our present study can be useful for the designing and development of novel drugs having better inhibitory activity against Keap1 protein. Further in vitro and in vivo studies are highly advised to check for the efficacy of this study.

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