Received: 21 December 2018 | Accepted: 4 March 2019

DOI: 10.1111/bcpt.13222

ORIGINAL ARTICLE

BCPT Basic & Clinical Pharmacology & Toxicology

Identification of compounds that inhibit the binding of Keap1a/Keap1b Kelch DGR domain with Nrf2 ETGE/DLG motifs in zebrafish

Azhwar Raghunath[1] | **Raju Nagarajan**[2] | **Kiruthika Sundarraj**[1] | **Kavipriya Palanisamy**[1] | **Ekambaram Perumal**[1]

[1]Molecular Toxicology Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, India

[2]Department of Biotechnology, Indian Institute of Technology Madras, Chennai, India

Correspondence
Ekambaram Perumal, Molecular Toxicology Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, India.
Email: ekas2009@buc.edu.in

Abstract

The Keap1-Nrf2-ARE system serves as a premier defence mechanism to curb oxidative stress, which remains as one of the major causes of ageing and pathogenesis in various diseases. Nrf2 is the principal master regulator of the cellular defence system, and its activation remains the prospective therapeutic approach against chronic diseases. One of the recent strategies is to disrupt Keap1-Nrf2 protein-protein interaction (PPI) that alters the docking of Keap1 with Nrf2 by compounds occupying a position in the Keap1 blocking the interface with Nrf2. In this study, we made an attempt to identify the compounds with anticancer, antioxidant and anti-inflammatory properties to disrupt Keap1a/b-Nrf2 PPI through in silico molecular docking in zebrafish. The phylogenetic analysis of Keap1 proteins revealed the existence of orthologous Keap1-Nrf2-ARE system in lower vertebrates that includes zebrafish. The DGR domains of zebrafish Keap1a and Keap1b were modelled with Modeller 9.19 using Keap1 of *Mus musculus* (PDB ID:5CGJ) as template. Based on the docking calculations, top hit compounds were identified to disrupt both Keap1a and Keap1b interaction with Nrf2 which include quercetin 3,4′-diglucoside, flavin adenine dinucleotide disodium salt hydrate, salvianolic acid A, tunicamycin and esculin. The LC_{50} of esculin in 3 dpf zebrafish larvae is 5 mmol/L, and the qRT-PCR results showed that esculin significantly increased the transcription of Nrf2 target genes—*Gstpi*, *Nqo1*, *Hmox1a* and *Prdx1* in 3 dpf zebrafish larvae. These potential hits could serve as safer Nrf2 activators due to their non-covalent disruption of Keap1-Nrf2 PPI and be developed into efficacious preventive/therapeutic agents for various diseases.

KEYWORDS

esculin, Keap1-Nrf2-ARE pathway, non-covalent interaction, oxidative stress, protein-protein interaction, zebrafish

1 | INTRODUCTION

It is ineludible that humans are constantly introduced to endogenous and exogenous chemicals.[1] Such exposure to these chemicals vitiates the redox homeostasis and leads to oxidative stress. Persistent oxidative stress inflicts damage to lipids, nucleic acids and proteins that result in cancer, cardiovascular, neurodegenerative and respiratory diseases.[2] All these diseases are the foremost cause for the deaths in humans

Raghunath and Nagarajan contributed equally to this work.

worldwide.[3] Chemoprevention is the strategy employed to alleviate these diseases with both natural and synthetic compounds.[4] Most eukaryotic systems have an intricate protective system called the Kelch-like ECH-associated protein 1—nuclear factor-erythroid 2-related factor 2—antioxidant response elements (Keap1-Nrf2-ARE) signalling pathway through which they orchestrate the regulation of an array of genes to hamper stress. This pathway regulates the synthesis of the phase II enzymes and antioxidant proteins, which detoxifies both the endogenous and exogenous deleterious chemicals in the eukaryotic cells.[5,6]

A basic leucine zipper (bZIP) transcription factor, Nrf2, ameliorates the oxidative stress and maintains redox homeostasis.[7] Thus, modulation of Nrf2 still remains as one of the therapeutic strategies for oxidative stress-related diseases.[8-10] Keap1 is an inhibitor of Nrf2 and a key negative regulator of Keap1-Nrf2-ARE signalling pathway.[11] Keap1 retains Nrf2 in the cytoplasm and targets Nrf2 for degradation via ubiquitin-proteasome pathway during unstressed conditions.[12] Two important motifs, the Glu-Thr-Gly-Glu (ETGE) and the Asp-Leu-Gly (DLG), in the Nrf2's Nrf2 extended homology 2 (Neh2) domain bind with Keap1's Kelch/double glycine repeat (DGR) domain in a hinge and latch fashion.[13] Upon stress, this interaction gets disrupted and allows the stabilization and translocation of Nrf2 into the nucleus. Once inside the nucleus, Nrf2 binds AREs and activates abundant Nrf2 target genes eventually offer cellular protection against oxidative insults.[14]

The identification of Nrf2 activators has enticed the attention of researchers around the world as they can be utilized as therapeutic agents against oxidative stress-related diseases. Two primary mechanisms elicit Nrf2 activation, one being the modification of cysteine residues in Keap1 and another being the disruption of protein-protein interaction (PPI) between Keap1 and Nrf2.[15-20] In this study, we made an attempt to identify Keap1-Nrf2 PPI inhibitors that disrupt PPI between Keap1a/b and Nrf2 in the zebrafish (*Danio rerio*) model system rather than identifying electrophilic activators, which covalently modify the cysteine residues in the Keap1. The former is preferred the most due to their less toxic nature over the latter, as the latter modifies thiol groups of other off-target proteins leading to unpredictable toxic effects.[21,22]

The zebrafish model system serves as a formidable pharmacological tool due to its high relevance with humans in disease-associated targets, drug metabolism, physiology and pharmacology.[23,24] In addition, zebrafish are immensely used as representative disease models in research.[25] The drug response in humans shows high resemblance in zebrafish, thus offers a quick way to develop therapeutics against human diseases.[26] Besides these above facts, the Keap1-Nrf2-ARE regulatory system is highly conserved among vertebrates from fish to mammals.[27] Novel Nrf2 activators are being identified and tested in zebrafish as their use in a disease model gets increased. Such identification of potential compounds in zebrafish will help scientists to test in humans. Hence, the zebrafish was used as the model system to identify the molecular activators of Keap1-Nrf2-ARE system. Molecular docking was carried to find the compounds with potential anticancer, antioxidant and anti-inflammatory properties that disrupt the PPI between Keap1a/b and Nrf2 in the zebrafish. Two types of Keap1, Keap1a and Keap1b, were present in the zebrafish. Both types regulate Nrf2 negatively in zebrafish. In this study, we investigated the binding of ligands with both Keap1a and Keap1b of zebrafish. This study sheds light on the use of zebrafish for the discovery of Nrf2 activators through in silico and in vivo approaches.

2 | MATERIALS AND METHODS

2.1 | Phylogenetic analysis of Keap1 proteins

The zebrafish amino acid sequences of both Keap1a (Q1ECZ2) and Keap1b (A9CP01) were obtained from UniProt, and these two sequences were used to retrieve available Keap1 sequences of other organisms through basic local alignment search tool (BLAST) in UniProt. The Keap1 amino acid sequences of the different organisms were aligned using Clustal Omega.[28] ESPript 3.0 was used to visualize the alignment of the Keap1 sequences.[29] All the 117 different organisms' Keap1 sequences were aligned by MUSCLE using the MEGA 7.0 for phylogenetic analysis.[30] The Poisson correction method was employed to compute the evolutionary distances among the Keap1 in MEGA 7.0. The tree was displayed and annotated using interactive tree of life (iTOL) v4.2.[31] The Newick file format was given as input to visualize and annotate the tree in iTOL.

2.2 | Structural modelling of Keap1a and Keap1b Kelch DGR domain of zebrafish

From zebrafish Keap1a (Q1ECZ2) and Keap1b (A9CP01), the sequences of Broad complex, Tramtrack and Bric-a-Brac (BTB), intervening region (IVR) and DGR domains were identified. The crystal structures of the DGR domain and whole Keap1a or Keap1b protein of zebrafish have not been available so far. In order to model the DGR domains of both zebrafish Keap1a and Keap1b proteins, BLAST was performed against the protein data bank (PDB).[32,33] So far, no crystal structure has been available for human Keap1. From the BLAST analysis, the crystal structure from *Mus musculus* (PDB ID: 5CGJ) was chosen as a template for homology modelling of DGR domains of both zebrafish Keap1a and Keap1b proteins. The 5CGJ crystal structure of *M. musculus* Keap1 complexed with (3S)-1-[4-[(2,3,5,6-tetramethylphenyl) sulfonylamino]-1-naphthyl] pyrrolidine-3-carboxylic acid (RA389) was retrieved from the PDB.[34] RA389 exhibits a non-covalent binding with Keap1. Using Modeller 9.19,[35] the DGR domains of Keap1a

and Keap1b were modelled as the experimentally derived structure was non-existent. The modelled structures were validated using the discrete optimized potential energy (DOPE) scores. Further, the homology modelled DGR domains were subjected to MolProbity analysis[36] to know the percentage of residues in favoured regions in the Ramachandran plot. The final model was validated and chosen based on the DOPE score and Ramachandran plot for further experimental procedures.

2.3 | Molecular docking

The Schrodinger software package was used to evaluate the mode of interaction of the compounds with the modelled DGR domains of zebrafish Keap1a and Keap1b separately and to better understand the inhibitory binding mode of the compounds; 472 compounds with potential pharmacological properties were collected from the literature, and SDF formats of all these compounds were downloaded from PubChem structure database.[37] The preparation of 3D format ligands and addition of polar hydrogens and energy minimizations of ligands were done using the LigPrep module. A maximum of 32 stereoisomers and tautomers were generated for each ligand (Figure S1). These generated ligands were used in Maestro formats. The DGR domains were prepared with protein preparation wizard tool. The DGR domains of both zebrafish Keap1a and Keap1b were optimized and minimized for docking calculations using the protein preparation wizard of the Schrodinger software which assigns addition of charges, proper bond order and protonation state prior to minimization. Ser363, Arg380, Asn382, Arg415, Arg483, Ser508, Tyr525, Gln530, Ser555 and Ser602 are the amino acid residues essential for the binding with ETGE, whereas Ser363, Arg380, Asn382, Arg415, Arg483, Ser508, Tyr525, Gln530, Ser555 and Ser602 are essential for DLG motif binding in Nrf2. All these residues were located as the active site for the docking of the ligands.[27,38-40] The receptor grid was created based on the DGR domain active site amino acids for the docking of each ligand. The 16Å square grid was generated from the centroid of all these selected amino acid residues and workspace ligand for docking calculations. Glide docking tool of Schrodinger was utilized for the identification of the interaction of all the ligands. The Schrodinger software package with OPLS_2005 force field was utilized for the calculations involved in protein and ligand preparations. For each ligand docking, a maximum 10 binding poses were generated, and the best pose was chosen based on the glide score and the interactions with active site amino acid residues.

2.4 | Zebrafish culture

Zebrafish (*D. rerio*) were obtained from local source (Zaman Aquarists, Kolathur, Chennai, India) and cultured in tanks. Fish were maintained at 28°C ± 0.5°C on a 14:10-hour light:dark cycle. Zebrafish were fed freeze-dried blood worms twice daily.

2.5 | Embryo collection

The evening before the day of embryo collection, zebrafish matings (2:1 male to female ratio) were set up in breeding tanks with perforated net that allowed embryos to fall out to the bottom of the tanks. The perforated net prevents the adults from preying on the fertilized embryos. The following day at dawn, the fertilized embryos were collected and staged according to Kimmel et al ; 60 to 100 embryos were maintained in 1× E3 medium pH 7.8 (E3 medium 60× composition: 5 mmol/L NaCl, 0.17 mmol/L KCl, 10 mmol/L HEPES, 0.33 mmol/L $MgSO_4 \cdot 7H_2O$, 0.33 mmol/L $CaCl_2 \cdot 6H_2O$, 0.00002% methylene blue as an antifungal agent) at 28°C in 90 × 15 mm sterile Petri dishes. The 1× E3 medium was renewed daily, and if found, the dead embryos or larvae were removed during renewal. The 3-day post-fertilized (dpf) embryos were utilized for both lethality and gene expression studies. The embryos were not fed throughout the experiment.

2.6 | Lethality effects of esculin

The compound esculin (catalogue No. sc-204744) was obtained from Santa Cruz Biotechnology, Inc, USA. The stock solution of esculin was prepared as per the instructions of the manufacturer and then diluted with E3 medium to make the required concentrations (1.5, 3, 4.5, 6 and 7.5 mmol/L). Two mL of these different concentrations of esculin was aliquoted in the 24-well plate. The 3 dpf larvae were exposed to these five different concentrations with one larva per well in 20 wells and the other four wells left empty.[41] As controls, larvae were exposed to E3 medium in a 24-well plate. The treatment and control plates were placed at 28 ± 0.5°C with a 14:10-hour light:dark photoperiod. The lethality was recorded at 24 hours after treatment using a microscope. The LC_{50} was determined based on the cumulative lethality recorded from three independent experiments and expressed in percentage. The study was conducted in accordance with the basic and clinical pharmacology and toxicology policy for experimental and clinical studies.[42]

2.7 | RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)

To determine the effective dose and exposure time of esculin to activate the transcription of *Nrf2* and its target genes—*Gstpi*, *Nqo1*, *Hmox1a* and *Prdx1*, qRT-PCR was carried out. Esculin at 50, 100 and 200 μmol/L concentrations along with control (E3 medium alone) was exposed to 3 dpf zebrafish larvae in

separate Petri dishes, and gene expression was analysed at 0, 3, 6 and 9 hours after exposure. Total RNA was isolated from the control and esculin-treated zebrafish larvae using Qiagen RNeasy Mini Kit protocol (# 74104, Qiagen, Germany) according to the manufacturer's instructions. The quantity and the quality of the extracted RNA were spectrophotometrically analysed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The integrity of the isolated RNA was checked by running in 2% agarose gel electrophoresis. cDNA was synthesized from 1 μg of the total RNA using RevertAid first-strand cDNA synthesis kit (# K1622 Thermo Fisher Scientific, MA, USA) as per the instructions provided by the manufacturer. Exon spanning type primers and GenBank accession numbers of genes are given in Table S1. The qRT-PCR was performed in Applied Biosystems 7500 using SYBR green fluorescence (# 208054 QuantiNova SYBR green PCR kit) as recommended by the manufacturer. The reactions were run in duplicate together with no-template control for each gene. The thermal cycle conditions include 2 minutes hold at 50°C, 2 minutes hot start at 95°C followed by the amplification for 40 cycles of 15-seconds denaturation at 95°C and 30-second annealing/extension at 60°C. A dissociation step (60°C-95°C) was performed at the end of amplification phase to determine the specificity of each primer set. The relative expression of the mRNA transcripts was quantified using the $\Delta\Delta CT$ method and normalized against *Gapdh* as reference gene.

2.8 | Statistical analysis

The animal experimental data were analysed using statistical package for the social sciences SPSS version 20 (SPSS, Chicago, IL, USA). The results were expressed as mean ± SD of three independent experiments and were analysed with one-way ANOVA followed by Tukey's multiple comparison test. The comparison between the groups was considered significant if $P < 0.05$.

3 | RESULTS

3.1 | Phylogenetic analysis of Keap1 proteins

Phylogenetic analysis of the amino acid sequences of Keap1 proteins was performed to comprehend the relationships of zebrafish Keap1 proteins with available Keap1 proteins of other organisms from the UniProt. The search for the Keap1 proteins in the UniProt showed the presence of Keap1 proteins in a wide variety of species ranging from lower invertebrates to primates. The multiple sequence alignment revealed the conserved residues in Keap1 orthologs (Figure S2). The Keap1 proteins present in the invertebrates represented that the Keap1-Nrf2-ARE defence system was conserved across species from lower invertebrates to higher mammals. The phylogenetic tree constructed was annotated using iTOL (Figure S3). The insects Keap1 proteins originated from the common ancestor and fell under the same unique clade. The sea squirt Keap1 protein displayed distinct position within the tree under a lone clade, whereas the Keap1 proteins of mammals, birds, reptiles and amphibians were grouped under another clade. The two types of Keap1, both Keap1a and Keap1b, are conserved among fishes and are absent in other animals. Yet, both these types exhibit their function in the Keap1-Nrf2-ARE pathway.[48] Though fishes Keap1a and Keap1b are the orthologues of Keap1 of other animals, only fish Keap1b displayed synteny with Keap1, not Keap1a. In comparison with Keap1 of different animals, zebrafish Keap1b remained the homologue of Keap1. Both the Keap1a and Keap1b sequences of zebrafish are grouped within clades consisting of other fishes. Zebrafish Keap1a and Keap1b proteins belonged to clades that contained paralogues from other fishes. Though most of the fish Keap1a and Keap1b proteins in the tree found to have diverged, the common ancestral clade revealed the genome duplication only in the pisces.

3.2 | Structural modelling of Keap1a and Keap1b Kelch DGR domain of zebrafish

The Keap1 of *M. musculus* showed high homology with zebrafish Keap1 proteins (Keap1b: Identity = 76% and Keap1a: Identity = 55%), and the Nrf2 binding domain of zebrafish Keap1 was also similar to that of the mouse (Figure 1A,B). The crystal structure of 5CGJ was used to build the DGR domain structure of both Keap1a and Keap1b of zebrafish. The 3.36Å high-resolution Keap1 template of the mouse ensured the quality of homology model built for DGR domain of Keap1a and Keap1b (Figure 1C). The best model was determined by the minimal DOPE score and the three-dimensional alignment. The superimposition of DGR of Keap1a and Keap1b revealed that the best-modelled structure did not differ with the template (Figure 1D,E). The overall conformations of Keap1a and Keap1b contain root mean square deviations (RMSD) of 0.240 and 0.143Å, respectively. The subtle differences in the RMSD values are due to the presence of the protruding tail region in the modelled structure, which is absent in the template structure. The RMSD of the atomic coordinates of the modelled structure reveal the accuracy of the model with the experimental structure of the target protein. The quality of the modelled structure was assessed through MolProbity analysis of the DGR domain of both Keap1a and Keap1b. The MolProbity score of the DGR domains of Keap1a and Keap1b was 94.55% and 97.39%, respectively, which fall under Ramachandran favoured regions.

3.3 | Molecular docking

In an effort to identify compounds that disrupt the Keap1-Nrf2 interaction and activate the Keap1-Nrf2-ARE signalling

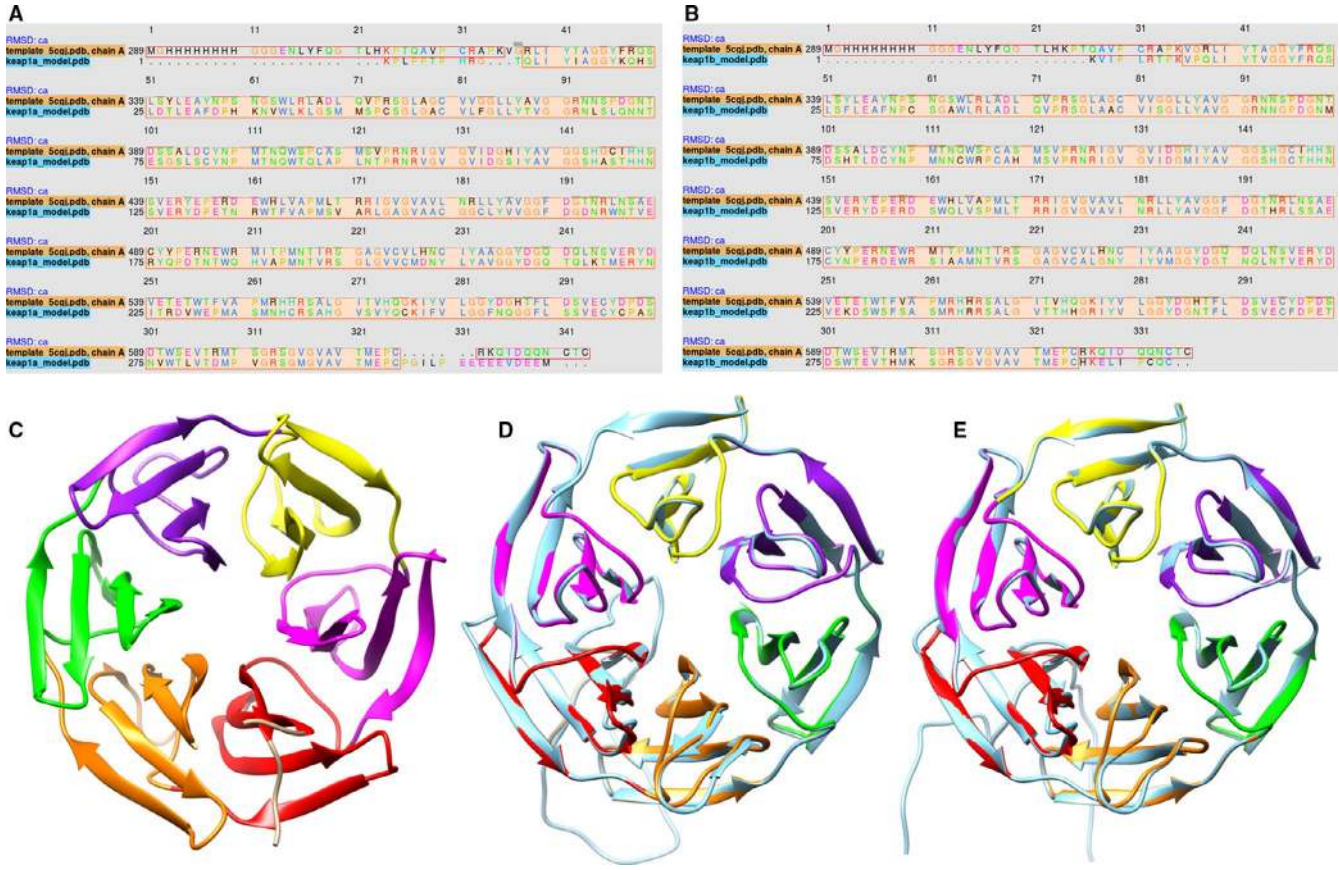

FIGURE 1 Structure-based sequence alignment of the DGR domains of Keap1a (A) and Keap1b (B) with DGR domain of Keap1 of *Mus musculus* (5CGJ.pdb.chain A). The Keap1a and Keap1b sequences share identical DGR domain architecture with 55% and 76% identities, respectively, with *M. musculus* DGR Keap1 sequence. The ribbon representation of the structure of 5CGJ.pdb.chain A (C), the superposed predicted structure of DGR domain of Keap1a (D) and Keap1b (E) with 5CGJ.pdb.chain A shown in top-down orientations. Both these modelled structures superimpose well in the six-bladed beta-propeller structure and four beta-strands in each blades. The template structure 5CGJ.pdb. chain A (C) is represented as follows: Blade 1—residues (598-609 and 327-358) in red, Blade 2—residues (359-409) in orange, Blade 3—residues (410-456) in green, Blade 4—residues (457-503) in purple, Blade 5—residues (504-550) in yellow and Blade 6—residues (551-597) in magenta

pathway in zebrafish, we performed molecular docking to recognize compounds that are putative Nrf2 activators. The hits are selected based on the glide score and energy, hydrogen bond contacts, distance and other interactions of all the ligands with DGR domain of both zebrafish Keap1a (Table S2) and Keap1b (Table S3). The top 15 best hit compounds with DGR domains of both Keap1a and Keap1b were evidenced by its low glide score (Figure 2). The docking poses of the intermolecular interactions of top 10 hits with Keap1a are represented in Figure 3. Quercetin 3,4′-diglucoside formed H-bonds with Ser49, Leu51, Arg101, Val102, Arg169, Ser194, Leu196 and Met290 of the Keap1a Kelch domain in addition to Pi-cation interaction with Arg101. FAD-Na_2 docking with Keap1a showed the formation of both a salt bridge and H-bond with Arg169 besides the H-bonds with Arg66, Asn100, Ala119 and Ser241 and salt bridge with Arg101. Due to the structural similarity among the three ligands—pelargonidin chloride, peonidin chloride and delphinidin chloride exhibited identical interaction with DGR domain of Keap1a at residues Ala149 and Val245. The docking pose of pentagalloyl glucose showed extended interactions with DGR domain of Keap1a. Pentagalloyl glucose was H-bonded to Ser49, Arg66, Asn100, Arg101, Ser194, Tyr211 and Gln260 in addition to Pi-cation interaction with Arg101. Piceatannol—a stilbene similar to resveratrol, showed an identical interaction pattern as that of resveratrol and formed H-bonds with Ser49, Asn100, Arg101, Leu196 and Ser288 of the Keap1a Kelch domain and Pi-cation interaction with Arg101. Piceatannol had an extra H-bond interaction with Leu196 than that of resveratrol. Keap1 accommodated sanguinarium chloride by establishing a lone H-bond with Arg101. Salvianolic acid A formed 2 H-bonds with Val102, a H-bond and salt bridge with Arg169 and a H-bond with Ser288 of the DGR domain of Keap1a.

The intermolecular interactions of top 10 hits with Keap1b DGR domain are shown in Figure 4. The visual inspection of MCULE-7242450970 aka Keap1b-4-O-(4-carboxy-3-hydroxy-3-methylbutanoyl)-3,5-di-O-caffeoylquinic

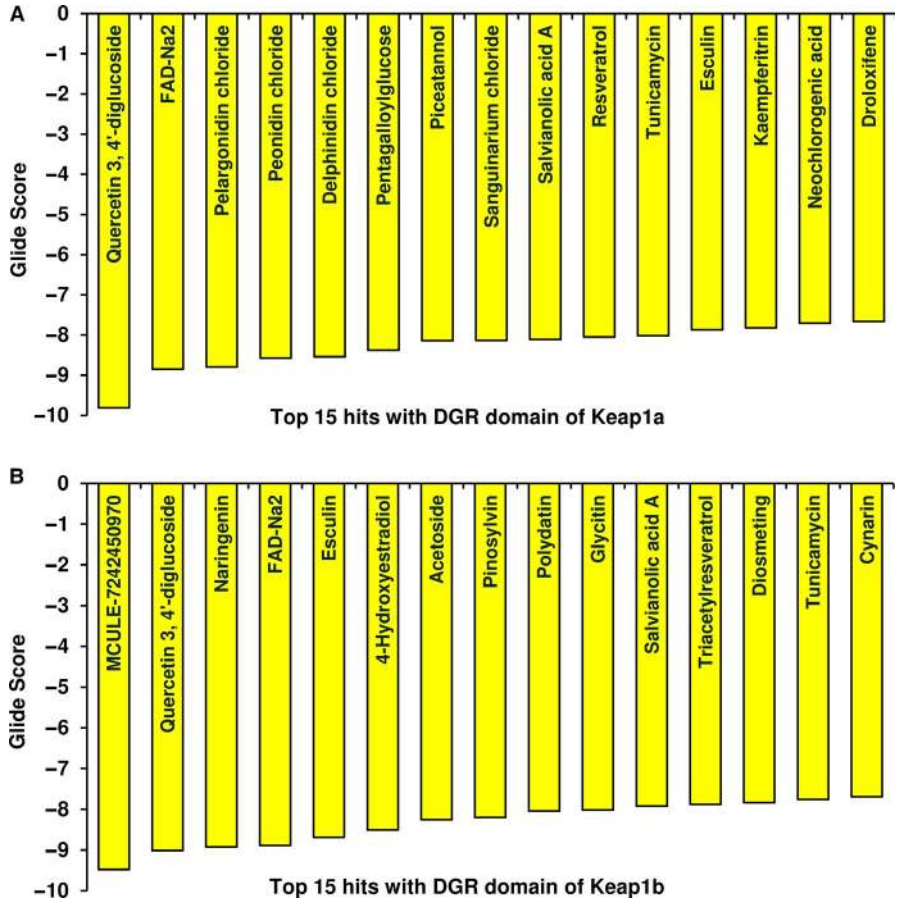

FIGURE 2 Glide score associated with best binding models of top 10 hits with the active site of DGR domain of both Keap1a (A) and Keap1b (B)

acid complex revealed that the ligand is co-ordinated by H-bonds with the following residues—Tyr20, Ser49, Leu51, Asn68, Asn100, Arg101, Arg169 and Leu243. Quercetin 3,4'-diglucoside established binding through H-bonds with Ile102, Val149, Arg169, Ser194, Ser241, Leu243 and Val290 residues of the DGR domain of Keap1b. Naringenin and pinosylvin made identical H-bond interactions with Ala53, Val198 and Val292 of the Keap1b Kelch domain. The extensive interaction was reported in FAD-Na_2:DGR domain complex through H-bonds, salt bridges and Pi-cation. The residues Ser49, Asn68, Asn100, Arg169, Ser194 and Ser288 formed H-bonds, where Arg101 established salt bridge and Pi-cation interaction in the complex. Keap1b accommodated esculin through H-bonds with Ala53, Val151, Ala196, Val198, Val290 and Val292. The interaction of 4-hydroxyestradiol with DGR domain of Keap1b was formed through 4 H-bonds: three with Val104 and one with Val151. Acetoside-Keap1b DGR complex exhibited H-bonds with Asn100, Ile102, Arg169, Ala196, and Ser288 and salt bridge with Arg101. Polydatin was stabilized by H-bonds with Asn68, Asn100, Arg101, Ser194 and Ser288 and Pi-cation interaction with Arg101 in Keap1b Kelch domain. Keap1b DGR domains held glycitin through a couple of H-bonds: one with Arg169 and another with Ser194. In the top 15 hits for both Keap1a and Keap1b, the common compounds found were quercetin 3,4′-diglucoside, FAD-Na_2, salvianolic acid A, tunicamycin and esculin. Though these compounds were common hits, their interactions with Keap1a and Keap1b were different due to a significant active site difference in the Keap1a and Keap1b.

3.4 | Lethality effects of esculin

The LC_{50} value of the esculin in 3 dpf zebrafish larvae after 24-hour exposure is 5 mmol/L (Figure 5). The mortality of the larvae after 24-hour treatment was found to increase gradually till the dose of 4.5 mmol/L, and a steep increase in mortality was observed between 4.5 and 6 mmol/L concentration of esculin. 100% mortality was observed at 7.5 mmol/L concentration.

3.5 | Potency of esculin to activate the expression of Nrf2-regulated cytoprotective genes (*Gstpi*, *Nqo1*, *Hmox1a* and *Prdx1*)

To inspect the potency of esculin to activate Nrf2 as a representative compound that disrupt the binding of Keap1a/Keap1b Kelch DGR domain with Nrf2 ETGE/DLG motifs,

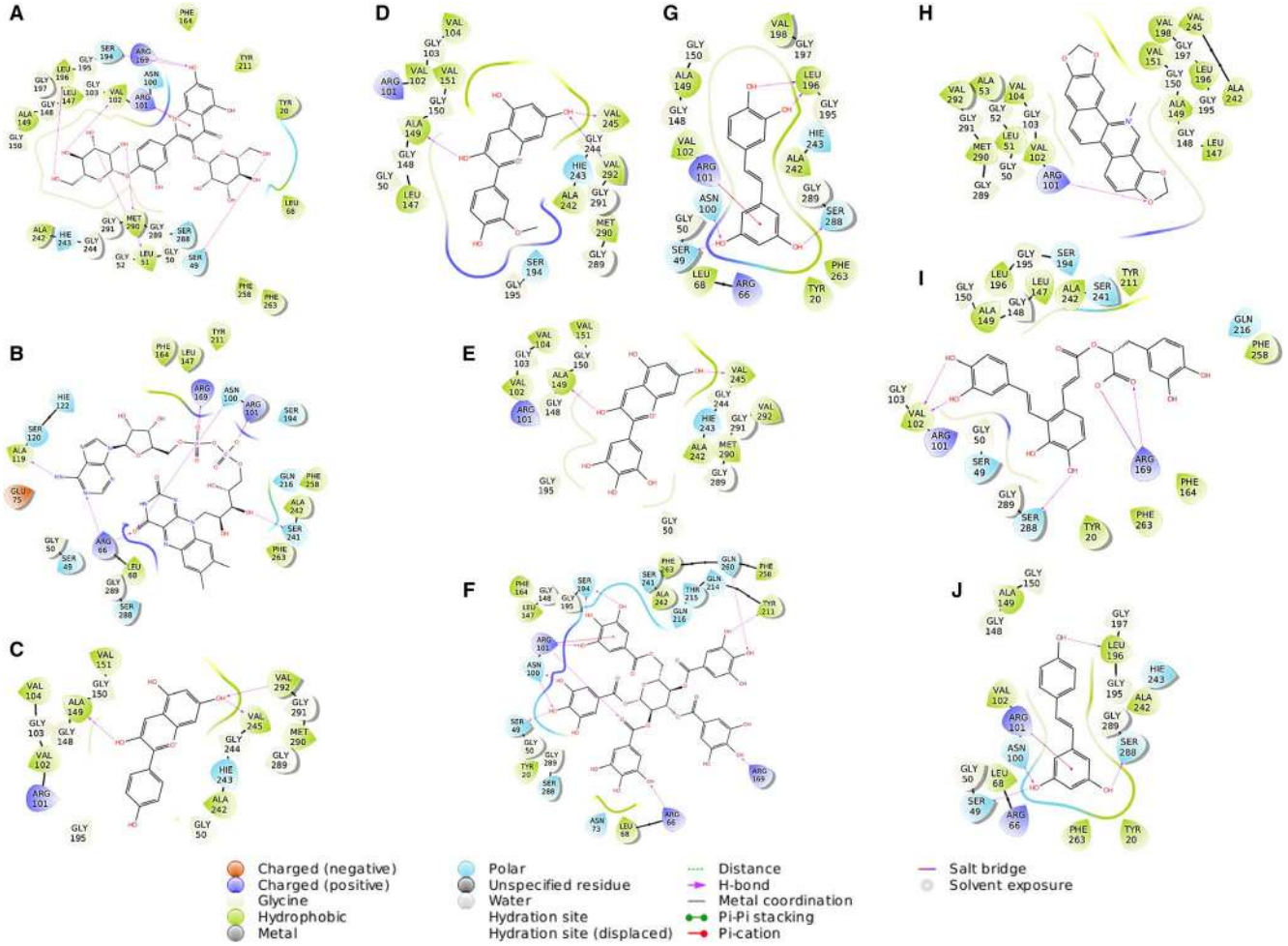

FIGURE 3 Map of 2D interactions of zebrafish Keap1a with ligands depicting the interaction pattern of quercetin 3,4′-diglucoside (A), flavin adenine dinucleotide disodium salt (B), pelargonidin chloride (C), peonidin chloride (D), delphinidin chloride (E), pentagalloyl glucose (F), piceatannol (G), sanguinarium chloride (H), salvianolic acid A (I) and resveratrol (J)

the expression of four Nrf2-regulated cytoprotective genes was carried out. The expression levels of Nrf2-regulated genes after treatment with esculin at three different doses (esculin 50, 100 and 200 µmol/L) and time-points (0, 3, 6 and 9 hours) were analysed. All the three tested concentrations exhibited elevated expression of *Nrf2* and its target genes—*Gstpi*, *Nqo1*, *Hmox1a* and *Prdx1* after 6 h of exposure in 3 dpf larvae (Figure 6). From the perspective of the activation of Nrf2 with time, esculin 100 µmol/L exhibited increased expression of Nrf2 and its four target genes with time up to 9 hours of exposure in 3 dpf zebrafish larvae. With respect to 50 µmol/L esculin, the activation of Nrf2 and its target genes expression started increasing as early as 3 hours and peaked at 6 hours and then declined at 9 hours In the case of 200 µmol/L esculin, the significant elevation of Nrf2 and its target genes expression was observed only at 6 hours after exposure in 3 dpf zebrafish larvae but did not show significant increased expression at both the 3- and 9-hour time-points.

4 | DISCUSSION

The use of zebrafish as disease models has increased in recent years, and also zebrafish exhibit high similarity with humans in many aspects right from development to metabolic pathways. Zebrafish serve as an excellent complementary vertebrate model system in place of mammalian models as the latter models have their limitations in drug development.[49] As several human disease models have been established in zebrafish and the Keap1-Nrf2 system is related to these diseases,[50] we selected zebrafish as a model organism for our study. The phylogenetic tree constructed from the Keap1 proteins available in the UniProt revealed that the regulation of Nrf2 and relevant proteins was identical among all organisms represented in the tree.[48] The Keap1 sequences from Uniprot revealed that Keap1 proteins are absent from archaea, bacteria and plants. The Keap1 phylogenetic tree was similar to that of the "Animal Tree of Life".[51] An interesting observation made in the tree was that all fish carried

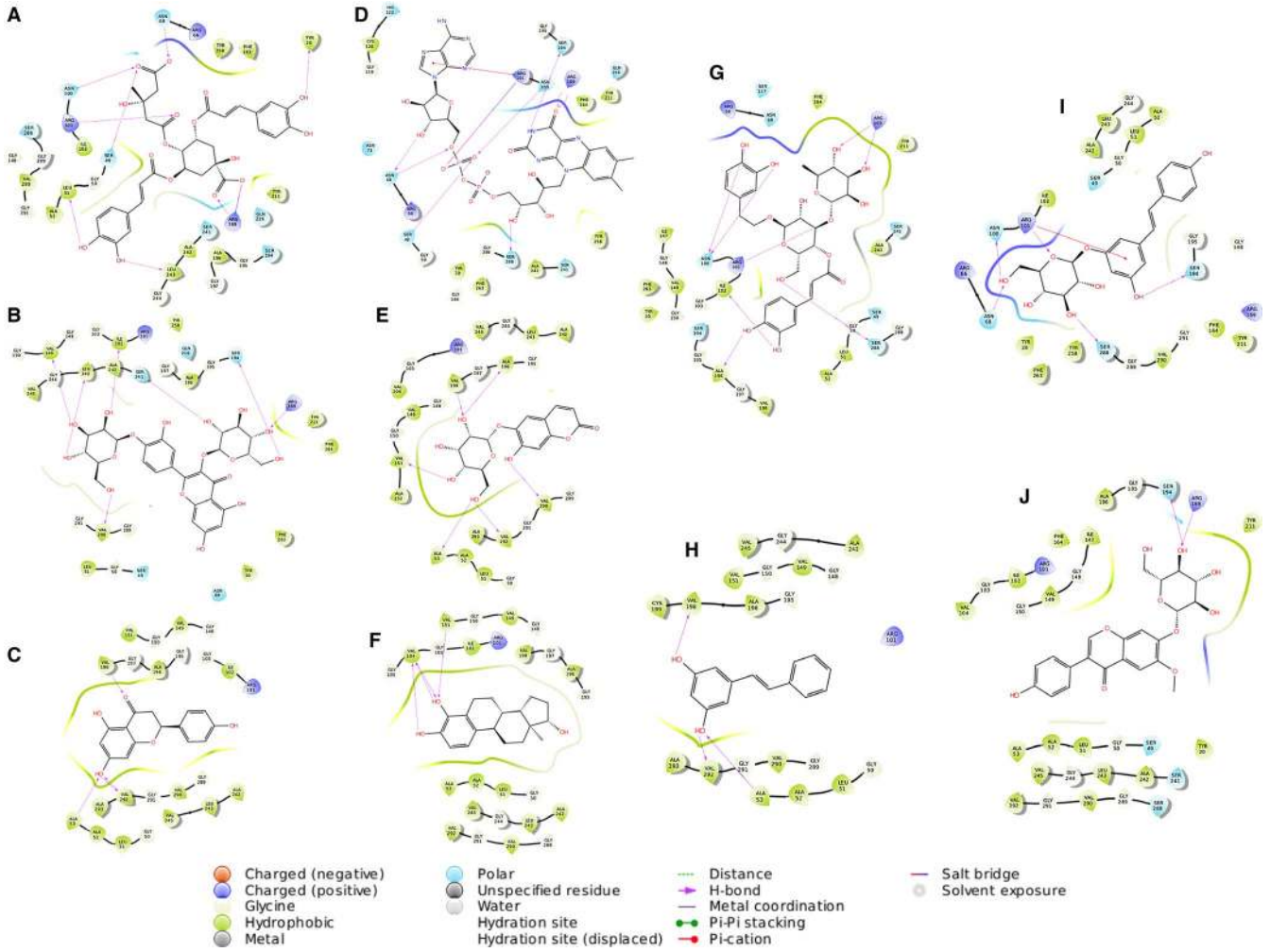

FIGURE 4 Map of 2D interactions of zebrafish Keap1b with ligands depicting the interaction pattern of MCULE-7242450970 aka 4-O-(4-Carboxy-3-hydroxy-3-methylbutanoyl)-3,5-di-O-caffeoylquinic acid (A), quercetin 3,4'-diglucoside (B), naringenin (C), flavin adenine dinucleotide disodium salt (D), esculin (E), 4-hydroxyestradiol (F), acetoside (G), pinosylvin (H), polydatin (I) and glycitin (J)

both Keap1a and Keap1b in them implying that fish regulate Nrf2 by both these co-ortholog Keap1 proteins. The co-orthologues appeared during the emergence of vertebrates and lost during the appearance of amniotes.[50] The synteny was exhibited between zebrafish Keap1b and human Keap1. The conditions that favour the homo- and heterodimerization of these Keap1a and Keap1b in fish remain elusive. Sea squirts—ascidians—have Keap1, which did not show synteny with vertebrates. The Keap1 phylogenetic tree revealed intriguing clades containing western clawed frog, brachiopod lamp shell and Japanese weathervane scallop. Further investigation of the Keap1 proteins in these three animals might reveal the complexity of the Keap1-Nrf2-ARE pathway. The existence of similar orthologous Keap1-Nrf2-ARE system was demonstrated in lower vertebrates that include zebrafish from the constructed phylogenetic tree. Hence, zebrafish remain as an excellent tool to study the Keap1-Nrf2-ARE system.

The above facts necessitate studying the compounds that activate Nrf2 in the zebrafish; 472 compounds exhibiting antioxidant properties were tested for their potential to disrupt Keap1-Nrf2 PPI through molecular docking. Though there are different strategies for targeting PPIs, molecular docking is preferred when the structure of the target is elucidated either through crystallography or nuclear magnetic resonance spectroscopy.[52] There is literature on the covalent modification of cysteine residues in Keap1 proteins leading to the activation of Nrf2, yet only few studies shed light on the disruption of interaction of Keap1 with Nrf2. As zebrafish carries two variants of Keap1 proteins, the interaction of all 472 ligands with DGR domains of both Keap1a and Keap1b was analysed through docking. Upon completion of the screening, the top 10 hits from DGR domains of Keap1a and Keap1b were selected based on the binding affinity, a more negative glide score meaning greater affinity. All the top 10 hits exhibited three modes of interaction with the Keap1 DGR domains, namely H-bonding,

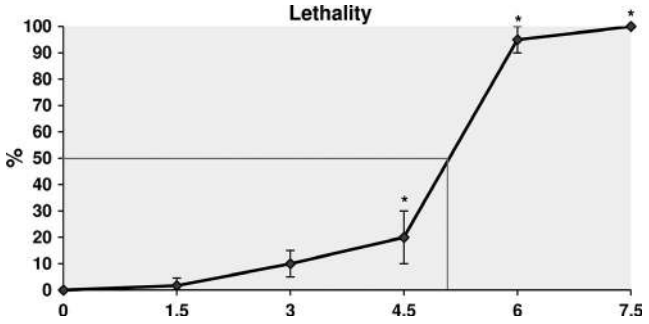

FIGURE 5 Lethality in 3 dpf zebrafish larvae after exposure to esculin. The x-axis represents the concentration of esculin in mmol/L. Data are represented as mean ± SD of three independent experiments. Asterisks indicate statistically significant differences compared to control (one-way ANOVA followed by Tukey's multiple comparison test)

Pi-cation and salt bridges. All these interactions formed by the top 10 hits are the residues that make the PPI between DGR domain of Keap1 and DLG/ETGE motifs of Nrf2.

In the top 15 hits for both Keap1a and Keap1b, the common compounds found were quercetin 3,4′-diglucoside, FAD-Na2, salvianolic acid A, tunicamycin and esculin which showed better glide score than other compounds. Though these compounds were common hits, the interaction of these compounds with Keap1a and Keap1b was different. This difference may be with difference in the sequence of Keap1a and Keap1b. Both the ETGE and DLG motifs must bind the Kelch domain in order to facilitate the Nrf2 ubiquitination,[13,53] and the compounds identified as disruptors of this interaction activate Nrf2. In case of zebrafish, two types of Keap1 proteins, Keap1a and Keap1b, form homodimers and heterodimers, thus interact with ETGE and DLG

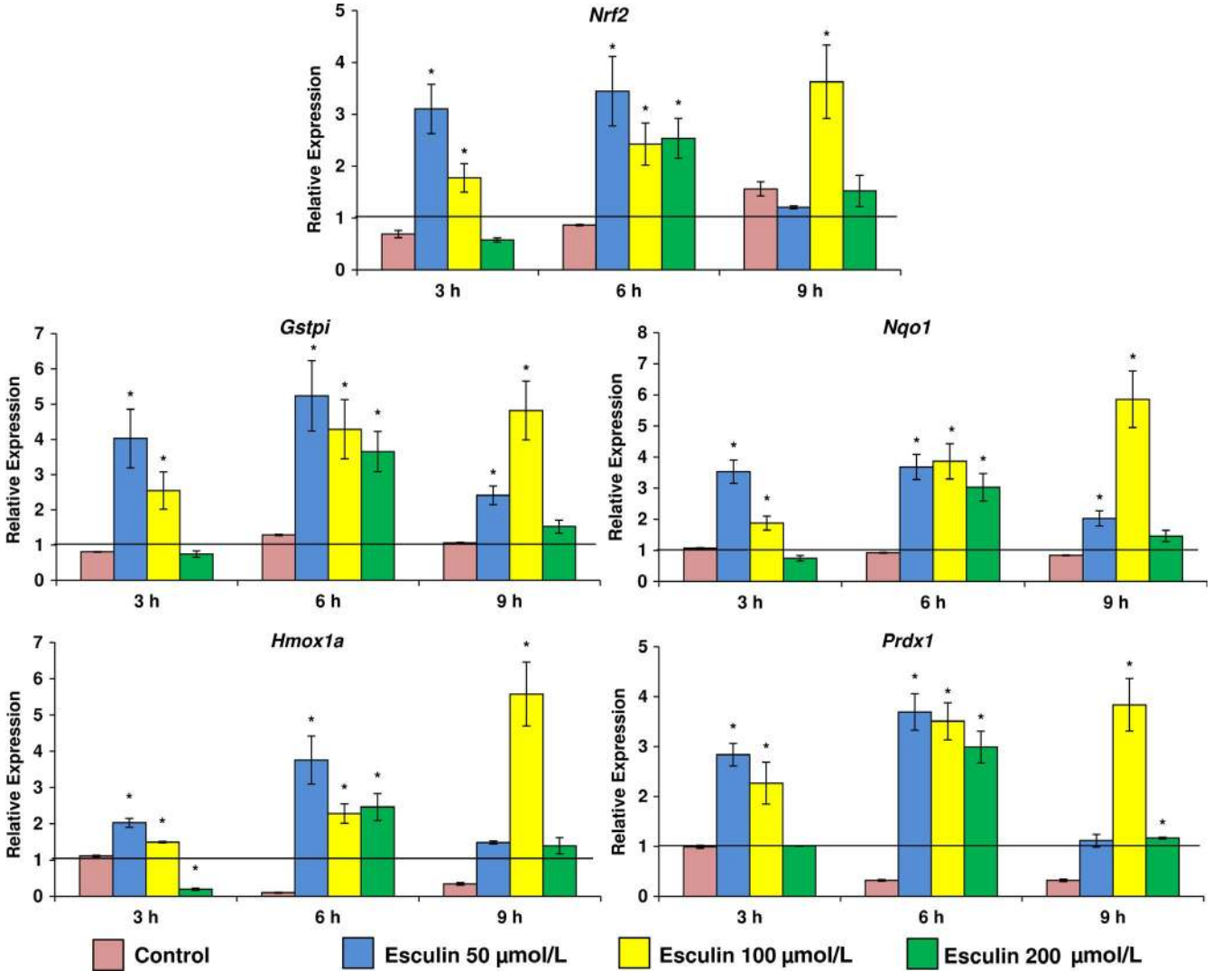

FIGURE 6 Esculin dose- and time-dependent expression of Nrf2 and its target genes (Gstpi, Nqo1, Hmox1a and Prdx1). Gene expression levels relative to an internal calibrator were determined using qRT-PCR. Control values are shown by the horizontal line. Data are represented as mean ± SD, $*p < 0.05$ compared to controls (one-way ANOVA followed by Tukey's multiple comparison test).

motifs of Nrf2.[48] This PPI is a major factor that regulates Nrf2 activity and offers ways to discover compounds that are potential Nrf2 activators by directly disrupting this interaction.[54] Ser49, Asn100, Arg101, Leu196 and Ser288 were the residues that formed the primary interactions with DGR domain of Keap1a, whereas Ser49, Ala53, Asn68, Asn100, Arg101, Arg169, Ser194, Val198 and Val292 contributed to the major part of the interaction with DGR domain of Keap1b. The identified top compounds interfered the binding of the Nrf2 with the Keap1a/Keap1b Kelch domain. These identified inhibitors corroborate with the principle that DLG and ETGE motifs of Nrf2 are obstructed to interact with Keap1a/Keap1b Kelch domains as demonstrated from structural and functional studies.[55] Yet another complexity in Keap1/Nrf2/ARE pathway is that different groups of compounds elicit this cytoprotective pathway via different mechanisms of activation.[56-58] This is due to the structural diversity in the compounds. Neurodegenerative diseases are one among the oxidative stress-mediated disorders, and most of the best compounds identified to disrupt Keap1a/b interaction with Nrf2 have the potential to cross the blood-brain barrier. A few of them are neuroprotectants, which could be utilized for the treatment of neurological diseases. If one of the top hits was known to be effective in zebrafish larvae, then the rest of the hits may still be efficacious and therefore worth testing further. The limitation of this study was that no solved crystal structure is available either for the full-length Keap1a/b or DGR domain of Keap1a/b in zebrafish. The results not only reveal the interactions of specific ligands with DGR domain of Keap1a and Keap1b but also provide insights into the design and discovery of novel potent Nrf2 activators.

In this study, esculin was identified to inhibit the interaction of both Keap1a and Keap1b Kelch domain: hence, esculin was selected as a representative hit for further experimental validation. Initially, lethality effect of esculin was determined using 3 dpf zebrafish larvae. 50 mmol/L esculin concentration exhibited 50% lethality. Our result sheds light on the mechanism of activation of Nrf2 by esculin in offering the protection against deleterious effects caused by oxidative stress. In recent years, different studies in mice and hamsters revealed the potential of esculin as a therapeutic agent for different toxicities, yet no mechanism has been revealed. The antioxidant and antiradical properties of esculin were documented in a mice model of epirubicin-induced bone marrow toxicity.[59] Esculin scavenged superoxide radical and diminished the lipid peroxidation. Esculin protected the genotoxic damage induced by mitomycin C in mice by decreasing the lipid peroxidation.[60] The free radical inflicted damage to biomolecules was inhibited by esculin in the pancreas of N-nitrosobis(2-oxopropyl)amine-exposed hamsters.[61] The gastroprotective activity of esculin was demonstrated in ethanol-induced gastric lesion mice model due to its antioxidant potential.[62,63] Esculin offered protection against pro-oxidant aflatoxin in mice through the activation of both enzymatic and non-enzymatic antioxidant systems.[64] The present study reveals that esculin activates Nrf2 by inhibiting the interaction between Keap1 and Nrf2 and has the potential to exhibit the properties demonstrated from the above studies. Therefore, the present finding provides evidence that esculin activates Nrf2 and can prevent damage due to oxidative stress.

5 | CONCLUSION

Through molecular docking method, we have identified top promising compounds that are potential Nrf2 activators. The top hit compounds identified have the potential to directly disrupt the binding of ETGE and DLG motifs of Nrf2 to the DGR domain of Keap1a/b. These top hit compounds exhibited favourable binding energy and interactions with the amino acid residues in the active site of DGR domains of both Keap1a and Keap1b. Encouraged by in silico results, esculin compound was explored through in vivo experiment using 3 dpf zebrafish larvae. Gene expression analysis showed that esculin activated Nrf2 and induced the expression of *Gstpi*, *Nqo1*, *Hmox1a* and *Prdx1* by inhibiting the interaction between Keap1 and Nrf2. Thus, this study provides novel lead molecules for further design and investigation of potent Keap1-Nrf2 disruptors, which could be developed as therapeutic agents for the diseases contributed by oxidative stress.

ACKNOWLEDGEMENTS

Azhwar Raghunath acknowledges the University Grants Commission—Basic Scientific Research (UGC-BSR), New Delhi, India, for the award of Senior Research Fellowship (UGC-BSR No. F7-25/2007). The authors gratefully acknowledge DBT, IIT-Madras, Chennai, for computation facility and Schrodinger software. The authors also thank Prof. M. Michael Gromiha and Dr. Chandrasekaran Ramakrishnan for their kind help during the study. The authors greatly appreciate the financial assistance provided by the University Grants Commission-Special Assistance Program (UGC-SAP-II:F-3-20/2013), Department of Science and Technology, Fund for Improvement of Science and Technology infrastructure in universities and higher educational institutions (DST-FIST:SR/FST/ LSI-618/2014) and DST-SERB (EEQ/2018/000633), New Delhi, India.

CONFLICT OF INTEREST

The authors declare that there were no competing interests.

REFERENCES

1. Aseervatham GS, Sivasudha T, Jeyadevi R, Arul AD. Environmental factors and unhealthy lifestyle influence oxidative stress in humans–an overview. *Environ Sci Pollut Res Int*. 2013;20:4356-4369.
2. Liguori I, Russo G, Curcio F, et al. Oxidative stress, aging, and diseases. *Clin Interv Aging*. 2018;13:757-772.
3. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the global burden of disease study 2010. *Lancet*. 2012;380:2095-2128.
4. Meier BP, Lappas CM. The influence of safety, efficacy, and medical condition severity on natural versus synthetic drug preference. *Med Decis Making*. 2016;36:1011-1019.
5. Lewis KN, Mele J, Hayes JD, Buffenstein R. Nrf2, a guardian of healthspan and gatekeeper of species longevity. *Integr Comp Biol*. 2010;50:829-843.
6. Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1-Nrf2 pathway. *Arch Toxicol*. 2011;85:241-272.
7. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem*. 2009;284:13291-13295.
8. Al-Sawaf O, Clarner T, Fragoulis A, et al. Nrf2 in health and disease: current and future clinical implications. *Clin Sci*. 2015;129:989-999.
9. O'Connell MA, Hayes JD. The Keap1/Nrf2 pathway in health and disease: from the bench to the clinic. *Biochem Soc Trans*. 2015;43:687-689.
10. Lu MC, Ji JA, Jiang ZY, You QD. The Keap1-Nrf2-ARE pathway as a potential preventive and therapeutic target: an update. *Med Res Rev*. 2016;36:924-963.
11. Canning P, Sorrell FJ, Bullock AN. Structural basis of Keap1 interactions with Nrf2. *Free Radic Biol Med*. 2015;88:101-107.
12. Katoh Y, Iida K, Kang M-IL, et al. Evolutionary conserved N-terminal domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. *Arch Biochem Biophys*. 2005;433:342-350.
13. Tong KI, Katoh Y, Kusunoki H, Itoh K, Tanaka T, Yamamoto M. Keap1 recruits Neh2 through binding to ETGE and DLG motifs: characterization of the two-site molecular recognition model. *Mol Cell Biol*. 2006;26:2887-2900.
14. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem*. 1991;266:11632-11639.
15. Hu C, Eggler AL, Mesecar AD, van Breemen RB. Modification of keap1 cysteine residues by sulforaphane. *Chem Res Toxicol*. 2011;24:515-521.
16. Marcotte D, Zeng W, Hus J-C, et al. Small molecules inhibit the interaction of Nrf2 and the Keap1 Kelch domain through a non-covalent mechanism. *Bioorg Med Chem*. 2013;21:4011-4019.
17. Hancock R, Schaap M, Pfister H, Wells G. Peptide inhibitors of the Keap1-Nrf2 protein-protein interaction with improved binding and cellular activity. *Org Biomol Chem*. 2013;11:3553-3557.
18. Jnoff E, Albrecht C, Barker JJ, et al. Binding mode and structure-activity relationships around direct inhibitors of the Nrf2-Keap1 complex. *ChemMedChem*. 2014;9:699-705.
19. Zhuang C, Narayanapillai S, Zhang W, Sham YY, Xing C. Rapid identification of Keap1-Nrf2 small-molecule inhibitors through structure-based virtual screening and hit-based substructure search. *J Med Chem*. 2014;57:1121-1126.
20. Satoh M, Saburi H, Tanaka T, et al. Multiple binding modes of a small molecule to human Keap1 revealed by X-ray crystallography and molecular dynamics simulation. *FEBS Open Bio*. 2015;5:557-570.
21. Hur W, Gray NS. Small molecule modulators of antioxidant response pathway. *Curr Opin Chem Biol*. 2011;15:162-173.
22. Magesh S, Chen Y, Hu L. Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents. *Med Res Rev*. 2012;32:687-726.
23. Goldsmith P. Zebrafish as a pharmacological tool: the how, why and when. *Curr Opin Pharmacol*. 2004;4:504-512.
24. MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. *Nat Rev Drug Discov*. 2015;14:721-731.
25. Dooley K, Zon LI. Zebrafish: a model system for the study of human disease. *Curr Opin Genet Dev*. 2000;10:252-256.
26. Langheinrich U. Zebrafish: a new model on the pharmaceutical catwalk. *Bioessays*. 2003;25:904-912.
27. Kobayashi M, Itoh K, Suzuki T, et al. Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. *Genes Cells*. 2002;7:807-820.
28. Sievers F, Wilm A, Dineen D, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 2011;7:539.
29. Robert X, Gouet P. Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res*. 2014;42(W1):W320-W324.
30. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016;33:1870-1874.
31. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res*. 2016;44(W1):W242-W245.
32. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Maddern TL. NCBI BLAST: a better web interface. *Nucleic Acids Res*. 2008;36:W5-W9.
33. Burley SK, Berman HM, Kleywegt GJ, Markley JL, Nakamura H, Velankar S. Protein data bank (PDB): The single global macromolecular structure archive. *Methods Mol Biol*. 2017;1607:627-641.
34. Winkel AF, Engel CK, Margerie D, et al. Characterization of RA839, a noncovalent small molecule binder to Keap1 and selective activator of Nrf2 signaling. *J Biol Chem*. 2015;290:28446-28455.
35. Webb B, Sali A. Comparative protein structure modeling using MODELLER. *Curr Protoc Bioinformatics*. 2016;54:5.6.1-5.6.37.
36. Chen VB, Arendall WB 3rd, Headd JJ, et al. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr D Biol Crystallogr*. 2010;66(Pt 1):12-21.
37. Kim S, Thiessen PA, Bolton EE, et al. PubChem substance and compound databases. *Nucleic Acids Res*. 2016;44(D1):D1202-D1213.
38. McMahon M, Thomas N, Itoh K, Yamamoto M, Hayes JD. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. *J Biol Chem*. 2006;281:24756-24768.
39. Tong Ki, Padmanabhan B, Kobayashi A, et al. Different electrostatic potentials define ETGE and DLG motifs as hinge and latch in oxidative stress response. *Mol Cell Biol*. 2007;27:7511-7521.

40. Padmanabhan B, Tong KI, Ohta T, et al. Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol Cell*. 2006;21:689-700.
41. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn*. 1995;203:253-310.
42. Tveden-Nyborg P, Bergmann TK, Lykkesfeldt J. Basic and clinical pharmacology and toxicology policy for experimental and clinical studies. *Basic Clin Pharmacol Toxicol*. 2018;123:233-235.
43. Raghunath A, Perumal E. Analysis of lethality and malformations during zebrafish (*Danio rerio*) development. *Methods Mol Biol*. 2018;1797:337-363.
44. Raghunath A, Nagarajan R, Sundarraj K, Panneerselvam L, Perumal E. Genome-wide identification and analysis of Nrf2 binding sites – antioxidant response elements in zebrafish. *Toxicol Appl Pharmacol*. 2018;360:236-248.
45. Hahn ME, McArthur AG, Karchner SI, et al. The transcriptional response to oxidative stress during vertebrate development: effects of tert-butylhydroquinone and 2,3,7,8-tetrachlorodibenzo-p-dioxin. *PLoS One* 2014;9:e113158.
46. Liu C, Su G, Giesy JP, et al. Acute exposure to tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) causes hepatic inflammation and leads to hepatotoxicity in zebrafish. *Sci Rep*. 2016;6:19045.
47. Holowiecki A, O'Shields B, Jenny MJ. Spatiotemporal expression and transcriptional regulation of heme oxygenase and biliverdin reductase genes in zebrafish (*Danio rerio*) suggest novel roles during early developmental periods of heightened oxidative stress. *Comp Biochem Physiol C Toxicol Pharmacol*. 2017;191:138-151.
48. Li L, Kobayashi M, Kaneko H, Nakajima-Takagi Y, Nakayama Y, Yamamoto M. Molecular evolution of Keap1. Two Keap1 molecules with distinctive intervening region structures are conserved among fish. *J Biol Chem*. 2008;283:3248-3255.
49. Kari G, Rodeck U, Dicker AP. Zebrafish: an emerging model system for human disease and drug discovery. *Clin Pharmacol Ther*. 2007;82:70-80.
50. Fuse Y, Kobayashi M. Conservation of the Keap1-Nrf2 system: an evolutionary journey through stressful space and time. *Molecules* 2017;22:E436.
51. Giribet G, Dunn CW, Edgecombe GD, Rouse GW. A modern look at the animal tree of life. *Zootaxa*. 2007;1668:61-79.
52. Shoichet BK, McGovern SL, Wei B, Irwin JJ. Lead discovery using molecular docking. *Curr Opin Chem Biol*. 2002;6:439-446.
53. Baird L, Lleres D, Swift S, Dinkova-Kostova AT. Regulatory flexibility in the Nrf2-mediated stress response is conferred by conformational cycling of the Keap1-Nrf2 protein complex. *Proc Natl Acad Sci USA*. 2013;110:15259-15264.
54. Jiang ZY, Lu MC, You QD. Discovery and development of Kelch-like ECH-associated protein 1. Nuclear factor erythroid 2-related factor 2 (KEAP1:NRF2) protein-protein interaction inhibitors: Achievements, challenges, and future directions. *J Med Chem*. 2016;59:10837-10858.
55. Abed DA, Goldstein M, Albanyan H, Jin H, Hu L. Discovery of direct inhibitors of Keap1-Nrf2 protein-protein interaction as potential therapeutic and preventive agents. *Acta Pharm Sin B*. 2015;5:285-299.
56. Zhang DD, Lo SC, Sun Z, Habib GM, Lieberman MW, Hannink M. Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway. *J Biol Chem*. 2005;280:30091-30099.
57. Hong F, Sekhar KR, Freeman ML, Liebler DC. Specific patterns of electrophile adduction trigger Keap1 ubiquitination and Nrf2 activation. *J Biol Chem*. 2005;280:31768-31775.
58. Raghunath A, Sundarraj K, Nagarajan R, et al. Antioxidant response elements: Discovery, classes, regulation and potential applications. *Redox Biol*. 2018;17:297-314.
59. Biljali S, Hadjimitova VA, Topashka-Ancheva MM, Momekova DB, Traykov TT, Karaivanova MH. Antioxidant and antiradical properties of esculin, and its effect in a model of epirubicin-induced bone marrow toxicity. *Folia Med*. 2012;54:42-49.
60. Mokdad Bzeouich I, Mustapha N, Maatouk M, Ghedira K, Ghoul M, Chekir-Ghedira L. Genotoxic and anti-genotoxic effects of esculin and its oligomer fractions against mitomycin C-induced DNA damages in mice. *Regul Toxicol Pharmacol*. 2016;82:48-52.
61. Kaneko T, Tahara S, Takabayashi F, Harada N. Inhibitory effect of esculin on oxidative DNA damage and carcinogenesis induced by N-nitrosobis(2-oxopropyl)amine in hamster pancreas. *Biofactors*. 2004;22:83-85.
62. Rios E, Rocha N, Venâncio ET, et al. Mechanisms involved in the gastroprotective activity of esculin on acute gastric lesions in mice. *Chem Biol Interact*. 2010;188:246-254.
63. Li W, Wang Yu, Wang X, et al. Gastroprotective effect of esculin on ethanol-induced gastric lesion in mice. *Fundam Clin Pharmacol*. 2017;31:174-184.
64. Naaz F, Abdin MZ, Javed S. Protective effect of esculin against pro-oxidant aflatoxin B1-induced nephrotoxicity in mice. *Mycotoxin Res*. 2014;30:25-32.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Raghunath A, Nagarajan R, Sundarraj K, Palanisamy K, Perumal E. Identification of compounds that inhibit the binding of Keap1a/Keap1b Kelch DGR domain with Nrf2 ETGE/DLG motifs in zebrafish. *Basic Clin Pharmacol Toxicol*. 2019;125:259–270. https://doi.org/10.1111/bcpt.13222