

## Original Research Article

# Evaluation of antioxidant activation by potential nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/Keap1 complex inhibitors

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### Abstract

**Purpose:** To investigate the binding affinities of forty-one (41) National Cancer Institute (NCI)-generated compounds, to the Nrf2 ligand, and possible activation of Nrf2 in the MCF-7 cell line.

**Methods:** To investigate the inhibition of the Nrf2/Keap1 complex, the MCF-7 cell line was treated with each of the 41 compounds, at a working concentration of 30  $\mu$ M. The extent of Nrf2 activation and corresponding Nrf2/Keap1 complex inhibition was evaluated in terms of Nrf2 expression and its antioxidant-associated enzyme gamma-glutamylcysteine synthetase (GCS), using western blot analysis.

**Results:** Twenty-nine compounds out of the 41 targeted compounds activated GCS, and some showed comparable or greater activation capacity than the standard Nrf2 activator tBHQ. To confirm that the activation of GCS was mediated via Nrf2 activation, cell lysates were tested for their Nrf2 protein expression, and it was found that Nrf2 was activated by the examined compounds for more than 24 h, indicating that the effect of the chosen compounds were not transient.

**Conclusion:** These results might be useful for identifying better targets for cytoprotection, and for oxidative stress alleviation through Nrf2 pathway activation. Further studies are required on the effects of these targets on the prevention and treatment of various oxidative stress disorders, including cancer.

**Keywords:** NCI compounds, Nrf2/Keap1, Cancer, Antioxidants, Gamma-glutamylcysteine synthetase (GCS), MCF7, Pharmacophore, Docking, Co-crystallized structure

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## INTRODUCTION

Cancer targeted therapy has developed immensely through targeting small molecules, which upon inhibition or activation, lead to cancer prevention or treatment. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic DNA-binding leucine zipper transcription factor. The erythroid-

derived 2-like2 gene (*NFE2L2*) encodes a protein that regulates cellular stress-induced metabolic and proteotoxic DNA damage, thereby helping cells to cope with stress-induced injury [1,2]. Moreover, Nrf2 is a defensive transcription factor that mitigates oxidative stress-induced inflammation [3]. It regulates the expression of antioxidant response elements (AREs) which

modulate the expressions of numerous antioxidant enzymes, primarily GCS, as part of the inflammation-control cascade. The enzyme GCS protects cells against inflammation-induced oxidative damage by activating additional downstream pathways [3,4]. Several drugs that stimulate the *NFE2L2* pathway have shown promising potential as new treatments for solid tumors such as thyroid and ovarian carcinomas [5].

Under resting conditions, Nrf2 is retained within the cytoplasm by complexing with Keap1 and Cullin3 (CUL3). The short half-life of Nrf2 (20 min) may be due to its continuous ubiquitination by Keap1 and CUL3 through regulation of proteasomal degradation of Nrf2. On the other hand, during oxidative stress, Nrf2 proteasomal degradation stops, and Nrf2 migrates to the nucleus and binds its DNA promoter. This is due to the disruption of critical cysteine residues in Keap 1, which ends up disrupting the ubiquitination process [5]. When Nrf2 is not ubiquitinated, it accumulates inside the cytoplasm and undergoes nuclear translocation where it combines with a small Maf protein to form a heterodimer which binds to AREs within the upstream promoter region of numerous antioxidative genes to initiate their transcription [6]. For that reason, the inhibition of Nrf2/Keap1 complex which finally ends up in Nrf2 activation, is a potential key goal in cancer prevention and therapy [5].

Considering the promising results of targeting the Nrf2/Keap1 complex in chemoprevention with antioxidant and anti-inflammatory drugs, studies on chemical substances with potential to bind and inhibit the Nrf2/Keap1 complex may be useful for identifying new potential chemoprotective agents.

The aim of the present study was to create compounds with high-affinity binding to Nrf2, using structure-based modeling and docking systems. Then, the expressions of Nrf2 and its housekeeping effector enzyme GCS were assayed in MCF7 cell line (a human breast cancer cell line) after treatment with 41 chosen compounds with various functional groups but with high binding affinities to Nrf2.

## EXPERIMENTAL

### Molecular modeling

A previously published in-house modeling station was used to map pharmacophores related to Nrf2 protein [7].

### Pharmacophore generation from co-crystallized structure

The pharmacophoric features of Nrf2 were investigated using a 4L7B co-crystallized structure [7]. The 3D coordinates of Nrf2 were recovered within the Protein Data Bank (Nrf2 PDB code: 4L7B, resolution: 2.4 Å) [8]. The DS 4.5 templates were used to feature hydrogen atoms into the protein residues. The parameters used to generate the receptor-ligand pharmacophore within DS 4.5 were set to get minimum quantity of the four features, and a maximum of ten. The resultant pharmacophores modeled were 10 in number, with co-crystallized water remaining inside the protein structure. The selective pharmacophore models based entirely on receptor-ligand interactions were created using Accelrys DS v4.5 and Receptor-Ligand Pharmacophore Generation Protocol with default settings. The following set of specified binding ligand properties is recognized: hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), hydrophobic (Hbic), negative ionizable (NI), positive ionizable (PI), and ring aromatic (RA) [7, 9,10].

### Molecular docking

Molecular docking was implemented within DS 4.5 so as to assign Gasteiger–Marsili charges to the protein atoms [9]. The required docking conditions retained the intrinsic water molecules. The subsequent docking experiments utilized protein construction without energy minimization. The docking parameters in LigandFit algorithm consider the ligand to be flexible while selecting the receptor's stiffness [11, 12]. The binding site was constructed and the selected hits were docked into it using co-crystallized ligand (1VV701 in Nrf2, 4L7B, resolution: 2.41 Å) in the presence of the indicated water molecules, under the following docking arrangements: Monte Carlo search parameters were set to 15000 trials, and attempts for rotations with polar hydrogen equalled 30.0°, the Root Mean Square threshold for ligand-to-binding-site shape matching was established at 2.0 Å, with a maximum of 1.0 binding-site partitions, and an electric grid that stretched 3.0 Å from the binding place was employed. DS 4.5 was used to investigate protein-ligand interactions which were assessed using consistent force field (v.1.02) and a distance-dependent dielectric with a non-bonded cutoff distance of 10.0 Å. The interaction energies were calculated using soft potential energy estimates and a trilinear interpolation value. For each docked ligand orientation, the rigid body ligand minimization parameters were 20 steepest descent iterations followed by 40

Broyden-Fletcher-Goldfarb-Shanno minimization iterations. The energy within the binding site of the best 10 poses was subsequently reduced to a maximum of 200 stiff body repetitions [9].

### Pharmacophore mapping

The value of fit for any drug is determined by the total mapped hypothesis features which reflect the total pharmacophore features superimposed on the analogous chemical moieties inside the fitted molecule. To match the mapped features with the docked posture, the screened National Cancer Institute (NCI) hits were matched with the virtual co-crystallized Nrf2 pharmacophore model 4L7B\_2\_03 using the “best fit” option inside CATALYST in DS 4.5 [10]. Then, the rigid-body docking feature was used to attach mapped compounds in the binding region of Nrf2 [7, 13].

### Reagents and chemicals used for cell culture assay

The National Cancer Institute (NCI, USA) was the source of the forty-one compounds, each with a specific Cancer Chemotherapy National Service Center (NSC) number. The other reagents were tert-butylhydroquinone (tBHQ; 30  $\mu$ M) as positive control; dimethylsulfoxide (DMSO), double distilled water, Roswell Park Memorial Institute (RPMI) media for cells, heat-inactivated fetal bovine serum (FBS), Trypsin-EDTA, L-glutamine, Dulbecco's phosphate-buffered saline (PBS), streptomycin, and penicillin were purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Dithiothreitol (DTT) and bovine insulin were products of Sigma Chemical Co. (St Louis, MO). Radioimmunoprecipitation assay (RIPA) lysis buffer was purchased from Cell signaling (Beverly, USA). Primary antibodies for Nrf2, GCS, and  $\beta$ -actin, and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while Amersham ECL detection reagent was provided by GE Healthcare (Buckinghamshire, UK). Bicinchoninic acid (BCA) protein test reagent was purchased from Pierce Biotechnology (Rockford, IL). Pall Gelman Laboratory (Ann Arbor, MI) provided polyvinylidene difluoride (PVDF) membranes. All other chemicals were either analytical or of the highest purity grade available.

### Preparation of the tested compounds for *in vitro* assay

The NCI compounds were supplied as dry powders weighing 5-10 mg. The compounds were solubilized in DMSO to make 0.02 M stock solutions. Each stock solution was diluted to final

working concentration of 30  $\mu$ M which was used in assay of protein expression levels of GCS and Nrf2 in the cell line studied.

### Cell culture

The MCF7 cells were grown in RPMI medium supplemented with 10 % FBS, 1 % penicillin-streptomycin, 1 % glutamate, and 10 % insulin (10  $\mu$ g/mL). The cells were treated for 24 h at 37 °C with tBHQ, DMSO, or NCI compounds, in a humidified environment containing 5 % CO<sub>2</sub> and 95 % air. Thereafter, the cells were lysed to get the whole lysate protein which was used for western blot assay.

### Western blot analysis

The cells were washed two times with PBS after drug treatment and then detached on ice with 1 x RIPA lysis buffer supplemented with 1 % phenylmethylsulfonyl fluoride for 1 h. After cell detachment, the cell lysates were centrifuged at 17,000 g for 15 min. Total protein contents of the supernatants were determined using BCA reagent. Equal amounts of protein (30-50  $\mu$ g) were resolved using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by transfer to PVDF membranes. After blocking for 1 h at room temperature (RT) with 5 % fat-free dry milk-PBST (Tris-buffered saline containing 0.1% Tween-20) buffer, the membranes were incubated overnight with specific primary antibodies diluted in PBS (1:1000) or 3 % fat-free dry milk-PBS. The blots were washed thrice with PBST, prior to incubation for 1 h at RT with horseradish peroxidase-labelled secondary antibodies diluted 1:2000 in 3 % fat-free dry milk-PBS. Then, blots were washed and incubated for 1 min with ECL Prime Western Blotting Detection Reagent, in accordance with the manufacturer's instructions. The images were observed with ultrasensitive chemiluminescence Western blot image model FCE (Proteinsimple®).

## RESULTS

Table 1 displays the generated pharmacophores using DS studio 4.5. The Nrf2 protein co-crystallized with 1VV701 (4L7B, resolution 2.41 Å) was used to generate the pharmacophores. 4L7B\_2\_03 was selected because of high ROC-AUC >80 % and the three diverse features; HBA, Hbic, and RA (Table 1). The 4L7B\_2\_03 (Tables 2 and 3; Figure 1A) was used as a search tool for selecting 534 compounds out of the 257,000 compounds in the NCI database. The chosen compounds were categorized based on their fit values, and the top 41 were evaluated *in vitro* for

GCS and Nrf2 activation using western blot analysis.

Figure 2 shows the potential interaction of co-crystallized structure (1VV701) inside the binding pocket of Nrf2 (PDB: 4L7B, resolution: 2.41 Å) after 2D analysis using the DS 4.5 visualizer. The results revealed the co-crystallized compound (1VV701) inside the binding region of Nrf2. The equivalent amino acids involved in hydrogen bond interactions were Arg415, Asn414, and Asn387; as well as those involved in *Van der Waal* and hydrophobic interactions i.e., Ala556, Gly364, Gly509, Tyr334, and Tyr572. These

amino acids created the binding pocket for the suitable co-crystallized ligand (1VV701). The mapping properties of 4L7B\_2\_03 (Figure 1 C) can be explained based on the following mappings: the isoquinoline group was mapped with two hydrophobic features that correlated with *Van der Waal* interactions with hydrophobic portions of Gly364, Gly509, and Ala556; the interaction of phenyl groups with electron-rich phenyl groups of Tyr334 and Tyr572 was mapped with the isoindole group and the aromatic ring group.

**Table 1:** Pharmacophores co-crystallized with Nrf2 structure (4L7B)

Pharmacophore	Model	Number of features	Feature set*	Selectivity score	ROC-AUC#
4L7B_2_01	Pharmacophore_01	4	HHNR	8.58	0.65
4L7B_2_03	Pharmacophore_02	5	AAHHR	7.88	0.50
4L7B_2_03	Pharmacophore_03	4	AHHR	6.37	0.833
4L7B_2_03	Pharmacophore_04	4	AHHR	6.37	0.53
4L7B_2_05	Pharmacophore_05	4	AAHR	6.37	0.56
4L7B_2_06	Pharmacophore_06	4	AAHR	6.37	0.61
4L7B_2_07	Pharmacophore_07	4	AAHH	6.37	0.88

\*A: HBA, N: NI, H: Hbic, R: RA; # Validation parameter using the default method

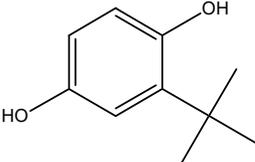
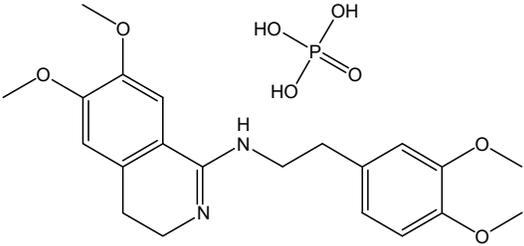
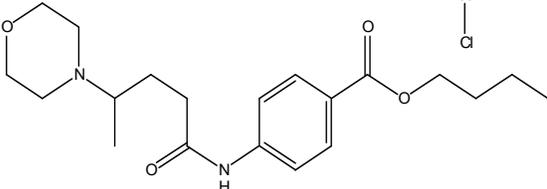
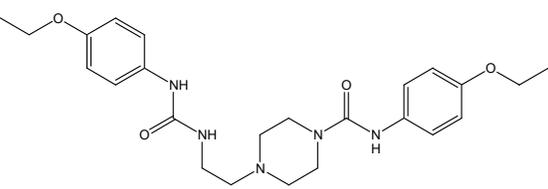
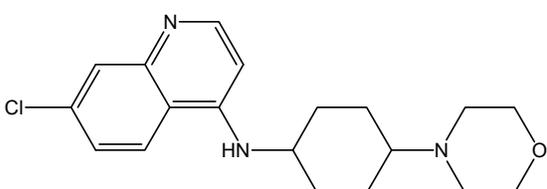
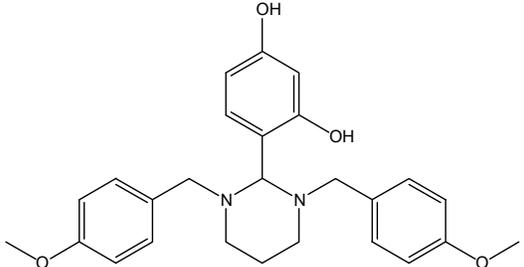
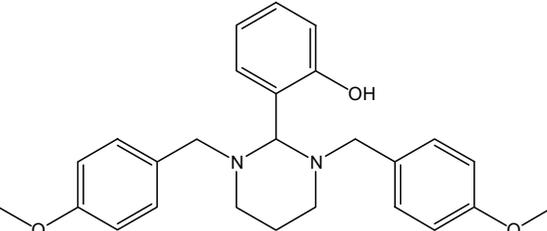
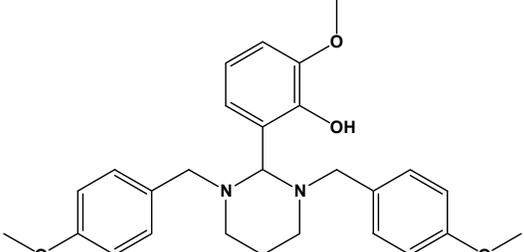
**Table 2:** Features of pharmacophores and corresponding weights, tolerances, and 3D coordinates of generated pharmacophore from co-crystallized Nrf2 (4L7B)

Model	Definition	Chemical feature						
		HBA	Hbic1	Hbic2	RA			
4L7B_2_03	Tolerances		1.6	2.2	1.6	1.6	1.6	2.2
		X	1.13	1.27	-3.4	-3.2	-6.1	-9.0
	Coordinates	Y	2.49	5.49	3.2	6.6	0.73	1.42
		Z	-26.78	-26.6	-30.4	-30.5	-24.6	-24.6

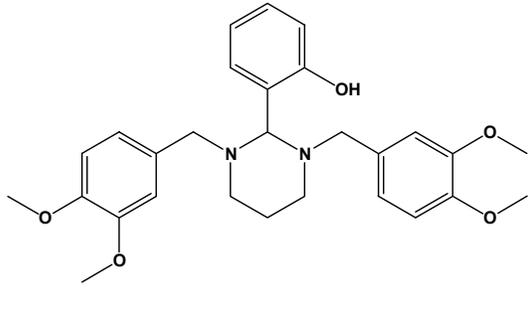
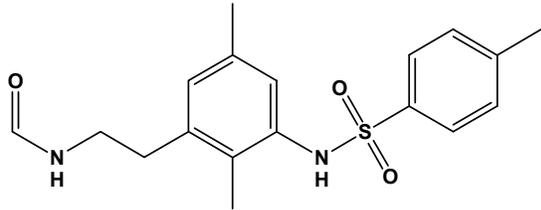
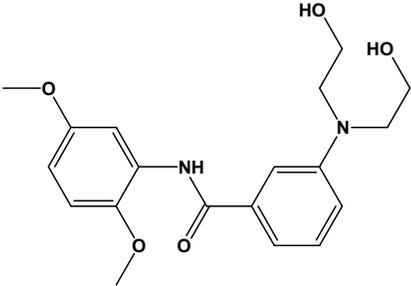
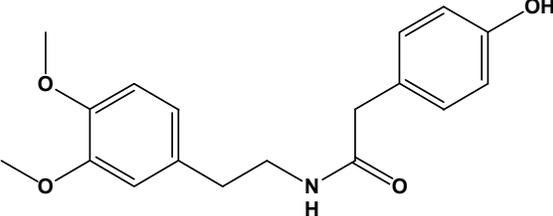
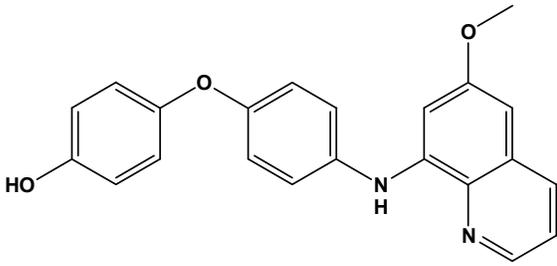
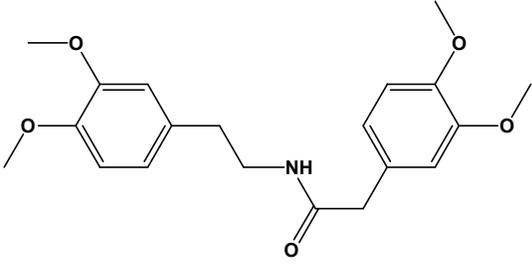
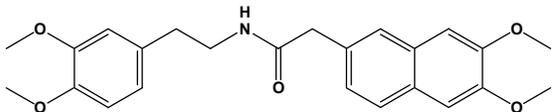
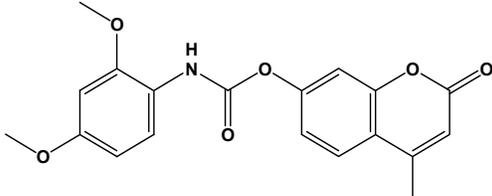
**Table 3:** Exclusion spheres added to the generated pharmacophore

Feature	X	Y	Z	Radius
ExcludedVolume_1.7	0.791	6.677	-29.766	1.724
ExcludedVolume_1.8	-9.632	0.62	-19.707	1.008
ExcludedVolume_1.9	-9.72	-2.9	-22.076	1.27
ExcludedVolume_1.10	1.385	-3.711	-26.942	1.27
ExcludedVolume_1.11	0.33	3.264	-34.284	1.27
ExcludedVolume_1.12	-9.042	-2.722	-27.925	1.27
ExcludedVolume_1.13	3.226	3.269	-30.447	1.154
ExcludedVolume_1.14	-1.371	11.268	-31.555	1.27
ExcludedVolume_1.15	-6.944	0.323	-32.432	1.368
ExcludedVolume_1.16	-9.375	6.394	-29.948	1.27
ExcludedVolume_1.17	0.11	-0.286	-33.055	1.368
ExcludedVolume_1.18	-9.81	1.808	-25.816	1.6
ExcludedVolume_1.19	4.726	0.095	-27.469	1.368
ExcludedVolume_1.20	-7.614	-3.791	-20.923	1.008
ExcludedVolume_1.21	-4.482	1.825	-34.727	1.27
ExcludedVolume_1.22	-3.501	-1.24	-19.984	1.454
ExcludedVolume_1.23	-6.96	-0.755	-18.968	1.27
ExcludedVolume_1.24	-5.507	12.055	-28.748	1.27
ExcludedVolume_1.25	-3.843	-3.714	-28.858	1.664
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ExcludedVolume_1.27	-6.2	10.26	-31.61	1.27

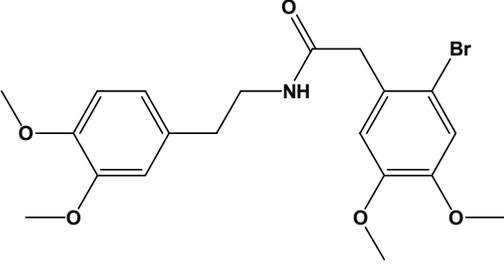
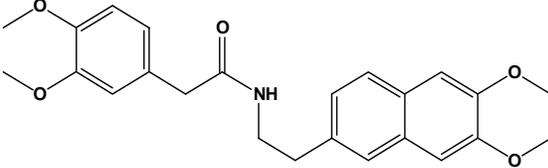
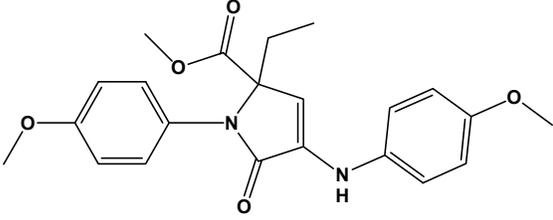
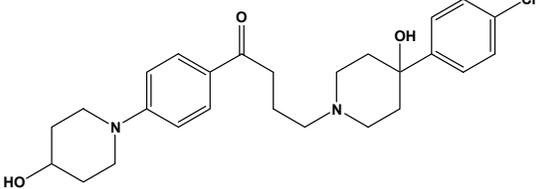
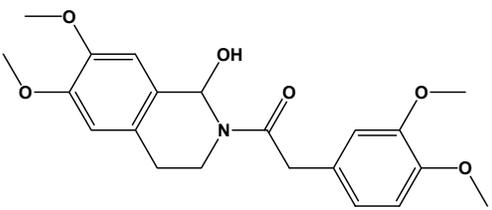
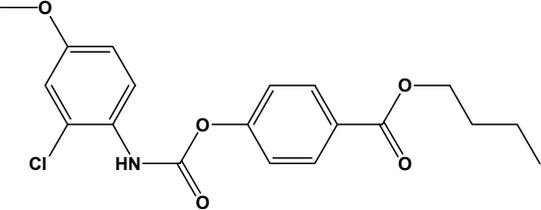
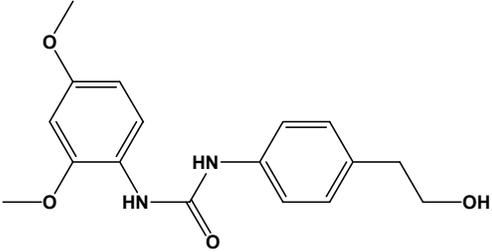
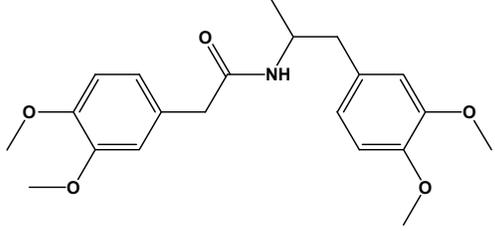


	
<b>tBHQ</b>	<b>NSC 25446 (1)</b>
	
<b>NSC 41492 (2)</b>	<b>NSC 54697 (3)</b>
	
<b>NSC 56626 (4)</b>	<b>NSC 71780 (5)</b>
	
<b>NSC 78586 (6)</b>	<b>NSC 83410 (7)</b>

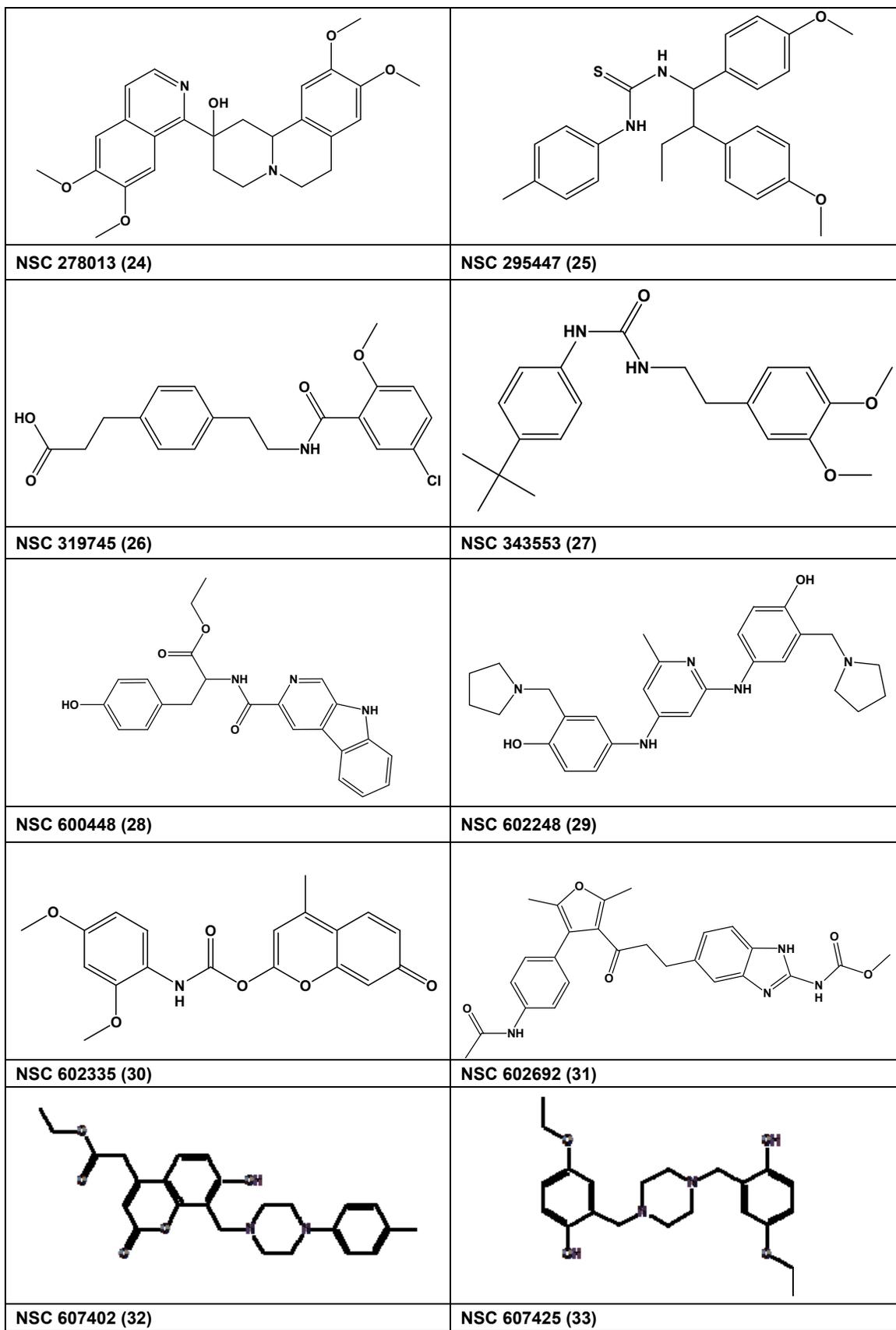
**Figure 3:** Chemical structures of tBHQ and NSC compounds (1-7) selected for Nrf2 activity

	
<b>NSC 87178 (8)</b>	<b>NSC 102639 (9)</b>
	
<b>NSC 109617 (10)</b>	<b>NSC 114979 (11)</b>
	
<b>NSC 130810 (12)</b>	<b>NSC 140013 (13)</b>
	
<b>NSC 146450 (14)</b>	<b>NSC 146556 (15)</b>

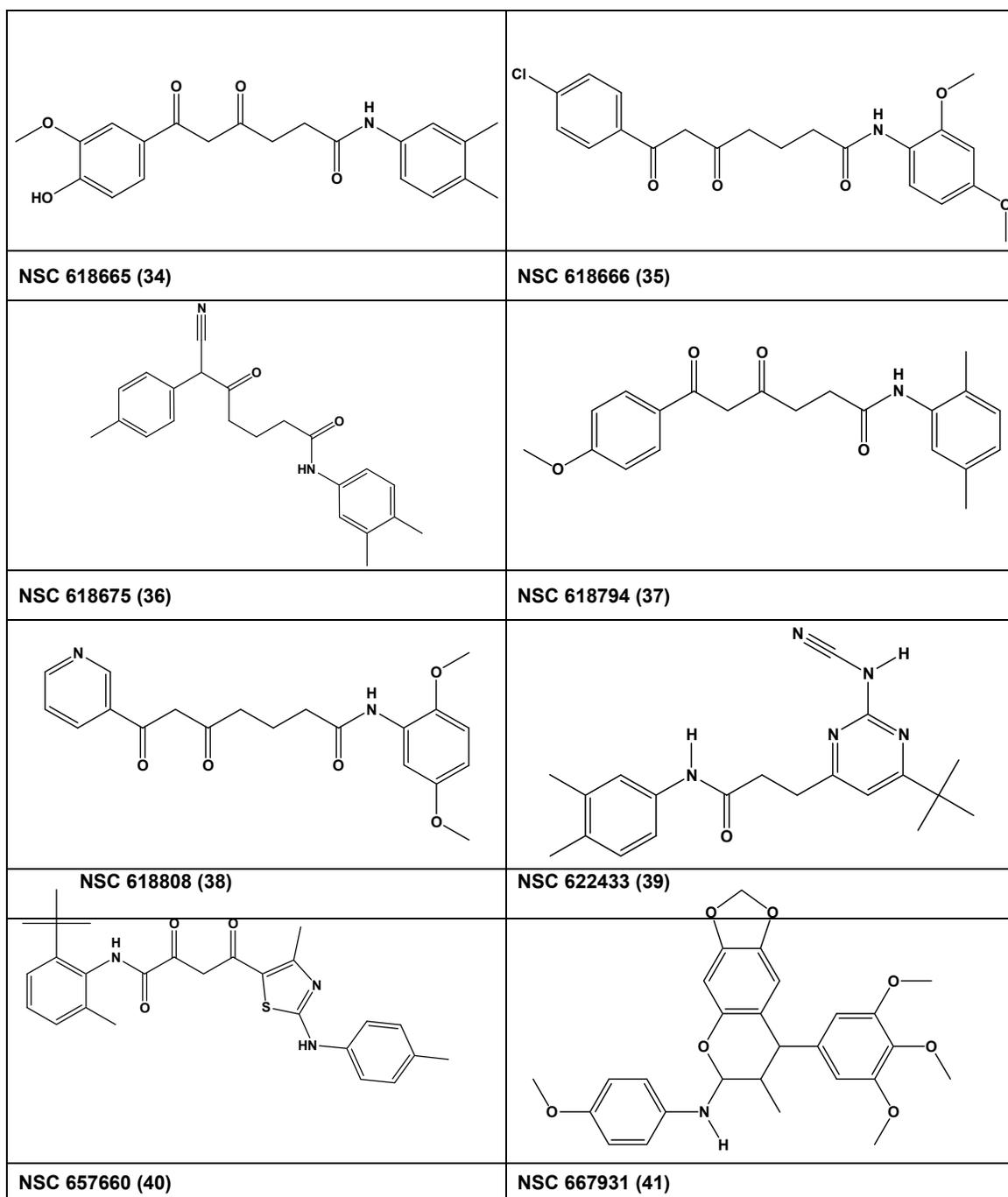
**Figure 4:** Chemical structures of tBHQ and NSC compounds (8-15) selected for Nrf2 activity

	
<b>NSC 149612 (16)</b>	<b>NSC 153424 (17)</b>
	
<b>NSC 159919 (18)</b>	<b>NSC 170475 (19)</b>
	
<b>NSC 194248 (20)</b>	<b>NSC 204092 (21)</b>
	
<b>NSC 204099 (22)</b>	<b>NSC 245459 (23)</b>

**Figure 5:** Chemical structures of tBHQ and NSC compounds (16-23) selected for Nrf2 activity



**Figure 6:** Chemical structures of tBHQ and NSC compounds (24-31) selected for Nrf2 activity



**Figure 7:** Chemical structures of tBHQ and NSC compounds (32-41) selected for Nrf2 activity

## DISCUSSION

Many studies have been carried out to find new drugs that can prevent or treat diseases or conditions caused by oxidative stress and/or inflammation. These diseases and conditions include cancer, aging, cardiovascular disease, atherosclerosis, Parkinson's disease, and Alzheimer's disease. Research has demonstrated that Nrf2 activation exerts

protective effect against oxidative stress-induced disorders [3, 15]. Thus, Nrf2 is considered an attractive target for the prevention and/or treatment of such diseases.

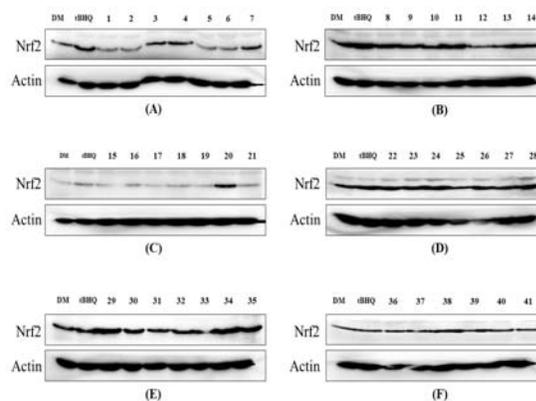
The transcription factor Nrf2 is encoded by erythroid-derived 2-like2 *NFE2L2* gene. The domain structure of Nrf2 contains six Nrf2-ECH homology (Neh) domains. Each Neh domain serves a special function that regulates its

activation, localization, degradation, and stabilization. It is known that Neh1 is important for the binding of Nrf2 to DNA. Studies have shown that Neh2 and Neh6 are important in targeting Nrf2 for proteasomal degradation by Keap1, while Neh4 and Neh5 domains are important in Nrf2-dependent transactivation [15].

Under normal conditions, the expression of Nrf2 mRNA is constitutively active, but the Nrf2 protein is subjected to continuous proteasomal degradation by complexing with Keap1, a process which is facilitated by its binding to ubiquitin ligase complex [5]. Upon disruption of

**Table 4:** Inhibition of sphere formation by Nrf2/Keap1 complex inhibitors

Compound no.	NSC no.	Inhibition of Nrf2 complex (%)
Negative control	DMSO	30.5
Standard Nrf2 activator	tBHQ	100.0
1	25446	88.3
2	41492	90.8
3	54697	111.2
4	56626	108.0
5	71780	70.7
6	78586	79.5
7	83410	71.7
8	87178	73.0
9	102639	72.3
10	109617	86.7
11	114979	182.7
12	130810	94.5
13	140013	34.1
14	146450	96.5
15	146556	66.9
16	149612	10.7
17	153424	24.0
18	159919	49.0
19	170475	77.0
20	194248	32.4
21	204092	80.7
22	204099	10.8
23	245459	53.7
24	278013	18.0
25	295447	10.6
26	319745	5.1
27	343553	25.5
28	600448	14.1
29	602248	17.2
30	602335	0.0
31	602692	1.1
32	607402	53.1
33	607425	73.5
34	618665	78.6
35	618666	133.2
36	618675	37.9
37	618794	31.8
38	618808	10.3
39	622433	47.1
40	657660	14.0
41	667931	0.0



**Figure 10:** Effect of Nrf2/Keap1 complex inhibitors on Nrf2 expressions in MCF7 cells. The MCF7 cells were treated for 24 h with DMSO (DM), 30  $\mu$ M of tBHQ, or 30  $\mu$ M of each of the NSC compounds 1-7 (A), (B) 8-14 (B), 15-21 (C), 22-28 (D), 29-35 (E), and 36-41 (F). Thereafter, Nrf2 protein expression was assayed with western blot analysis, with F-actin as loading control

the Nrf2/Keap1 complex by oxidative stress conditions (which affect Neh2 or Neh6 domains), Keap1 is degraded, thereby allowing Nrf2 to build up in the cytosol. After escaping from its complexation with Keap1, Nrf2 migrates to the nucleus where it binds to the AREs or electrophile-response element (EpRE) and induces the expression of antioxidant enzymes [6]. The expressions of the antioxidant enzymes superoxide dismutase, catalase, peroxiredoxin, glutathione peroxidase, *HO-1*, *NQO-1*, and *GCS*, are induced via the Nrf2/Keap1 pathway [16]. The activation of Nrf2 is strongly associated with cytoprotection from the oxidative insults caused by the accumulation of reactive oxygen species (ROS) which are implicated in aging and age-related diseases [17].

Under normal circumstances, MCF7 is a breast cancer cell line with low levels of Nrf2 protein. However, when treated with tBHQ and Nrf2-activating targets, Nrf2 protein levels are increased, leading to upregulation of the expression of the cytoprotective antioxidant enzyme GCS. Interestingly, 29 out of the 41 tested compounds (about 71%) produced positive activation of GCS (Figure 4). Compounds **1-4**, **11**, **12**, **14** and **35** produced activation levels similar to, or higher activation than that of the standard Nrf2 activator tBHQ. This underscores the importance of inhibiting the formation of Nrf2/Keap1 complex, as a strategy for stabilizing and activating Nrf2. Another interesting finding is that the effect of Nrf2 activation, and subsequently, GCS activation was effective even after 24 h. This indicates that these compounds might be useful in fighting

oxidative stress conditions and associated diseases. Most of the tested compounds activated Nrf2.

## CONCLUSION

In the search for new anticancer compounds, many potential targets have been identified in this study using the virtual co-crystallized Nrf2 pharmacophore model. Many of the NCI samples tested were good inhibitors of Nrf2/Keap1 complexation, and they resulted in disruption of Nrf2 degradation, causing Nrf2 to be stabilized and unregulated. The most interesting result is that Nrf2 activation is long-acting (> 24 h). Thus, the disruption of the Nrf2/Keap1 complex is likely to be an effective cytoprotective approach for combating elevated ROS levels and oxidative stress-related disorders and diseases. Although most of the tested compounds upregulate Nrf2 protein expression, some of these compounds do not upregulate GCS. This can be explained by the fact that Nrf2 is involved in the upregulation of many antioxidant enzymes, not just GCS. However, there is need for further investigations to confirm this.

## DECLARATIONS

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### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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