A Complementary Chemical and Genomic Screening Approach for Druggable Targets in the Nrf2 Pathway and Small Molecule Inhibitors to Overcome Cancer Cell Drug Resistance

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Abstract

Resistance to chemotherapy is a major obstacle in the treatment of a wide array of different types of cancer. Chemotherapeutic drug resistance is achieved by cancer cells by a variety of different mechanisms, which can be either compound specific or general. An emerging mechanism for non-specific chemotherapeutic drug resistance relies on hyperactivity of the transcription factor Nrf2. Normally Nrf2 levels are tightly regulated by the ubiquitin-proteasome system, however mutations in genes responsible for this regulation are common in many cancer types, resulting in increased expression of Nrf2, activation of its downstream target genes and resistance to a variety of chemotherapeutic agents. For this reason, there has been considerable interest in the discovery of small molecule inhibitors of Nrf2 capable of attenuating this resistance mechanism. To this end, we have screened two commercially available libraries of known biologically active small molecules to identify potential Nrf2 inhibitors. To increase the breadth of this screen we have also screened an RNAi library that targets the majority of the druggable genome to also identify Nrf2-inhibitor targets that are not currently targeted by small molecules. To complement the commercial chemical and genomic library screening, we screened a small collection of proprietary natural products isolated from marine cyanobacteria, which included actin targeting and uncharacterized but biologically active compounds. Through these efforts, we have identified three classes of compounds: cardiac glycosides; Stat3 inhibitors; and actin disrupting agents as Nrf2 inhibitors that are able to attenuate Nrf2 activity and synergize with chemotherapeutic agents in the non-small

cell lung cancer cell line A549. In addition, we found that grassypeptolide A exerts Nrf2 modulatory activity via a thus far uncharacterized mechanism. In addition, we have identified a set of putative Nrf2-targets comprising the transcription factors TWIST1 and ELF4, the protein kinase NEK8, the TAK1 kinase regulator TAB1 and the dual specific phosphatase DUSP4. This study broadens the range of mechanisms through which inhibition of Nrf2 activity can be achieved, which will facilitate the characterization of novel Nrf2 inhibitors, and allow the design of target specific screening procedures with which to identify more.

Graphical Abstract
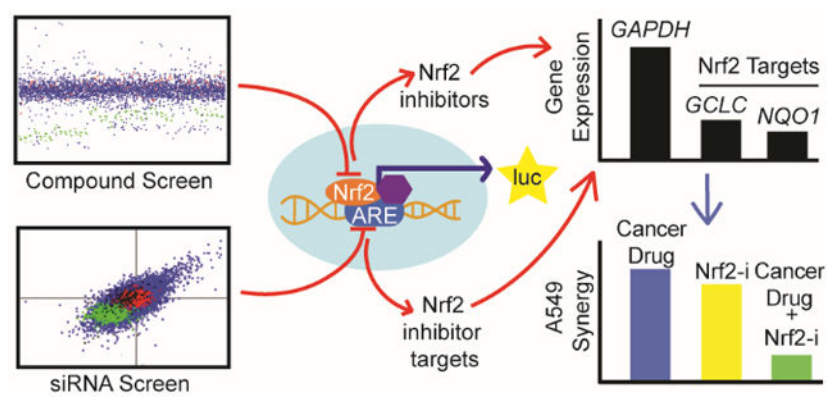
INTRODUCTION

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a cap-'n'-collar type transcription factor that is responsible for expression of genes whose promoters contain the antioxidant responsive element (ARE) sequence[1]. Many genes have been identified as Nrf2 targets and their functions can be broadly categorized into four groups: (1) Resistance to oxidative stress; (2) Oxidant signaling; (3) Drug metabolism and transport; and (4) Intermediary metabolism[2]. In the absence of oxidative stress, Nrf2 is constitutively degraded by the ubiquitin-proteasome system due to its association with Kelch-like ECH-associated protein 1 (KEAP1) and an E3 ubiquitin ligase. Oxidation of key cysteine thiol side chains by reactive oxygen species (ROS) or alkylation by electrophilic compounds causes Nrf2 to dissociate from KEAP1, thereby preventing its ubiquitination and stabilizing its intracellular concentration[3]. The increase in cytoplasmic Nrf2 levels causes increased nuclear import and transcription of genes that are controlled by the ARE (Figure 1a). Once cellular redox balance has been restored by the action of Nrf2 target genes and the bulk of the oxidized KEAP1 has been replaced with its newly synthesized reduced equivalent, Nrf2 ubiquitination resumes and Nrf2 levels decrease to normal[4].

The role of Nrf2 in cancer cell biology is stage dependent and apparently antithetical[5]. Enhancing Nrf2 activity in normal cells helps prevent neoplastic transformation by enhancing detoxification of ROS, limiting the pro-mutagenic damage that high levels of these reactive compounds can induce. In contrast, enhancing Nrf2 activity in transformed cells leads to increased cellular proliferation and tissue invasion and increased anti-neoplastic drug resistance[6, 7]. It is therefore not surprising that a number of different cancer

types express high levels of Nrf2 and are more refractory to clinical chemotherapy. A significant proportion of lung cancers in particular have been found to harbor mutations in KEAP1, Nrf2 or other genes that affect Nrf2 function, such as CUL3 and PTEN[8, 9].

While the major regulatory mechanism of Nrf2 activity by the ubiquitin-proteasome systems is mediated by the oxidation state of KEAP1 this step is also subject to regulation by the presence of proteins that compete with Nrf2 for KEAP1 binding. In this manner the p62/SQSTM1 (sequestosome1), IKKβ (IκB kinase), Palb2 (partner and localizer of Brca2), WTX (Wilms tumor gene on the X chromosome) and phosphoglycerate mutase 5 (PGAM5) have been implicated in the integration of Nrf2 activity with autophagy, DNA damage and intermediary metabolism (Figure 1a)[2]. Gene products that modulate Nrf2 activity can be identified through genomic screening. For example we identified *DPP3* and *SQSTM1* as top hits in a genomic screen for cDNA that enhance Nrf2 activation in IMR-32 cells[10]. DPP3 was later shown to physically interact, via its ETGE domain, with the KELCH domain of KEAP1, confirming the role of DPP3 in the regulation of Nrf2 activity[11]. Both Nrf2 and KEAP1 are also regulated at the post-translational level by phosphorylation by an array of different kinases which can act to increase[12], decrease[13] or have unknown effects[14] on the expression of Nrf2 target genes[2]. This broad range of proteins and pathways that are able to exert a regulatory effect on Nrf2 suggests the existence of a similarly broad array of potential Nrf2 inhibitor targets.

Given the role of Nrf2 in anti-neoplastic drug resistance, there is a sustained effort to identify small molecule inhibitors of Nrf2. To date the most well characterized Nrf2 inhibitor is the natural product brusatol (Figure 1b). Treatment of A549 cells, an adenocarcinomic human alveolar basal epithelial cell line, with brusatol causes down-regulation of numerous Nrf2-target genes[15].Brusatol is a translation elongation inhibitor that causes a decrease in Nrf2 protein levels by decreasing its synthesis[16]. This effect occurs at concentrations that are non-cytotoxic, however pretreatment of A549 cells with brusatol enhances the toxicity of various chemotherapeutic agents in both *in vivo* mouse xenograft tumor growth and *in vitro* cell culture assays [15-17]. Aside from brusatol a small number of other Nrf2 inhibitors have been reported, the majority of which are analogues of the natural product flavonoid chrysin[18, 19] (Figure 1b). However, the recent reports of the synthetic Nrf2 inhibitors AEM1[20] and ML385[21], which also synergize with current chemotherapeutic agents, are a testament to how attractive the prospects of inhibiting this pathway are (Figure 1b).

The exciting potential of Nrf2 inhibitors to constitute part of an anti-neoplastic combination therapy certainly warrants efforts to discover these compounds. It is reasonable to suggest, given the ever evolving and already multi-faceted nature of the regulatory network that surrounds Nrf2, that a wide array of potential Nrf2 inhibitor targets remain to be discovered. Knowledge of these targets and the mechanism by which they regulate Nrf2 activity would greatly enhance efforts to identify the targets of novel Nrf2 inhibitors in the future. These assertions motivated us to screen two libraries of commercially available, biologically active small molecules to identify potential inhibitors of Nrf2. In order to broaden the scope of this screen, and due to our previous success in using a genomic screening approach to identify

novel Nrf2 regulators[10], we also screened a library of siRNA that targets the majority of the known or predicted druggable genome to identify potential Nrf2-inhibitor drug targets.

RESULTS AND DISCUSSION

Small molecule library screen

In order to identify putative inhibitors of Nrf2, the MDA-MB-231 reporter cell line expressing luciferase under the control of the ARE-containing NQO1 promoter[22] (referred to hereafter as MDA-MB-231 ARE-luc) was used to screen the Sigma LOPAC and Spectrum Collection libraries of biologically active small molecules as we previously did to identify indirect inhibitors of the HIF pathway in colon cancer cells[23]. We used a two-step screening process since any Nrf2 inhibitor could also be cytotoxic at concentrations greater than those that modulate Nrf2 activity, and that the relative concentrations at which these two distinct phenotypes occur will likely differ in a compound dependent manner, as previously seen with brusatol[15]. The primary screen for Nrf2 inhibitors was performed in quadruplicate at a single concentration of 10 μM in 384-well plates for 24 h and luciferase expression was quantified using the BriteLite reagent. The raw luciferase data was processed and hit compounds identified as outlined in the methods (MAD score   −2). Hit compounds from the primary screen were retested in a secondary screen that consisted of 24-h parallel dose-response luciferase and viability assays (Figure 2b and Figure 3a). Dose-response curves were plotted and compounds that showed a range of concentrations at which the luciferase activity was inhibited to a greater extent and at lower concentrations than the viability assay were further investigated. The primary screen yielded 31 and 102 structurally distinct potential Nrf2 inhibitors from the Sigma LOPAC and Spectrum Collection libraries, respectively, plus 10 hit compounds present in both libraries (Figure 2a and Table S1). This overlap is greater than what would be expected by random chance given the overlap between the two libraries screened (Figure S1c). In total 77 compounds were retested in the secondary screen (Figure 2b). The selection of these compounds aimed to maximize diversity of biological activities, targets, and chemical structures within the secondary screen. Of all the compounds retested, 15 showed a clear window of specific Nrf2 inhibitory activity (Figure 2b). Due to target and mechanism redundancy, 12 of these compounds were selected for further characterization based on their drug-like properties (Figures 3b and S1).

Nrf2 protein levels and therefore expression of ARE controlled genes is higher in MDA-MB-231 cells than in non-transformed cell lines despite the presence of wild type copies of *NFE2L2*, *KEAP1* and *CUL3*. Overexpression of Nrf2 still acts to enhance drug resistance in MDA-MB-231 cells, indicating that this cell line has not reached the maximal capacity for Nrf2 expression[24]. To assess whether the putative Nrf2 inhibitors identified using the MDA-MB-231 ARE-luc reporter cell line would act in the same way in a cell line with considerably higher basal Nrf2 protein levels and ARE gene expression, they were tested for the ability to inhibit Nrf2-target gene expression in the non-small cell lung cancer A549 cell line. The A549 cell line, which was derived from an adenocarcinoma of the human alveolar basal epithelium, expresses high levels of Nrf2 due to a somatic mutation of the KEAP1 gene, in combination with hypermethylation in the KEAP1 promoter, and has been used extensively to study the role of Nrf2 in cancer biology[25-28]. In this cell line Nrf2 has been

found to be required for long term acquired resistance to cisplatin[29], and attenuating Nrf2 activity by shRNA knockdown or pharmacologically enhances chemotherapeutic sensitivity both *in vitro* and *in vivo*[15, 24, 30] by enhancing cellular ROS levels[17]. Compounds were first tested for their ability to inhibit the expression of the Nrf2-target genes *GSTM2*, *NQO1*, *HMOX1* and *GPX2* (Figure 3b). The translation elongation inhibitor emetine inhibited the expression all of Nrf2 target genes tested to varying extents (Figure 3b). This result was expected since the inhibitory effect of brusatol on cellular Nrf2 activity is due to preferential inhibition of Nrf2 translation and lends validity to the screen design used[16, 31]. However, compounds that generally perturb transcription such as the topoisomerase I and II inhibitors topotecan, and mitoxantrone failed to specifically inhibit the expression of Nrf2 target genes in A549 cells (Figure S2). This discrepancy could indicate cell type specificity with regards to Nrf2 inhibitor mechanism or more likely it is due to the transcription centered mechanism of all of these compounds.

The hit compound with the largest Nrf2 inhibitory window aside from the protein synthesis inhibitors emetine[32] and anisomycin was the Stat3 inhibitor stattic[33]. Stattic was the third most potent inhibitory hit in the Sigma LOPAC library screen and showed minimal toxicity at concentrations that almost completely inhibited expression of the ARE-luc reporter (Figure 3a). Stattic modestly decreased the expression of Nrf2 target genes in A549 cells (Figure 3a) as well as a slight effect on the expression of *NFE2L2* itself that did not translate to a decrease in Nrf2 protein levels (Figure 3d). Stattic displayed statistically significant cooperativity with vinblastine in A549 cell cytotoxicity and a trend towards cooperativity with cisplatin (Figure 3e and Figure S3).

One interesting class of compound identified as putative Nrf2 inhibitors was the cardiac glycosides. While this class is particularly over-represented in the Spectrum Collection library, all of the five cardiac glycosides (lantoside C, strophanthidin, peruvoside, proscillaridin and ouabain) tested showed a window of specific ARE-luc inhibition in the MDA-MB-231 reporter cell line, and also inhibited the expression of Nrf2 target genes to varying extents in A549 cells (Figures 3a, 3b and Figure S1) including genes involved in glutathione biosynthesis and recycling. Furthermore, exposure to ouabain also causes a dose dependent decrease in A549 cellular glutathione levels (Figure 3c), consistent with inhibition of Nrf2 target gene expression[20, 34, 35]. Furthermore, ouabain treatment causes a significant decrease in cellular Nrf2 protein levels in A549 cells without effecting *NFE2L2* gene expression (Figure 3d). The cardiac glycosides are well characterized inhibitors of the plasma membrane sodium-potassium ATPase pump (Na^+,K^+-ATPase)[36], however the structurally unrelated Na^+,K^+-ATPase inhibitor 3,4,5,6-tetrahydroxyxanthone[37] failed to inhibit ARE-luc expression in MDA-MB-231 cells, nor does it inhibit the expression of the Nrf2 target genes tested in A549 cells (Figure S4). Finally, to investigate the potential for cardiac glycoside drugs to be used as a part of an anti-cancer drug combination treatment regime, the ability of ouabain to potentiate the *in vitro* cytotoxicity of vinblastine was assessed. Figure 3e shows that 40 nM ouabain[38] is able to significantly enhance the *in vitro* cytotoxicity of vinblastine towards A549 cells when pretreated for 6 h () and potentially augment the cytotoxicity of cisplatin (Figure S3). The potential mechanisms responsible for the Nrf2 modulatory activity of the cardiac glycosides are discussed further in the supplementary discussion (Supporting Information). Of note is the fact that effects of both

ouabain and stattic appear to be relatively specific towards cancer cells since they display considerably less cytotoxicity towards the non-tumorigenic cell lines MCF10a and NIH 3T3 (Figure S5).

siRNA Library Screen

In order to identify potential Nrf2 inhibitor targets neglected by the libraries containing pharmacologically validate compounds, a library of siRNA targeting 7,784 druggable targets, with four independent siRNA per gene, was screened for those that inhibit expression of the ARE-luc reporter in MDA-MB-231 cells. The primary screen was performed by reverse transfection in 384-well plates at a final siRNA concentration of 20 nM (Figure 4a and 4b) and luciferase expression and viability were measured in parallel luminescence assays using the BriteLite and ATPlite plus reagents, respectively, 72 h post transfection (48 h to allow target knockdown plus 24 h to allow the downstream effect to occur). The web application CARD was used to normalize the raw luciferase data and identify likely hit siRNAs as outlined in the methods[39]. The summary statistics of the normalized luminescence scores were equivalent across all of the plates comprising the siRNA library in both screen replicates (Figure S6). An off-target analysis was also performed using CARD, this is an important step in the analysis of the siRNA library screen data since the library has not been extensively validated with respect to target specificity. This analysis searches the hit siRNA sequences for a common seed sequence. The prevalence of such a sequence among hit siRNA that are supposed to target different genes would indicate their effects are likely mediated through a common, phenotype-relevant off-target gene. Such an effect has been noted in several previously published siRNA library screens, however, no such common seed sequence was found amongst the siRNA hit sequences from either of the screen replicates. In total, 157 and 224 gene targets were identified in the two screen replicates as being potential Nrf2 inhibitor targets in MDA-MB-231 breast cancer cell (Figure 4b), with 40 gene targets common between both replicates (Figure 4b and 4c). The knockdown efficiency of a selection of five of these overlapping hits was assessed and suggests a correlation between the knockdown efficiency of the hit siRNA and the level of ARE-luc inhibition (Figure S7) lending to the validity of the hits from the siRNA primary screen. The only gene hit which was targeted by more than two different siRNAs was *ACTR10*. There is little literature regarding *ACTR10*, however this actin related protein has been implicated in axonal mitochondrial transport in zebrafish by constituting part of the dynein-associated dynactin complex[40]. This indicates a potential role of either the dynactin complex or the actin cytoskeleton itself, in the regulation of Nrf2 activity in MDA-MB-231. Due to the lack of actin targeting compounds in either of the commercial chemical libraries screened this possibility was addressed by screening a proprietary collection of marine natural products of bacterial origin which included two actin disrupting agents dolastatin12 and lyngbyabellin A described in the following section.

The result of the siRNA screens were analyzed for canonical pathway and network enrichment using Ingenuity Pathway Analysis (IPA). The list of overlapping hits from both the screen replicates was not enriched for any one particular pathway, however, the network analysis revealed the enrichment of two networks both of which are centered on known regulators of Nrf2. The most significantly enriched network contains hit genes linked to

peroxisome proliferator receptor gamma (*PPARG*) and *BRCA1* and the next most significant network contains the well-characterized post-translational Nrf2 regulatory kinases Akt, Erk and p38/MAPK as well as NF-κB (Figure S8 and Table S3). Given the modest but encouraging overlap between the two screen replicates, the hit lists were combined and analyzed using IPA. The results of this analysis show the most significantly enriched canonical pathway was the protein kinase A signaling pathway (P = 0.000138) however this was due to the presence of only 17 of the 384 pathway components in the hit list (Table S4 and Figure S9). The enrichment in this case is likely driven by the presence of siRNAs that target *PRKACG*, which encodes the gamma regulatory subunit of protein kinase A, which is a component of the central hub of this pathway. The role of this pathway is discussed further in the supplementary material however since this pathway is known to regulate Nrf2 activity in various different cell types and lends confidence to the primary screen results.

Despite a lack of pathway enrichment amongst the siRNA inhibitors, the IPA analysis hinted at the involvement of the Stat1/3/5 pathway in ARE-luc expression (Figure 4d). The Stat3 inhibitor stattic was a hit in the small molecule screen, and showed a significant ARE-luc inhibition window in MDA-MB-231 cells and decreased the expression of Nrf2 target genes in A549 cells (Figures 2c-e). A regulatory interaction between Stat3 and Nrf2 was predicted by an *in silico* network analysis that combined genome-wide data regarding predicted and known physical interactions of Nrf2 with its list of target genes, transcription factors that control *NFE2L2* transcription and miRNA data[41]. Furthermore, it has been recently proposed that the Nrf2 inhibitor, wogonin, reverses adriamycin resistance in myelogenous leukemia cells and decreases *NFE2L2* gene expression by inhibiting Stat3 and NF-κB signaling[42]. These results are further evidence that despite the lack of enrichment of many canonical pathways' central components, enrichment of a peripheral pathway component is enough to implicate the involvement of the pathway in Nrf2 activity.

Selected potential novel Nrf2-inhibitor targets from the siRNA screen were chosen for individual validation. Two independent siRNA for each putative target gene, differing in sequence from those present in the siRNA library, were tested in a dose-response parallel luciferase and viability assay. Of the siRNAs tested in this manner, six potential Nrf2-inhibitor targets (*TWIST1*, *ELF4*, *TAB1*, *NEK8*, *DUSP4* and *NDUFS4*) were identified by virtue of their ability to inhibit expression of the ARE-luc reporter without a significant effect on cell viability in the MDA-MB-231 ARE-luc reporter cell line (Figure 5a). The high degree knockdown efficiency of these independent siRNAs indicates that the inhibitory effect on the ARE-luc reporter is due to knockdown of the specific target gene expression and not an off target effect (Figure S10). The siRNA of targeting five of the six putative Nrf2 inhibitor genes (*TWIST1*, *ELF4*, *TAB1*, *NEK8* and *DUSP4*) also decreased Nrf2 target-gene expression in A549 cells (Figure 5b), suggesting that these Nrf2-inhibitor targets are conserved between at least these two cell types. To date none of these genes have been empirically determined to modulate Nrf2 activity however there is substantial circumstantial evidence for their role and is discussed further in the supplementary discussion however we suggest that the transcription factors *TWIST1* and *ELF4* could directly modulate *NFE2L2* gene expression as well as the expression of its target genes (Figure 5c).

Identification of Marine Natural Products as Nrf2 Inhibitors

We then screened a focused library of natural products from marine cyanobacteria. Since there is an absence of actin targeting compounds in either of the compound libraries screened, we also included two known cyanobacteria-derived actin disruptors, using the MDA-MB-231 ARE-luc reporter cell line. As a result, three known natural products, lyngbyabellin A[43], grassypeptolide A[44, 45] and dolastatin 12[46] were identified to display a specific window of ARE-luc inhibitory activity at 24 h without significant cytotoxicity at the same time point (Figure 6a). To validate the Nrf2 inhibitory effect of these compounds, Nrf2 target gene expression was measured in MDA-MB-231 cells after 24 h exposure. Cells were treated with a concentration of each compound which suppressed luciferase activity to less than 20% of the solvent treated control, whilst maintaining cell viability at more than 70% based on MDA-MB-231 ARE-luc reporter assay results. To establish whether the Nrf2 inhibitory effect observed with the three marine natural products can be translated into another cell type, the effect of these compounds on Nrf2 activity in the human lung carcinoma cell line A549 was also tested. Considering the similarity of the dose-response patterns of these natural product hits on MDA-MB-231 and A549 cells after 24 h treatment (Figure S11), A549 cells were treated with the same doses as MDA-MB-231 cells. All the natural products tested effectively decreased the transcription of Nrf2 target genes *NQO1*, *HMOX1*, *GPX2* and *GCLC* (Figure 6b) in both cancer cells. Moreover, lyngbyabellin A, grassypeptolide A and dolastatin 12 reduced protein expression level of Nrf2 and its target NQO1 (Figure 6c) and the total cellular levels of glutathione in A549 cells after 24 h exposure (Figure 6d) as well, which is also consistent with reported Nrf2 inhibitor activities[20,33] and is discussed further in the supplementary material.

Grassypeptolide A has been reported to have moderate cell cytotoxicity toward HT29 cells, cause G_0/G_1 phase cell cycle arrest at lower concentrations while induce G_2/M arrest at higher concentration and exhibit apoptosis-inducing activity[47]. Interestingly, grassypeptolide A was found to preferentially inhibit dipeptidyl peptidase 8 (DPP8) over dipeptidyl peptidase 4 (DPP4)[44, 48]. As mentioned in the introduction, DPP3 protein can compete with Nrf2 for binding to KEAP1 and prevent ubiquitination of Nrf2, which increases its cellular concentration and triggers the expression of Nrf2 target genes[11]. Given the functionality of DPP3, it is tempting to speculate that DPP8 may also be able to compete with Nrf2 to associate with KEAP1. And by inhibiting the KEAP1 binding activity with DPP8, grassypeptolide A may be able to stimulate the degradation of Nrf2, thus inhibiting the expression of Nrf2 target genes and sensitizing cancer cells.

To verify the practical effect of natural product inhibitor hits, a combination study was carried out in A549 cells line. Due to the potential novelty of grassypeptolide A target and mode-of-action, this assay was performed with grassypeptolide A. As shown in Figure 6e, co-treatment of A549 cells with grassypeptolide A and vinblastine proved to be synergistic at certain dose as determined by changes in Bliss dependence calculations. . Moreover, co-treatment of A549 cells with grassypeptolide A and cisplatin also show potential synergy (Figure S12).

CONCLUSION

Through our compound screening efforts we have identified promising Nrf2-inhibitors of diverse mechanism. Likewise, by screening a library of siRNA that target the druggable genome, we have also identified novel Nrf2-inhibitor targets for which there are currently no inhibitors. The combination of these screening strategies has enabled us to define a broader set of Nrf2-inhibitor targets than either in isolation would be capable of. Screening of a novel natural product library focused on marine cyanobacterial compounds revealed novel activities for compounds with known and unknown targets. The breadth of diversity of the inhibitors and targets identified herein reflects the multifaceted aspects of Nrf2 activity regulation, which will facilitate the mechanistic delineation of novel Nrf2 inhibitors in the future and enable the development of specific Nrf2 targeted drug screens as well.

MATERIALS AND METHODS

Details of the experimental procedures are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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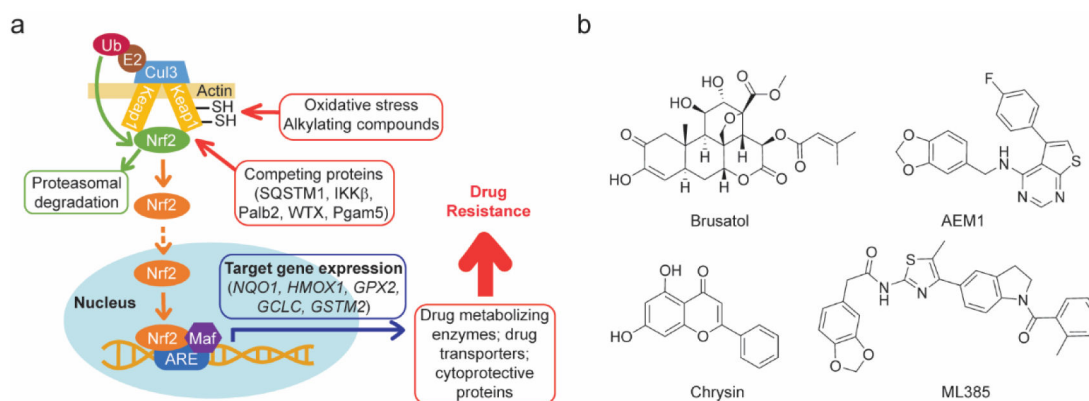
Figure 1.
(a) The dominant control mechanism by which Nrf2 activity is regulated occurs through its association with the E3 ubiquitin ligase KEAP1 and subsequent ubiquitination and proteasomal degradation. Reactive oxygen species and alkylating compounds react with key thiol resides on KEAP1 and promote dissociation of Nrf2 from the KEAP1 complex. Dissociation of Nrf2 from this complex can also be achieved by the expression of mutated form of KEAP1 as is common in numerous different cancer types and through the increased expression or availability of proteins that compete with Nrf2 for KEAP1 binding. Nrf2 translocates to the nucleus where it positively regulates the expression of genes required for resistance to oxidative stress, including drug metabolizing enzymes and transporters capable of detoxifying and effluxing anti-neoplastic agents, the combination of which gives rise to drug resistance. **(b)** Representative Nrf2 inhibitors previously reported.
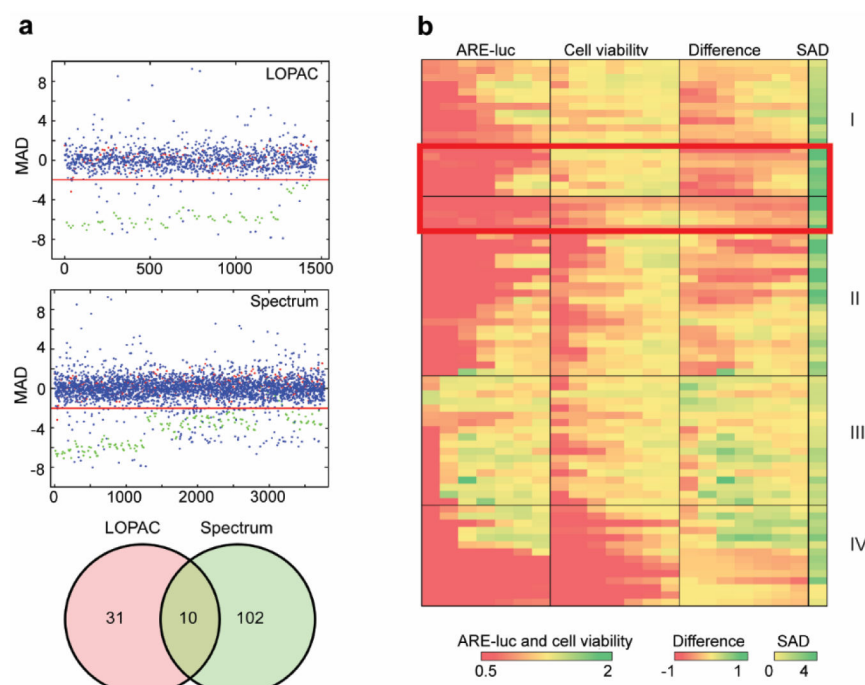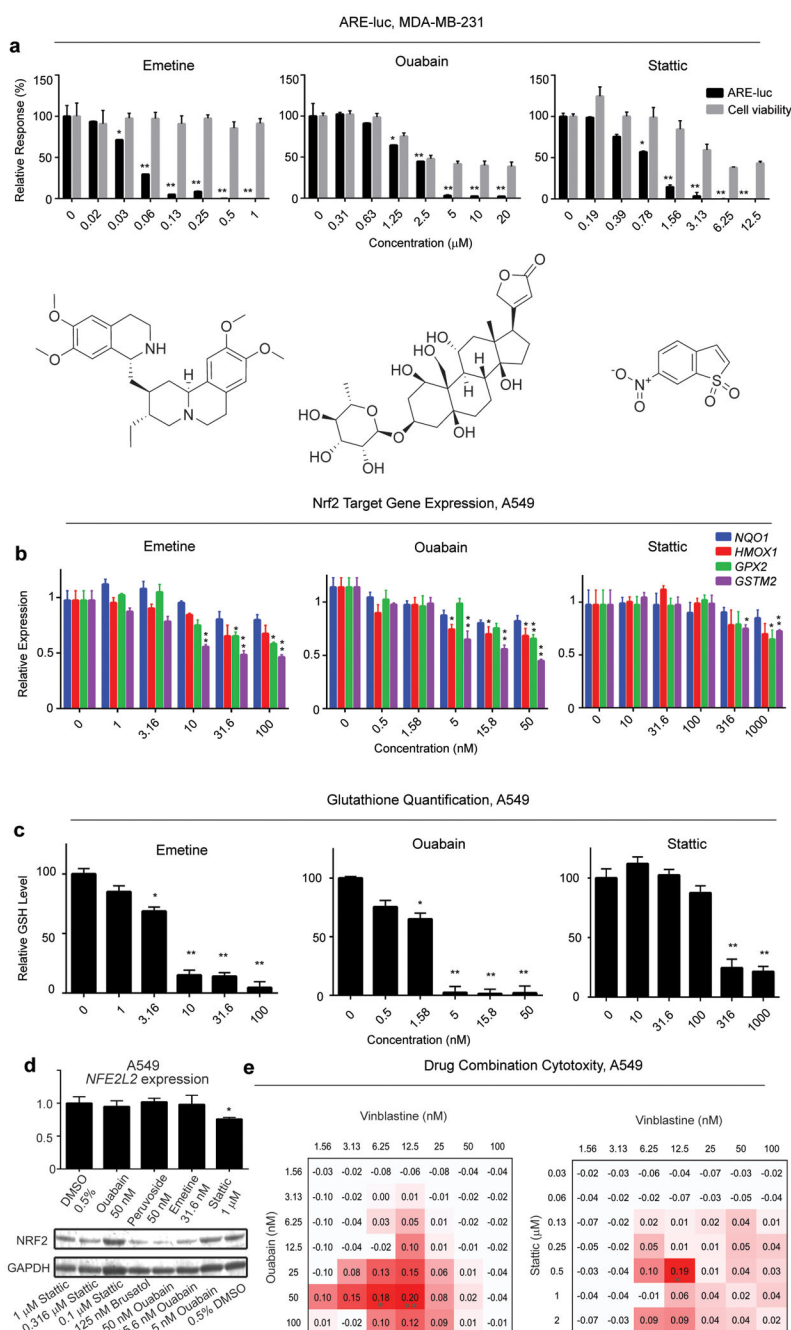
Figure 2.
Screen for small molecule modulators of Nrf2 activity. **(a)** Primary screen for Nrf2 modulators from the Sigma LOPAC and Spectrum Collection libraries using the MDA-MB-231 ARE-luc reporter cells line. Compound index number plotted against the median absolute deviation (MAD) from quadruplicate samples, the red line indicates the Nrf2 inhibitor MAD cutoff value (MAD −2). Blue: library compounds tested at 10 μM. Red: Negative control (0.5% DMSO). Green: Nrf2 inhibitor positive control (125 nM brusatol). The Venn diagram summarizes the common and unique Nrf2 inhibitor hits from each library. **(b)** Secondary screen for Nrf2 inhibitors. Primary screen hits were retested in parallel ARE-luc and cell viability seven-point dose-response assays. The ARE-luc and cell viability assay raw values are expressed relative to the negative control (0.5% DMSO) and the sum of the absolute difference across all seven concentrations was used to identify: non-toxic Nrf2 inhibitors (group I); Nrf2 inhibitors that are also toxic (group II); compounds with negligible activity (group III); toxic compounds (groups IV). The red box indicates the compounds that were further characterized.

Figure 3.
(a) Representative secondary screen dose-response plots for the putative ARE inhibitors emetine, stattic and ouabain in the MDA-MB-231 ARE-luc reporter cell line expressed relative to the negative control (0.5% DMSO). **(b)** Nrf2 target gene expression in A549 cells after exposure to emetine, stattic and ouabain. A549 cells were exposed to the putative Nrf2 inhibitors for 24 h and the expression of the indicated Nrf2 target genes was quantified by RT-qPCR using *GAPDH* as the reference gene. **(c)** Total glutathione content of A549 cells after exposure to putative Nrf2 inhibitors. A549 cells were exposed to emetine, stattic and

ouabain for 24 h and total glutathione was quantified with the colorimetric DTNB assay. Error bars represent ± SEM, * P 0.05, ** P 0.01 relative to negative control (One way ANOVA followed by Dunnett's test). **(d)** *Upper: NFE2L2* gene expression quantification by RT-PCR in response to ARE inhibitor treatment in A549 cells using *GAPDH* as the endogenous control. Error bars represent ± SD, *P 0.05, relative to 0.5% DMSO treatment (one way ANOVA followed by Dunnett's test). ***Lower*** Nrf2 protein expression in response to 24 h treatment of the indicated Nrf2 **(e)** Bliss independence calculations for A549 cells co-treated with oubain (***left***) and stattic (***right***) and vinblastine. A549 cells were treated with ouabain or stattic at the indicated concentrations for 6 h, followed by treatment with vinblastine at the indicated concentrations and viability was quantified using the MTT assay after a total of 72 h drug exposure. " Bliss independence" is the difference between observed growth inhibition and Bliss expectation. Values greater than zero represent a synergistic response. Bliss expectation equals to C = (A+B) - (A×B), where A and B are the growth inhibition fractions of two compounds at a given dose and expressed relative to the negative control (0.5% DMSO). * P 0.05, ** P 0.01 relative the expected inhibitory effect of the two compounds (Student's t-test).
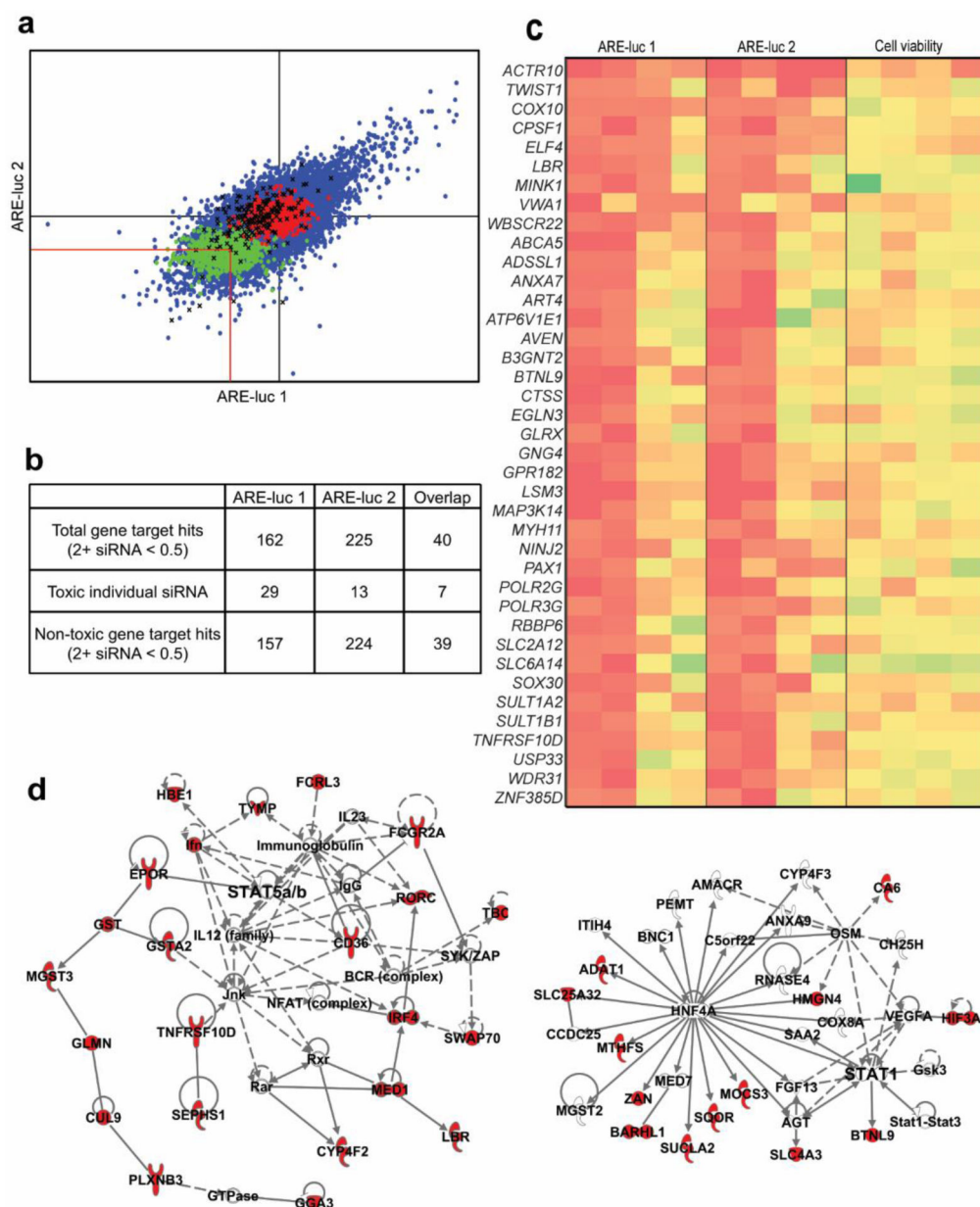
Figure 4.
Screen for siRNA modulators of Nrf2 activity. **(a)** Primary screens for siRNA Nrf2 modulators using the MDA-MB-231 ARE-luc reporter cell line. Raw luciferase output for each siRNA was expressed relative to the median luciferase values of the negative control present on each plate and the standardized scores of the two replicate screens are plotted against each other. The impact on viability of each siRNA was assessed in parallel and siRNA that caused greater than 50% toxicity were excluded from the downstream analyses. Red lines indicate the cutoff for hit siRNAs that decrease luciferase output by greater than 50%. Blue dots: test siRNA. Green dots: positive control siRNA (*NFE2L2* SMARTpool). Red dots: negative control siRNA. Black X: toxic test siRNA. **(b)** Hit summary of the two siRNA ARE-luc inhibitor screens. The total number of gene hits is the number of genes

targeted by more than two different siRNA from each screen. The total number of toxic individual siRNA is the number of single siRNA ARE-luc inhibitor hits that cause a greater than 50% decrease in viability. The number of non-toxic gene target hits is the number of genes targeted by more than two different siRNAs after the removal of the toxic individual siRNA hits. **(c)** Heatmap representing the standardized ARE-luc and viability scores for the overlapping set of siRNA ARE-luc inhibitor hits from the two replicate siRNA modulator screens. Each column represents a single siRNA targeting the indicated gene **(d)** Two examples of enriched networks resulting from the IPA analysis of the combined list of ARE-luc inhibitor gene hits from both ARE-luc screens. Red nodes are genes present in the combined list of ARE-luc inhibitor genes hits, colorless nodes are genes that interact with the ARE-luc inhibitor gene hits. Solid edges represent physical interaction between the protein products of the indicated genes. Dashed edges represent non-physical, functional interactions between the indicated genes or their protein products.
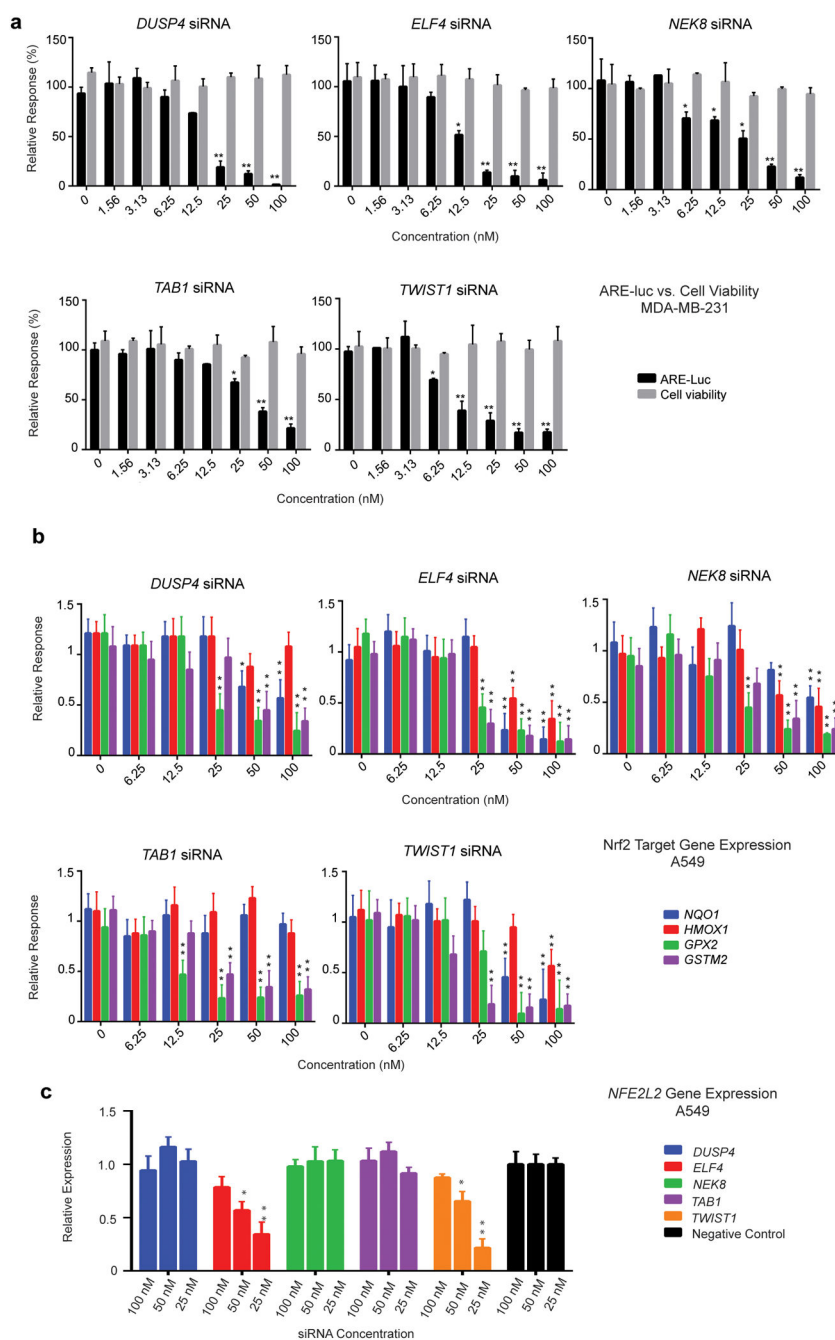
Figure 5.
Validation of selected ARE-luc inhibitor gene hits using two different siRNAs targeting genes identified in the high-throughput MDA-MB-231 ARE-luc modulator screen. **(a)** Dose-response ARE-luc and cell viability assay. The two different siRNA targeting the indicated genes were transfected at the indicated concentrations into the MDA-MB-231 ARE-luc reporter cell line. ARE-luc output and cell viability was quantified 72 h post-transfection using the BriteLite reagent and the MTT assay. The assay was performed in triplicate with two different siRNA, raw luciferase and MTT assay values are expressed relative to the non-

targeting siRNA negative control. The mean and standard deviation of the relative luciferase and MTT assay values were calculated. **(b)** Nrf2 target gene expression in response to transfection with two different siRNA targeting the indicated genes. The expression of four Nrf2 target genes (*NQO1*, *HMOX1*, *GPX2* and *GSTM2*) was quantified by RT-qPCR using *GAPDH* as the reference gene. Error bars represent ± SEM, * P   0.05, ** P   0.01 (One way ANOVA followed by Dunnett's test) relative to negative control. (c) *NFE2L2* gene expression in A549 cells in response hit siRNA treatment. The effect of treatment with three concentrations of two independent siRNA for each putative ARE inhibitor gene on *NFE2L2* gene expression was quantified by RT-qPCR. NFE2L2 expression in response to hit siRNA is expressed relative to that of the negative control siRNA treatment and the average of the two independent siRNA is depicted. *GAPDH* was used as the endogenous control gene. Error bars represent ± SEM, * P   0.05, ** P   0.01 (One way ANOVA followed by Dunnett's test) relative to negative control siRNA.
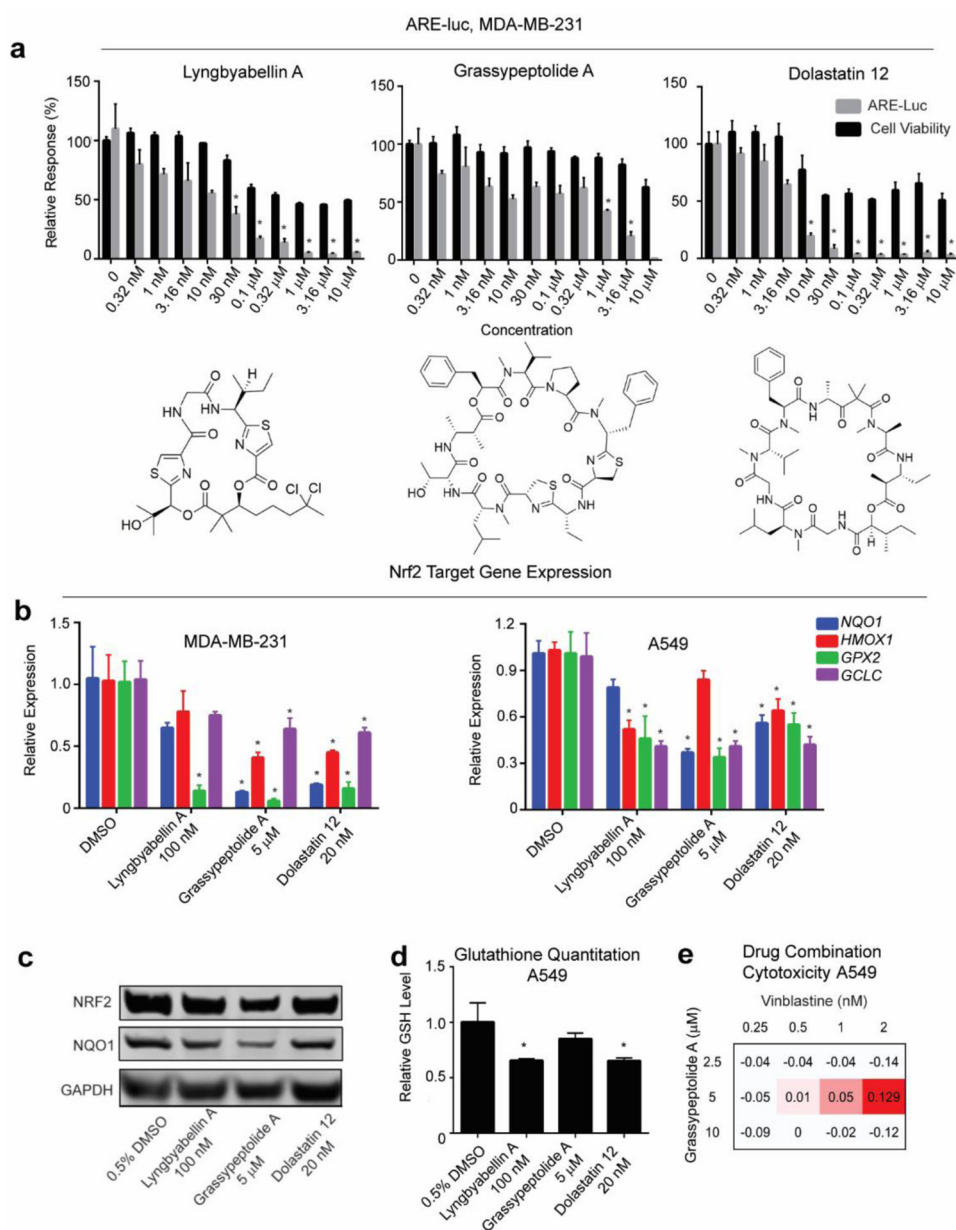
Figure 6.
Inhibitor hits from natural product library screening. **(a)** Structure of lyngbyabellin A, grassypeptolide A and dolastatin 12 with their differential ARE inhibitory effects versus cell viability from MDA-MB-231 ARE-luc reporter assay. Error bars represent ± SEM. **(b)** Transcriptional expression levels of *NQO1*, *HMOX1*, *GPX2* and *GCLC* decreased in MDA-MB-231 and A549 cells upon treatment of natural product inhibitor hits for 24 h, with GAPDH as endogenous control. Error bars represent ± SEM. **(c)** Quantification of Nrf2 and its target NQO1 protein expression by western blot with GAPDH as endogenous control after 24 h treatment with natural products Nrf2 inhibitors in A549 cells. **(d)** A549 cellular reduced glutathione (GSH) content level decreased after 24 h natural product Nrf2 inhibitor hits treatment. In each case error bars represent ± SEM * P < 0.05 relative to vehicle control

(One way ANOVA followed by Dunnett's test). **(e)** Bliss independence calculations for A549 cells co-treated with grassypeptolide A and vinblastine. A549 cells were treated with grassypeptolide A in serial concentrations for 24 h, followed by treatment with vinblastine in serial concentrations for 24h. " Bliss independence" is the difference between observed growth inhibition and Bliss expectation. Values greater than zero represent a synergistic response. Bliss expectation equals to C=(A+B)-(A×B), where A and B are the growth inhibition fractions of two compounds at a given dose. Cell viability was quantified using MTT assay.