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FULL PAPER

Differentiating antiproliferative and chemopreventive modes of activity for electron-deficient aryl isothiocyanates against human MCF-7 cells

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Abstract: Consumption of *Brassica* vegetables provides beneficial effects due to organic isothiocyanates (ITCs), a resultant product of the enzymatic hydrolysis of glucosinolate secondary metabolites. The ITC L-sulforaphane (L-SFN) is the principle agent in broccoli that demonstrates several modes of anticancer action. While the anticancer properties of ITCs like L-SFN have been extensively studied and L-SFN has been the subject of multiple human clinical trials, the scope of this work has largely been limited to those derivatives found in nature. Previous studies have demonstrated that structural changes in an ITC can lead to marked differences in a compound's potency to (1) inhibit growth of cancer cells, and (2) alter cellular transcriptional profiles. This study describes the preparation of a library of non-natural aryl ITCs and the development of a bifurcated screening approach to evaluate the dose- and time-dependence on antiproliferative and chemopreventive properties against human MCF-7 breast cancer cells. Antiproliferative effects were evaluated using a commercial MTS cell viability assay. Chemopreventive properties were evaluated using an antioxidant response element (ARE)-promoted luciferase reporter assay. The results of this study have led to the identification of (1) several key structure-activity relationships and (2) lead ITCs for continued development.

Introduction

The *Brassica* vegetables, which include broccoli, cabbage, cauliflower, Brussels sprouts, kale, collard greens, pak choi and kohlrabi, are rich sources of glucosinolates (β-thioglucoside-*N*-hydroxysulfates); tissue damage to the plant induces enzymatic hydrolysis of glucosinolates, resulting in evolution of various secondary metabolites.[1] At physiological pH, the principle products of glucosinolate hydrolysis are isothiocyanates (ITCs),[2] which are believed to be primarily responsible for the observed cancer chemoprevention that results from diets rich in these vegetables.[3] Although dietary glucosinolates have no known direct bioactivity, many of their resultant ITCs are well-studied anticancer agents; several of the most highly-studied ITC natural products include L-sulforaphane (L-SFN, **1**, Figure 1), benzyl isothiocyanate (BITC, **3**), phenethyl isothiocyanate (PEITC, **4**), and allyl isothiocyanate (AITC, **5**). L-SFN (**1**) is particularly abundant in broccoli and has been the subject of numerous clinical trials.[4] The unsubstituted aryl ITCs **3** and **4** have received significant attention for their ability to inhibit chemically-induced cancer in animal models at low micromolar concentrations,[5] well below the observed threshold for toxicity against noncancerous cells.[6] Enthusiasm for the aliphatic ITC **5** has decreased in part due to preclinical reports citing toxicity in the bladder and hematological tissues.[3i, 6] The literature is rich with many specific examples describing the effects of these (and other) dietary ITCs against cancer cell types and in specific model systems.[3i, 6] One of the most attractive features of using ITCs as anticancer agents is the ability of one agent to affect multiple mechanistic points involved in cancer pathogenesis, through a combination of cytostatic,[7] cytotoxic,[8] and chemopreventive mechanisms.[3c, 8c, 9]

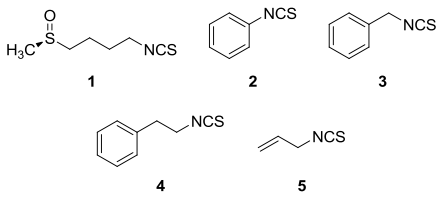


Figure 1. L-sulforaphane (L-SFN), phenyl isothiocyanate (PITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC) and allyl isothiocyanate (AITC).

Dietary ITCs demonstrate chemotherapeutic properties through their ability to damage and inhibit proliferation of cancerous cells. These agents are capable of directly modulating signaling pathways which promote cell proliferation[3i] and inhibiting features of cell division, leading to cell cycle arrest. The most common points of arrest are G_0–G_1 and G_2–M, dependent on both the identity of the ITC and cellular mechanisms specific to a cancer cell type.[3i, 6] ITCs can promote apoptosis and autophagic cell death through diverse mechanisms,[3i] modulate epigenetic marks through inhibition of histone deacetylase (HDAC),[7a, 10] promote anti-angiogenic effects through downregulation of vascular endothelial growth factor (VEGF), and elicit anti-metastatic effects through suppression of ERK kinase.[3i] ITCs have also demonstrated anti-inflammatory and immunomodulatory activity[11] and can sensitize cancer cells to other anticancer agents.[12]

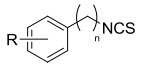
The second major mode of ITC activity relates to their ability to inhibit carcinogenesis through chemopreventive mechanisms. ITCs block tumorigenesis caused by various chemical carcinogens by reducing expression levels of phase I drug-metabolizing cytochrome P450s.[3i, 13] Arguably, the most significant chemopreventive activity of ITCs results from their potent induction of phase II enzymes that assist in clearing chemical carcinogens and reactive oxygen species from the body; noteworthy phase II enzymes include glutathione *S*-transferases (GST), NAD(P)H:quinone oxidoreductase (NQO1), epoxide hydrolase, and UDP-glucuronosyl-transferases.[4c, 14] The most well-studied phase II chemopreventive mechanism involves the activation of nuclear factor E2 p45-related factor 2 (Nrf2).[3d, 15] Under basal conditions, cytoplasmic Nrf2 is associated with Kelch-like ECH-associated protein 1 (Keap1), a multidomain, cysteine-rich protein bound to the actin cytoskeleton that promotes Cul3-based ubiquitination of Nrf2 and its subsequent degradation by the proteasome.[3a, 3d] ITCs contain an electrophilic carbon (R–N=C=S) capable of reaction with thiols, including specific cysteine residues on the surface of Keap1.[16] Thiocarbamoylation of Keap1 at Cys151 by ITCs disrupts the Keap1-Cul3 interaction, thus preventing Nrf2 ubiquitination, and resulting in Nrf2 release and its subsequent nuclear accumulation.[4a, 16b, 17] In the nucleus, heterodimerized Nrf2 binds to antioxidant response elements (ARE), regulatory DNA sequences upstream of chemopreventive genes, where it activates transcription.[18] The large number of genes impacted through ITC-mediated stimulation of Nrf2 transcriptional activity indicates the importance of this interaction in dietary cancer chemoprevention.[19]

Although the effects and mechanisms of naturally-occurring, dietary ITCs have been heavily investigated, fewer studies have described the anticancer properties of non-natural ITCs. A 2008 study described the preparation and evaluation of 35 non-natural ITCs and several of their non-natural glucosinolate precursors.[20] In this study, 5 of the 35 analogues screened demonstrated enhanced antiproliferative activity versus **1**; each of these analogues were substituted aryl ITC variants. The percentage of hits produced by this panel (14%) was high compared to what is typically observed during a preliminary screen (0.1-5%),[21] suggesting that the structure of the parent compound **1** may be sub-optimal for antiproliferative activity. Several key structure–activity relationships (SARs) were identified, indicating that ITCs may be amenable to drug development strategies. Importantly, this study also demonstrated that synthetic, non-natural glucosinolates can serve as precursors for their analogous, improved non-natural ITCs.[3d, 22] This feature has been validated by more recent work[23] and may serve as an opportunity to circumvent the problem of aqueous instability of ITCs, which has plagued formulation strategies during clinical trials.[24] Together, this supports the premise that non-natural ITCs are viable targets for the design of novel anticancer agents, a field that has not been extensively explored.

The central motivation for this study was to test the hypothesis that unsubstituted, dietary aryl ITCs are not optimal as anticancer agents and that the structure of a substituted variant will impact it's observed anticancer properties. Despite the specific mechanistic differences between the action of **3** and **4** across various cancer cell types, both compounds demonstrate broad similarities in their ability to inhibit cancer cell growth, promote cell cycle arrest, stimulate apoptosis, generate ROS, inhibit phase I enzymes, and induce expression of ARE-dependent phase II genes. These trends are a stark contrast to the properties exhibited by their non-natural methylene homologue, phenyl isothiocyanate (PITC, **2**), which has significantly reduced activity.[3h, 20] The anticancer properties of substituted aryl ITCs have not been thoroughly investigated and would prove useful in developing a more comprehensive understanding of SARs for this class of compounds. Since natural ITCs are known to thiocarbamoylate cellular proteins,[16a] it is reasonable to expect that the steric and electronic properties of non-natural, substituted aryl ITCs may impact an analogue's observed anticancer properties.

To test this hypothesis, a panel of 36 substituted aryl ITCs were selected for preparation (Figure 2). Since the greatest difference in activity between natural aryl ITCs occurs between 2 and 3, this panel included derivatives of both PITC (n = 0) and BITC (n = 1). Substituted PEITC analogues (n = 2) remain an interest toward the larger hypothesis and were not included in this panel due to the increased synthetic complexity required for their preparation; these studies are ongoing and will be reported in due course. With the expectation of preparing *ortho*-, *meta*-, and *para*-regioisomers for each substituent variant, six R groups were selected for inclusion. Previous SARs noted improved antiproliferative properties for aryl ITCs bearing electron-deficient groups;[20, 22] for this reason, trifluoromethyl (R = CF_3) and nitro (R = NO_2) groups were selected. Since fluorine is a bioisostere for hydrogen, methyl analogues (R = CH_3) were included to directly contrast any electronic effects displayed by trifluoromethyl analogues.[25] Methylsulfanyl (R = SCH_3), methylsulfinyl (R = $S(O)CH_3$), and methylsulfonyl (R = $S(O_2)CH_3$) analogues were selected based on both previous SAR which noted the importance of the sulfinyl group in **1**[20] and the goal of evaluating the effect of electron-deficient groups (e.g. sulfinyl, sulfonyl). Furthermore, these latter analogues served as an opportunity to hybridize the key structural features of **1** with aryl ITCs **3** and **4**; even for such relatively simple compounds, most of these 18 sulfur-containing analogues have novel structures.

In an attempt to accommodate and differentiate the diverse anticancer modes exhibited by natural ITCs, it was desirable to develop a systematic and efficient screening process to broadly determine the properties of candidates against cancer cells. Preliminary screening efforts were conducted against MCF-7 human breast cancer cells; the selection of MCF-7 cells was based on a combination of factors, including: (1) its regard as one of the most highly-studied breast cancer cell lines,[26] (2) its established use as an in vitro model for proliferation and chemoprevention studies with ITCs,[12b, 20, 24, 27] and (3) its reduced levels of cytosolic Nrf2 and subsequent chemosensitivity.[28] The dose-response for each ITC candidate would be determined following variable incubation time with MCF-7 cells (t = 24 h, 72 h). Antiproliferative properties would be evaluated using a commercial MTS cell viability assay which measures the reductive capacity of viable cells.[20, 29] Chemopreventive properties would be evaluated using an in vitro reporter assay which correlates ARE activation to a spectrophotometric response against human MCF-7 cells stably transfected with an ARE-luciferase reporter plasmid vector (MCF-7-ARE cells).[30] Although in vitro assays exist for many biological endpoints, recent analysis has identified limited options to evaluate candidate ARE induction without use of an animal model;[3h] consequently, development and validation of this latter assay would greatly complement the body of available in vitro screening methods.



R = CH_3, CF_3, NO_2, SCH_3, $S(O)CH_3$, $S(O_2)CH_3$
n = 0, 1

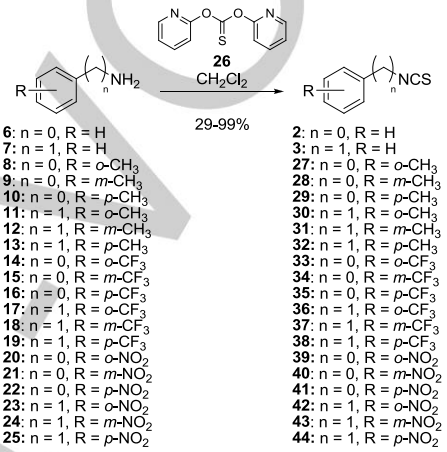
Figure 2. Target aryl ITC analogues.

Results and Discussion

Preparation of aryl ITC analogues

Approximately half of the target aryl ITCs were prepared directly from reaction of their corresponding, commercially-available primary amines (**6**–**25**, Scheme 1) with *O*,*O*-di(pyridin-2-yl) carbonothioate (**26**).[23a, 31] While many of the substituted PITC analogues (n = 0) have been described within the literature or are, in some cases, commercially-available, all compounds were synthetically prepared, purified, and characterized to ensure uniformity among the analogues to be evaluated. It was observed that the highest yields of ITC were obtained when using > 3.0 equivalents of **26**. Although **26** is commercially-available, it was more cost-efficient to prepare **26** in-house using a modified version of the synthesis described by Kim, et. al.[31]

Sulfane-, sulfoxide-, and sulfone-derived aryl ITCs were prepared using the divergent synthesis described in Scheme 2. Since two of the target ITC scaffolds lacked commercially-available primary amines, they were prepared from benzaldehydes **45**–**46** using reductive amination. Treatment of **45**–**46** with hydroxylamine hydrochloride provided oxime intermediates in high yield (data not shown), which were immediately reduced to amines with $LiBH_4$.[32] It was observed that the reduction was most often responsible for the greatest loss in yield, due to a combination of sluggish reaction and the difficulty in isolating the free amine products. Passing gaseous HCl through the solution of the amines in dry MeOH induced selective precipitation of the amine hydrochloride salts;[29b] intermediate **52** was obtained in 27% yield over three steps. Amines **47**–**52** were reacted with **26** to generate ITCs **53**–**58**.[23a, 33] Sulfoxide- (**59**–**64**) and sulfone-analogues (**65**–**70**) were prepared through treatment of **53**–**58** with *m*CPBA.[20, 34] While sulfone-variants could be prepared in high yield using excess (> 3 equivalents) oxidant, it was challenging to obtain sulfoxide analogues in similar yield; even with careful, controlled addition of one equivalent *m*CPBA, some unreacted sulfane and sulfone were observed. Since both sulfoxide- and sulfone-analogues were desired for evaluation, reactions were intentionally performed with 1-2 equivalents *m*CPBA to provide a mixture of both sulfoxide and sulfone products which were obtained using flash chromatography separation. This approach provided high combined yields of both products (67–94%).

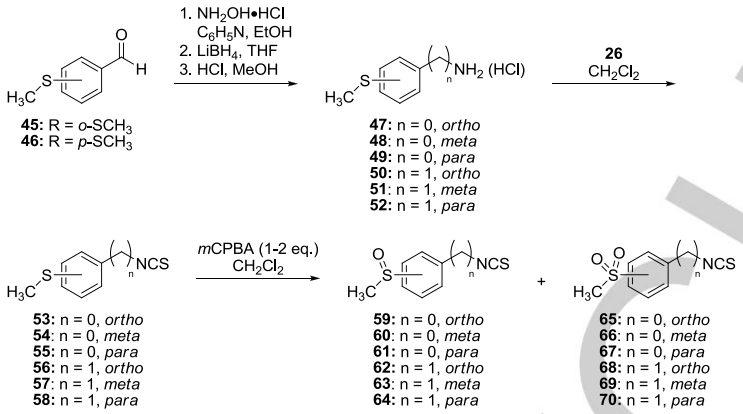


Scheme 1. Preparation of methyl-, trifluoromethyl-, and nitro-derived aryl ITCs.

Evaluation of antiproliferative activity for aryl ITC analogues

The antiproliferative properties of each non-natural aryl ITC candidate was evaluated against human MCF-7 breast cancer cells over the concentration range 200 to 0.78 µm. Although a candidate incubation time of 72 h was selected to maintain consistency with previous methods,[20, 29] parallel evaluation of ARE-induction required shorter incubation periods; consequently, in order to maintain congruency between the datasets for both endpoint assays, candidates were evaluated after both 24 h and 72 h incubation. The commercial MTS assay was employed to assess the number of viable cells relative to control, similar to previous reports.[35] Representative antiproliferation dose-response curves for aryl ITC analogues are depicted in Figures 3-5, with calculated GI_{50} values in Table 1. In this study, the graphical depiction of data provides the means to draw parallels between antiproliferation and ARE-induction modes of evaluation, as well as to illustrate unexpected features within the antiproliferation dataset.

The antiproliferative activity of **1** after 72 h was consistent with previous data (3.1 ± 0.9 µm), despite the differences in the method used to determine cell viability and the nature of the dosing regimen.[20] However, antiproliferation data for **1** after 24 h unexpectedly demonstrated a significant non-sigmoidal relationship (NSR) in the dose-response curve, which was unable to be fit to the nonlinear log(inhibitor) vs. response (variable slope, 4 parameters) function in GraphPad 6.0. The deviation from an expected sigmoidal response was observed at concentrations between 50 to 200 µm and was consistent across individual trials obtained over a five year-span (see Figure SI-1 in the Supporting



Scheme 2. Preparation of sulfane-, sulfoxide-, and sulfone-derived aryl ITCs.

Information). Since the MTS assay was used to determine percent viability relies on mitochondrial function to provide a colorimetric measurement of living cells, these data alone do not directly provide insight to rationalize the presence of the NSR. While the antiproliferative properties of **1** against a variety of cancer cell lines have been previously examined,[36] few reports have even addressed a possible relationship between **1** and pro-viability effects.[37] Consequently, the literature is unclear whether a pro-viability NSR has been similarly observed and documented. It is plausible that this deficiency results from a combination of (1) the infrequency of reporting dose-response curves, (2) the increased dose-response resolution afforded by iterative, 2-fold serial dilutions used in this current study, and (3) the range of concentrations tested. Furthermore, an attenuated NSR for **1** also appears to be reproducibly present over the same concentration range after 72 h incubation; the slight deviation from sigmoidal response at [**1**] = 50 μm was initially believed to result from normal experimental variation and does not significantly impact the ability to fit the data to a nonlinear curve. However, with the additional context of the 24 h incubation response, it seemed more likely that the presence of a NSR is time dependent, decreasing between 24 h and 72 h incubation.

Expanding the body of antiproliferation results to include the panel aryl ITCs draws attention to several comparisons, trends, and structure-activity relationships (SARs). Out of the 38 ITCs evaluated, 14 demonstrated non-sigmoidal dose-response curves after 24 h incubation; only three analogues demonstrated a NSR after 72 h incubation. All but one of the compounds with a NSR were substituted derivatives of BITC (**3**); the sole exception was **33**, *ortho*-CF_3-substituted PITC. Similar to the trends for **1**, the NSR effects were reproducible across trials (data not shown) and were largely attenuated at 72 h incubation. While it is remains unclear why BITC and its analogues exhibit a greater propensity to have a NSR, the alkyl linker between the isothiocyanate functional group and the aromatic ring (n = 0-1) appears to play an important role. Throughout the remainder of this account, compounds demonstrating NSR have been excluded from quantitative comparisons of GI_{50} trends; this designation most significantly affects analysis of certain analogue subsets after 24 h incubation. For most analogues, antiproliferative activity increased between 24 h and 72 h, consistent with previous observations.[36a] Of the 10 aryl analogues demonstrating the opposite qualitative trend, three were inactive across the dose range (**59**–**61**) and five had 24 h activity within error of 72 h activity (**2**, **27**–**29**, **40**); the anomalies (**33** and **39**) were both *ortho*-substituted PITC analogues bearing a strongly electron withdrawing group (CF_3 and NO_2, respectively). On average, PITC analogues (n = 0) demonstrated greater than 5-fold reduction in antiproliferative properties versus BITC analogues (n = 1) after both 24 h and 72 h incubation; this calculation was made using GI_{50} = 200 μm for inactive compounds (GI_{50} > 200 μm).

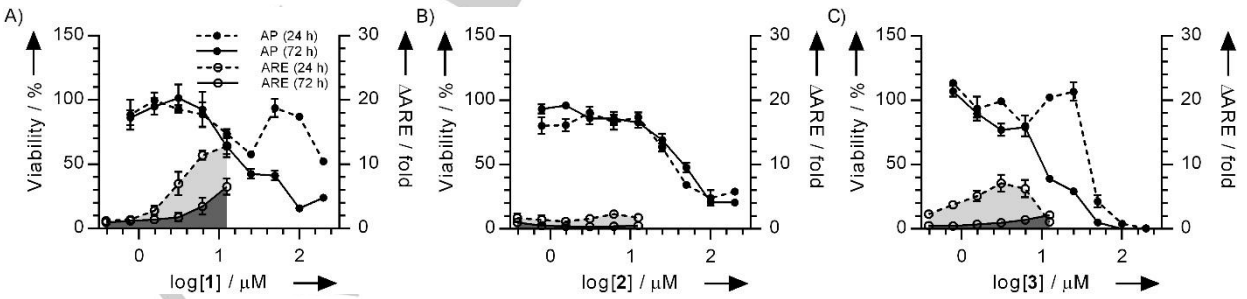


Figure 3. Dose-response anticancer properties of ITCs **1**, **2**, and **3** against MCF-7 cells. Trials were conducted with 24 h (dashed lines) and 72 h incubation (solid lines) for each ITC candidate. Plots depict representative data acquired via antiproliferation (AP, left axis) and ARE-induction assays (ARE, right axis), arranged systematically with regard to candidate structure; the area below ARE-induction data is shaded to improve clarity (t = 24 h, light gray; t = 72 h, dark gray). Error bars represent standard deviation within a representative trial (*n* = 3). A) ITC **1**. B) ITC **2**, C) ITC **3**.

Comparison of substituted aryl ITCs to the unsubstituted **2** and **3** provided insight toward the impact of functional group on antiproliferation activity. All aryl ITCs bearing a methyl group or a trifluoromethyl group had lower GI_{50}s relative to the unsubstituted aryl ITC analogue, with the trifluoromethyl group providing the larger reduction; the magnitude of this reduction ranged from a statistically-insignificant 0.1 μm (**28**, 72 h) to 24.4 μm (**35**, 72 h). The presence of a nitro substituent also generally lowered the GI_{50} (range of reduction = 3.0 μm to 20.1 μm); the sole exception to this trend was *ortho*-nitro PITC analogue **39**, which had significantly reduced antiproliferative activity. Structure-activity trends for the three sulfur-containing substituents (methylsulfanyl, methylsulfinyl, and methylsulfonyl) differed between the PITC scaffold and the BITC scaffold. PITC analogues bearing methylsulfinyl, and methylsulfonyl groups were inactive (GI_{50} > 200 μm), as was the *p*-methylsulfanyl analogue **55** after 24 h incubation. The other methylsulfanyl-substituted PITC analogues (**53**, **54**) exhibited no discernible pattern to their antiproliferation activity. In contrast, all of the sulfur-containing BITC analogues (**56**–**58**, **62**–**64**, **68**–**70**) demonstrated decreased GI_{50} after 72 h, relative to unsubstituted **3**. Unfortunately, most comparisons between *ortho*-, *meta*-, and *para*-regioisomers were unable to provide conclusive, statistically-significant trends; the most noticeable difference between regioisomers was observed for the *ortho*-nitro PITC analogue **39**, as previously noted.

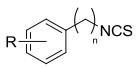


Table 1. Antiproliferation activities of aryl ITC analogues against human MCF-7 breast cancer cells. Reported GI_{50} values represent the mean ± standard deviation of at least two separate experiments, each conducted in triplicate. NSR = non-sigmoidal relationship.

No.	R	n	GI_{50}, 24 h (μM)	GI_{50}, 72 h (μM)	No.	R	n	GI_{50}, 24 h (μM)	GI_{50}, 72 h (μM)
1			NSR	15.5 ± 1.4[a]					
2	H	0	27.2 ± 1.1	33.5 ± 1.2					
3	H	1	NSR	9.8 ± 1.2[a]					
27	*o*-CH_3	0	20.9 ± 1.2	32.5 ± 1.2	**53**	*o*-SCH_3	0	14.6 ± 1.0	17.6 ± 1.2
28	*m*-CH_3	0	19.0 ± 1.0	33.4 ± 1.0	**54**	*m*-SCH_3	0	18.8 ± 1.3	34.6 ± 1.3
29	*p*-CH_3	0	19.4 ± 1.1	24.5 ± 1.2	**55**	*p*-SCH_3	0	> 200	44.1 ± 1.1
30	*o*-CH_3	1	NSR	NSR	**56**	*o*-SCH_3	1	NSR	9.6 ± 1.1
31	*m*-CH_3	1	NSR	NSR	**57**	*m*-SCH_3	1	20.3 ± 1.1[a]	12.3 ± 1.2[a]
32	*p*-CH_3	1	NSR	NSR	**58**	*p*-SCH_3	1	28.1 ± 1.0	10.4 ± 1.1
33	*o*-CF_3	0	NSR	NSR	**59**	*o*-$S(O)CH_3$	0	> 200	> 200
34	*m*-CF_3	0	14.5 ± 1.1	10.8 ± 1.0	**60**	*m*-$S(O)CH_3$	0	> 200	> 200
35	*p*-CF_3	0	15.3 ± 1.0	9.1 ± 1.3	**61**	*p*-$S(O)CH_3$	0	> 200	> 200
36	*o*-CF_3	1	NSR	5.5 ± 1.0[a]	**62**	*o*-$S(O)CH_3$	1	NSR	5.1 ± 1.2
37	*m*-CF_3	1	NSR	NSR	**63**	*m*-$S(O)CH_3$	1	NSR	8.2 ± 1.2[a]
38	*p*-CF_3	1	10.1 ± 1.1	6.1 ± 1.1	**64**	*p*-$S(O)CH_3$	1	NSR	12.3 ± 1.1
39	*o*-NO_2	0	67.0 ± 1.2	142.9 ± 1.4	**65**	*o*-$S(O_2)CH_3$	0	> 200	> 200
40	*m*-NO_2	0	19.0 ± 1.0	15.3 ± 1.1	**66**	*m*-$S(O_2)CH_3$	0	> 200	> 200
41	*p*-NO_2	0	16.0 ± 1.1	13.4 ± 1.2	**67**	*p*-$S(O_2)CH_3$	0	> 200	> 200
42	*o*-NO_2	1	NSR	3.9 ± 1.3[a]	**68**	*o*-$S(O_2)CH_3$	1	NSR	4.4 ± 1.2
43	*m*-NO_2	1	13.3 ± 1.1	5.5 ± 1.2	**69**	*m*-$S(O_2)CH_3$	1	NSR	NSR
44	*p*-NO_2	1	18.9 ± 1.3	6.8 ± 1.2[a]	**70**	*p*-$S(O_2)CH_3$	1	30.8 ± 1.0[a]	6.3 ± 1.1

[a] Exhibited minor traces of NSR which did not appear to impact calculation of GI_{50} values.

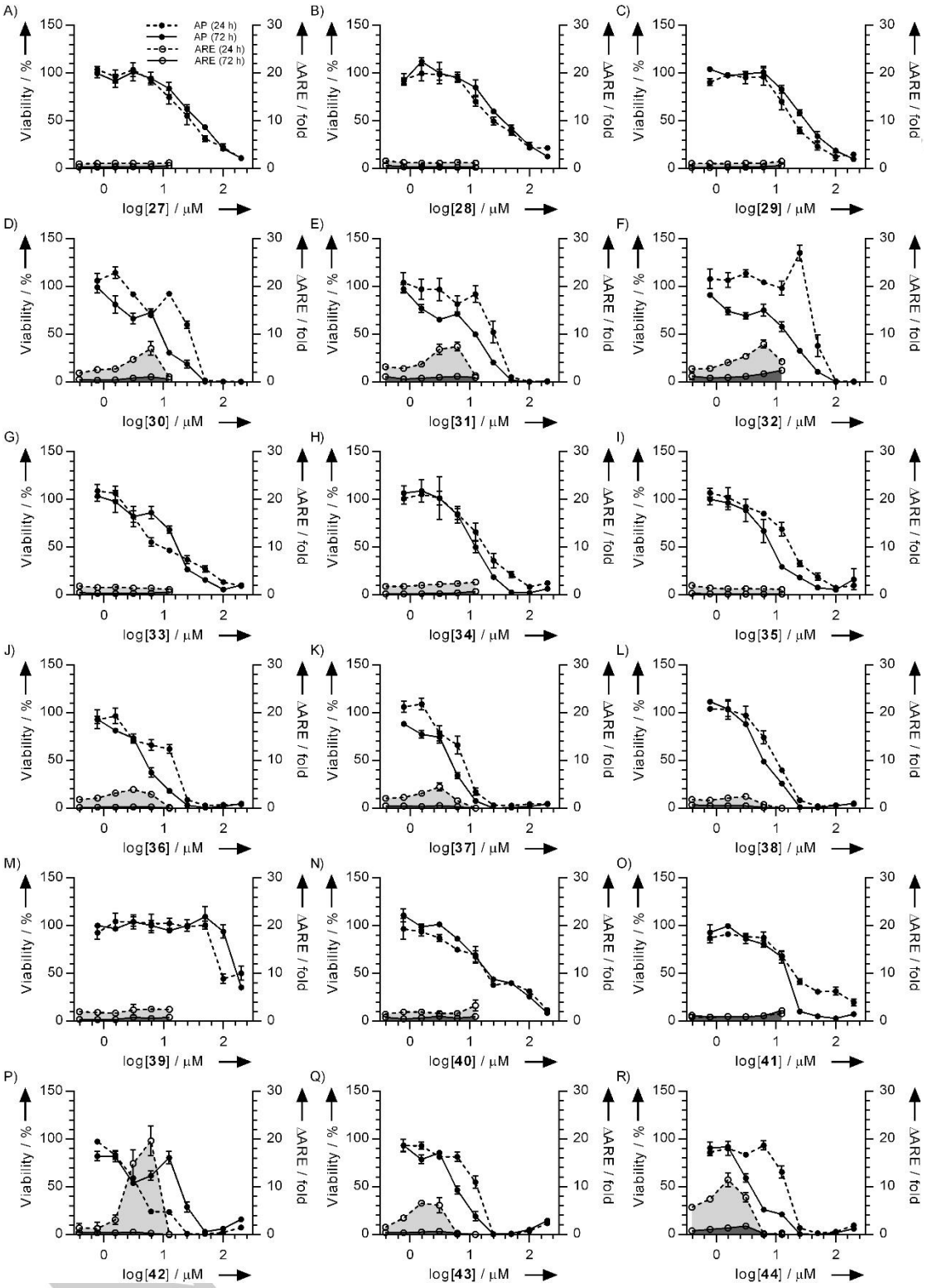


Figure 4. Dose-response anticancer properties of aryl ITC analogues bearing methyl, trilfluoromethyl, and nitro substituents against MCF-7 cells. Trials were conducted with 24 h (dashed lines) and 72 h incubation (solid lines) for each ITC candidate. Plots depict representative data acquired via antiproliferation (AP, left axis) and ARE-induction assays (ARE, right axis), arranged systematically with regard to candidate structure; the area below ARE-induction data is shaded to improve clarity (t = 24 h, light gray; t = 72 h, dark gray). Error bars represent standard deviation within a representative trial (*n* = 3). A) ITC **27**. B) ITC **28**. C) ITC **29**. D) ITC **30**. E) ITC **31**. F) ITC **32**. G) ITC **33**. H) ITC **34**. I) ITC **35**. J) ITC **36**. K) ITC **37**. L) ITC **38**. M) ITC **39**. N) ITC **40**. O) ITC **41**. P) ITC **42**. Q) ITC **43**. R) ITC **44**.

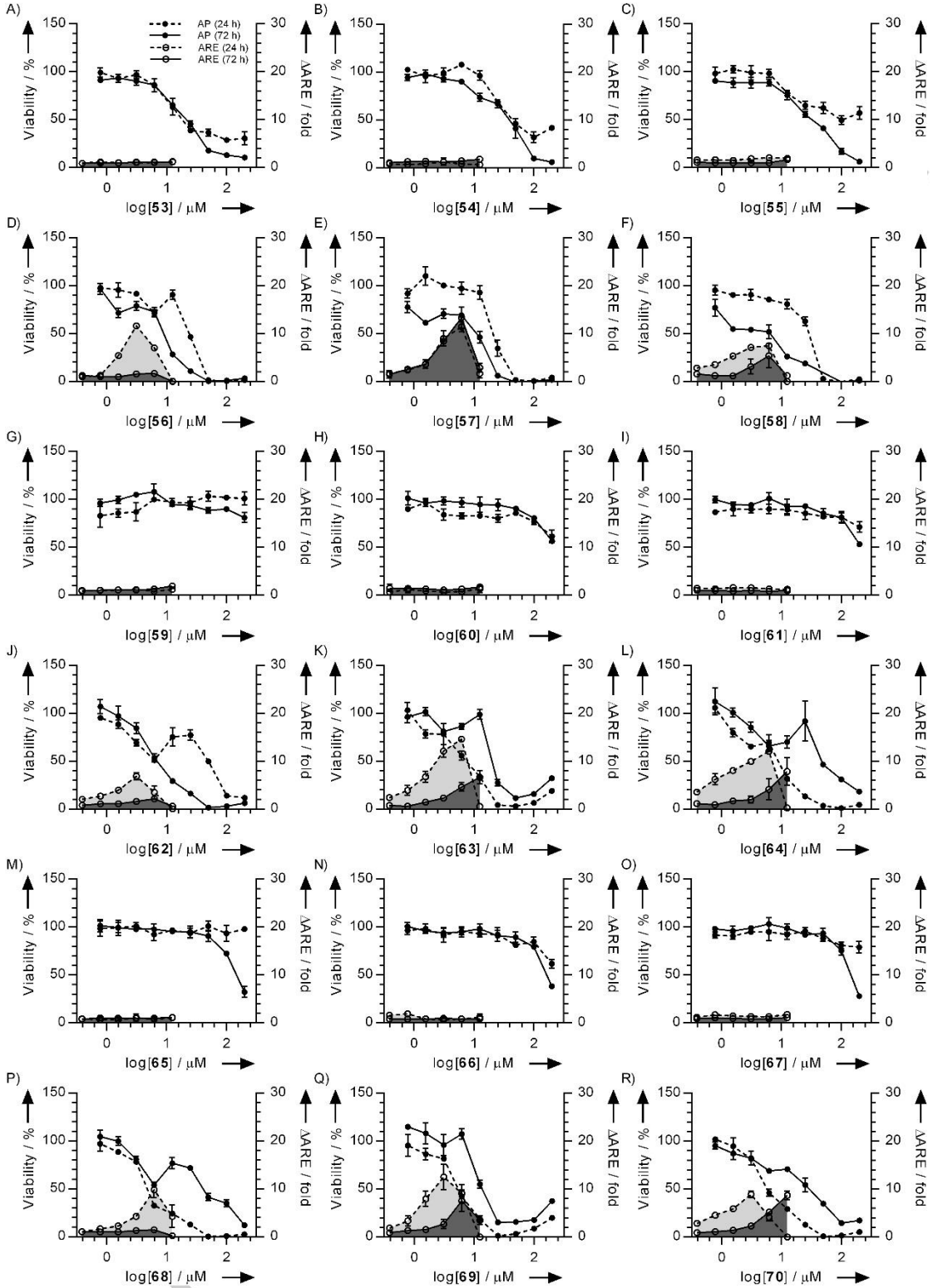


Figure 5. Dose-response anticancer properties of aryl ITC analogues bearing methylsulfanyl, methylsulfinyl, and methylsulfonyl substituents against MCF-7 cells. Trials were conducted with 24 h (dashed lines) and 72 h incubation (solid lines) for each ITC candidate. Plots depict representative data acquired via antiproliferation (AP, left axis) and ARE-induction assays (ARE, right axis), arranged systematically with regard to candidate structure; the area below ARE-induction data is shaded to improve clarity (t = 24 h, light gray; t = 72 h, dark gray). Error bars represent standard deviation within a representative trial (*n* = 3). A) ITC **53**. B) ITC **54**. C) ITC **55**. D) ITC **56**. E) ITC **57**. F) ITC **58**. G) ITC **59**. H) ITC **60**. I) ITC **61**. J) ITC **62**. K) ITC **63**. L) ITC **64**. M) ITC **65**. N) ITC **66**. O) ITC **67**. P) ITC **68**. Q) ITC **69**. R) ITC **70**.

Evaluation of ARE-induction activity for aryl ITC analogues

A primary goal of this work was to develop an accurate and efficient method to screen the chemopreventive capacity of non-natural ITC candidates. To facilitate rapid evaluation and identification of promising lead ITCs, a reporter assay which correlates ARE activation to a spectrophotometric response was utilized. The reporter vector contained eight ARE motifs upstream of a SV40 promoter and the gene for firefly luciferase.[38] Induction of Nrf2 would result in the production of luciferase which could be quantified using a commercial luciferase assay, then normalized to lysate protein concentration. To maintain consistency between antiproliferation studies and ARE-induction studies, human MCF-7 cells stably transfected with a luciferase reporter vector (MCF-7-ARE cells) were utilized.[30]

To evaluate the effectiveness of this reporter construct, MCF-7-ARE cells were treated with **1** over the concentration range 200–0.1 μm and were evaluated between 3–48 h after treatment (Figure 6). The normalized response increased with the concentration of **1** and peaked between 5–10 μm; at higher concentrations, the normalized response sharply declined. This decline appears to be due to a reduction in the detected relative light units (RLU), the quantitative measure of luciferase activity that is proportional to the concentration of luciferase (see Figure SI-2B); the protein concentration remained constant over the range of concentrations evaluated (see Figure SI-2A). It is plausible that higher concentrations of **1** could promote a stress response in MCF-7-ARE cells which affects the stability, longevity, and activity of any luciferase produced by the reporter vector. Consequently, subsequent ARE studies were limited to ITC concentrations below 12.5 μm. Experiments conducted using MCF-7-ARE and parental MCF-7 cell in parallel demonstrated that an observed luciferase response was specific to MCF-7-ARE cells containing the vector and luciferase gene (see Figure SI-3). The data in Figure 6 also suggests that the maximal reporter response occurred 24 h after inoculation with **1**; prior to 12 h, minimal ARE-activation was observed. These findings are consistent with previous reports describing the dose- and time-dependence of Nrf2-activation following treatment with **1**,[39] further supporting the use of this reporter construct to screen the chemopreventive capacity of aryl ITC analogues.

Despite the semi-quantitative nature of data from the ARE reporter assay, a number of SARs for aryl ITC analogues have been identified. A clear trend was observed concerning the chain length between the isothiocyanate functional group and the aryl ring; after 24 h, none of the PITC analogues (n = 0) demonstrated significant capacity for ARE induction over the dose range, while BITC analogues (n = 1) all demonstrated some degree of ARE induction. While (1) the magnitude of ARE induction, (2) the concentration at which maximal ARE induction was observed, and (3) the presence of a sharp decline in ARE induction at higher concentrations differed between individual BITC analogues, it is unclear whether most of these differences are statistically significant. Qualitatively, it appears that trifluoromethyl-substituted BITC analogues (**36**–**38**) demonstrated consistently-reduced capacity for ARE induction. Although **1** demonstrated diminished (but measurable) ARE induction after 72 h, many of the BITC analogues demonstrated little to no capacity for ARE induction after 72 h. Those with noticeable ARE induction after 72 h included *meta* and *para* isomers of methylsulfanyl- (**57**–**58**), methylsulfinyl- (**63**–**64**), and methylsulfonyl-substituted BITC analogues (**69**–**70**); the activity of *ortho* isomers (**56**, **62**, and **68**) were diminished after 72 h. Interestingly, analogues **63**, **64**, and **70** were the only aryl ITC analogues that paralleled the ARE induction profile for **1**, which lacked an observed decline in ARE induction over the concentrations tested. It is plausible that this similarity may be due to the presence of a methylsulfinyl or methylsulfonyl group, which is a common structural feature among these ITCs. Analogue **57** was unique among the ITCs evaluated, as its significant ARE induction did not noticeably change between 24 h and 72 h incubation. Given the prominent and well-characterized capacity of **1** to act as an ARE inducer, aryl ITCs **57**, **63**, **64**, and **70** were identified as potential lead compounds due to their strong similarity in ARE-induction profile.

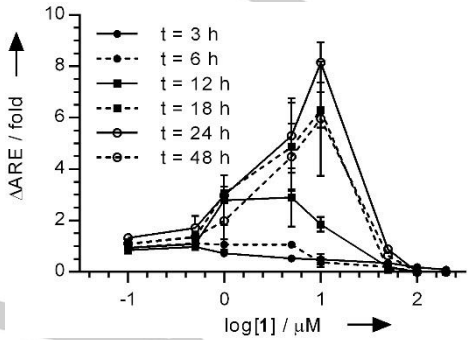


Figure 6. Time-dependence of the dose-response curves for the ARE induction activity of **1** against MCF-7-ARE cells. Trials were conducted over a range of incubation times (3–48 h) for 200–0.1 μm ITC concentration. Error bars represent standard deviation within a trial (*n* = 3).

Integrated analysis of antiproliferation activity and ARE-induction activity for aryl ITC analogues

A secondary outcome of evaluating both the antiproliferative and chemopreventive modes of activity exhibited by aryl ITC analogues was to determine if there were any correlating factors between the two types of data. This underlying analysis served as a motivation to depict the time- and dose-dependence of each ITC analogue on a single plot (Figures 3–5). One of the most interesting features of the antiproliferation data for ITC analogues was the significant and unexpected presence of NSRs. With the exception of ITC **33**, every analogue which demonstrated an antiproliferation NSR after 24 h or 72 h also demonstrated ARE induction through the luciferase reporter assay; the opposite correlation does not appear valid, as several compounds (**38**, **43**, **44**, **56**, **58**) elicited ARE induction without an evident non-sigmoidal antiproliferation response. Furthermore, the data suggest that these two effects appear to occur over a similar concentration range. This qualitative observation is complicated by the sharp reduction in ARE induction exhibited by many ITC analogues, which, as previously mentioned, may be an undesired artifact of the reporter assay. It is also possible that these two responses result from different cellular mechanisms, similar to conclusions drawn in a previous study.[37]

From a mechanistic perspective, cellular induction of the ARE by ITCs are known to result in increased transcription of several phase II genes and a chemopreventive response. It is plausible that ARE induction may be related to the activation of other, pro-survival responses that lead to increased relative proliferation, observed as a NSR. Based on the data, a NSR response only appears to occur over a relatively narrow concentration window (1–2 two-fold dilutions = approximately 2–4-fold change in [ITC]). Beyond this range, antiproliferation activity is reasserted, suggesting that an ITC-induced pro-survival response has limited capacity. Were these NSR effects only observed after treatment with substituted aryl ITC analogues, it would be easier to speculate that these non-natural agents were eliciting different

cellular responses than their natural analogues. However, both natural analogues **1** and **3** also demonstrate similar trends in antiproliferation dose-response, ARE induction, and a NSR. Additional studies to more fully understand the interplay between these types of cellular responses are ongoing and will be reported in due course.

Validation of ARE-induction by qPCR

In order to validate the screening effectiveness of the luciferase ARE reporter assay, a subset of five ITCs were selected for evaluation of their capability to induce expression of ARE promoted genes. L-SFN (**1**) was included as a well-described ARE inducer and positive control. Phenyl isothiocyanate (**2**) was a non-natural aryl ITC that demonstrated negligible capacity for ARE induction by the luciferase reporter assay. ITC **37** was selected as a representative substituted BITC analogue which elicited a non-sigmoidal antiproliferative response and a low capacity for ARE induction. ITC **57** was included for its strong and consistent ARE induction after both 24 h and 72 h incubation. ITC **64** was selected as a representative analogue which demonstrates ARE induction after both 24 h and 72 h and a non-sigmoidal antiproliferative response, criteria which are qualitatively similar to the activity profile of L-SFN (**1**). Human MCF-7 cells were treated with the selected ITCs over the same concentration range used in the luciferase reporter assay (12.5–0.4 μm) and gene expression was determined by quantitative polymerase chain reaction (qPCR). The levels of four well-described redox-sensitive, ARE-promoted genes were analyzed in parallel to provide a more-complete picture of the changes in ARE-promoted transcriptional response: NAD(P)H:quinone oxidoreductase (NQO1), heme oxygenase 1 (HMOX1), glutathione *S*-transferases α1 (GSTα1), and thioredoxin reductase 1 (TXNRD1). Transcriptional responses were normalized to glyceradehyde-3-phosphate dehydrogenase (GAPDH).

Dose-response curves for the transcriptional expression of genes following treatment by the five selected ITCs are depicted in Figure 7. As a positive control, treatment with L-SFN (**1**) increased expression of all four genes in a dose-dependent fashion. Treatment with ITC **2** did not elicit significant transcription of any of the four genes, consistent with the ARE induction data from the luciferase reporter assay. Treatment with ITC analogues **37**, **57**, and **64** increased expression of all four analyzed genes. Unlike the data resulting from the evaluation of these analogues through the luciferase reporter assay, gene expression increased over the dose range that was tested; the sharp decline in ARE-induction from the reporter assay was not observed through the qPCR analysis, an observation which supports the premise that this decline may be an artifact of the reporter system. The increases in gene expression for **37** and **57** were similar in magnitude, both between the four gene targets and in comparison to the effects of **1**. The major exception to this trend related to the significantly increased expression of HMOX1 following treatment with **37**. The underlying rationale for this large and gene-specific difference is unclear and the subject of ongoing investigation. Of the ITC analogues evaluated, **64** was found to be an incredibly strong inducer of all four ARE-promoted genes. The magnitudes of the transcriptional responses elicited by **64** are especially compelling when normalized to the expression profile for L-SFN (**1**), a well-described ARE inducer. Expression of NQO1 (10-21 fold), HMOX1 (0.7-15 fold), GSTα1 (1.4-8.6 fold), and TXNRD1 (0.8-3.8 fold) were all significantly larger following treatment with **64** relative to **1**, especially at the higher ITC concentrations.

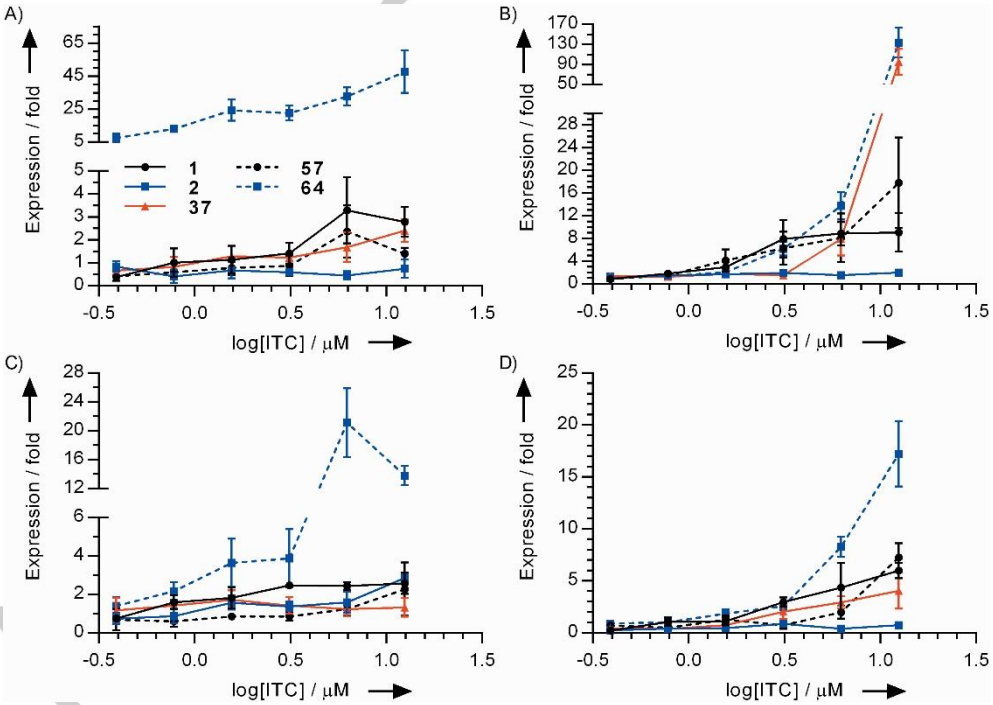


Figure 7. Dose-response curves for the transcription of ARE-promoted genes following treatment of ITCs against MCF-7 cells, analyzed by qPCR. Experiments were conducted after 48 h incubation with ITCs, over a concentration range 12.5–0.4 μm. Fold-increases in transcribed genes are reported relative to vehicle-only controls. Error bars represent the standard deviation (n = 3). A) NQO1. B) HMOX1. C) GSTα1. D) TXNRD1.

Taken together, the qPCR data provides support to validate the use of the luciferase reporter assay during initial evaluation of ITC candidates. It appears that the greatest strength of the luciferase reporter lies in discerning compounds which are capable of inducing expression of ARE-promoted genes (e.g. 1, 37, 57, 64) from those that are incapable (e.g. 2). However, there appears to be minimal correlation between both the magnitude and gene specificity of a response in the luciferase reporter assay and the magnitude of actual gene transcription assessed by qPCR

Conclusions

The central hypothesis of this study was that the anticancer properties of naturally-occurring, unsubstituted aryl ITCs are not optimized and that substituted aryl ITC variants will demonstrate differential (and hopefully improved) anticancer properties. To test this hypothesis, a panel of 36 substituted variants of PITC and BITC were identified and prepared. The time- and dose-dependence of ITC candidates were systematically evaluated against human MCF-7 breast cancer cells using two differential screening methods; one assay evaluated antiproliferative activity while the second utilized a luciferase reporter construct to indirectly measure induction of ARE-promoted genes. Together, the combined body of data resulting from these studies identified key several structure-activity relationships for both potential modes of aryl ITC bioactivity. In relation to the original hypothesis, these screening methods have provided the means to differentiate each candidate's anticancer activity profile and, in part, to identify structural features which confer improved modal selectivity. Several of the ITC analogues demonstrated antiproliferative properties with a non-sigmoidal dose-response, a unique feature that has not previously been documented for this class of compounds. Efforts to understand and rationalize this effect are ongoing and will be reported in due course. While the described luciferase reporter assay provided an efficient method to evaluate ARE induction, further efforts and revisions to this methodology will continue to address some of its limitations, including the need for improved dynamic range. Finally, *p*-methylsulfinyl BITC (**64**) was identified as a potent inducer of ARE-promoted genes, approximately an order of magnitude stronger than the natural product L-SFN (**1**). The anticancer properties of ITC **64**, and other related analogues, will continue to be evaluated and explored as a new class of non-natural aryl ITC variants.

Experimental Section

All reactions were carried out under nitrogen unless indicated otherwise. All reagents were obtained from available commercial sources and were used without further purification unless otherwise noted. Melting point analyses were conducted using an open capillary tube, unless otherwise noted. The silica gel used in flash chromatography was 60 Å, 230–400 mesh. Analytical TLC was performed on Uniplate 250 µm silica gel plates with detection by UV light. NMR spectra were acquired on a JEOL ECS-400 400 MHz NMR spectrometer with multinuclear capability and 24-sample autosampler, with solvent as internal reference; the chemical shifts are reported in ppm, in δ units. Infrared spectra were acquired on a Nicolet Avatar FTIR. High resolution mass spectroscopic data were obtained at the Mass Spectrometry & Analytical Proteomics Laboratory at the University of Kansas (Lawrence, KS). Cell viability absorption was measured using a SpectraMax M2 Multi-Mode Microplate Reader. Firefly luciferase activity was measured using a Glomax 96-well Luminometer (Promega). RNA integrity was determined using an Agilent 2100 Bioanalyzer. Quantitative real-time PCR analysis was conducted using an Applied Biosystems 7500 Real Time PCR System.

***O*,*O*-Di(pyridin-2-yl) carbonothioate (26):**[31] To a solution of 2-hydroxypyridine (19.05 g, 200 mmol) and triethylamine (29.3 mL, 210 mmol) in dry CH_2Cl_2 (300 mL) at 0 °C was slowly added thiophosgene (7.60 mL, 99 mmol) over 5 min. After warming to rt over 3 h, the reaction was diluted with saturated aqueous sodium bicarbonate (300 mL). The organic layer was collected, was washed with saturated aqueous sodium chloride (300 mL), dried (Na_2SO_4), and concentrated. Recrystallization from CH_2Cl_2/hexanes afforded **26** as a colorless solid (17.80 g, 77%): m.p. 109.6–113.1 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.46 (d, *J* = 4.6 Hz, 2H), 7.86 (tt, *J* = 7.3, 1.8 Hz, 2H), 7.30 (m, 2H), 7.20 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 192.3, 159.4 (2C), 148.9 (2C), 140.1 (2C), 123.2 (2C), 116.9 (2C); IR (film) ν_{max} 3075, 2993, 2883, 2825, 1722, 1702, 1678, 1672, 1658, 1650, 1643, 1612, 1573, 1468, 1433, 1372, 1302, 1280, 1224, 1170, 1095, 1046, 994, 907, 853, 774, 728, 562 cm^{-1}.

Isothiocyanatobenzene (2): ITC **2** was prepared as previously described.[20]

(Isothiocyanatomethyl)benzene (3): ITC **3** was prepared as previously described.[20]

1-Isothiocyanato-2-methylbenzene (27):[40] To a solution of *o*-toluidine (140 µL, 1.34 mmol) in dry CH_2Cl_2 (19.5 mL) at rt was added **26** (413 mg, 1.72 mmol). The reaction was stirred for 20 h and the solvent was concentrated. Flash chromatography (silica gel, 25:1 hexanes:EtOAc) afforded **27** as a colorless oil (172 mg, 86%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.24-7.16 (m, 4H), 2.40 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 135.4, 135.2, 130.9, 130.5, 127.6, 127.1, 126.2, 18.6; IR (film) ν_{max} 2923, 2854, 2175, 2086, 1598, 1580, 1501, 1485, 1460, 1381, 1290, 1115, 1037, 929 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for C_8H_7NS, 149.0299; found, 149.0293.

1-Isothiocyanato-3-methylbenzene (28):[41] To a solution of *m*-toluidine (0.12 mL, 1.05 mmol) in dry CH_2Cl_2 (11.0 mL) at rt was added **26** (268 mg, 1.16 mmol). The reaction was stirred for 24 h and the solvent was concentrated. Flash chromatography (silica gel, hexanes) afforded **28** as a colorless oil (93 mg, 59%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.24 (t, *J* = 7.3 Hz, 1H), 7.12-7.01 (m, 3H), 2.35 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 139.9, 135.0, 131.2, 129.5, 128.4, 126.5, 122.9, 21.4; IR (film) ν_{max} 2921, 2229, 2136, 2099, 1604, 1583, 1484, 1484, 1453, 964, cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for C_8H_7NS, 149.0299; found, 149.0277.

1-Isothiocyanato-4-methylbenzene (29):[42] To a solution of *p*-toluidine (158 mg, 1.03 mmol) in dry CH_2Cl_2 (15.0 mL) at rt was added **26** (292 mg, 1.26 mmol). The reaction was stirred for 12 h and the solvent was concentrated. Flash chromatography (silica gel, 25:1 hexanes:EtOAc) afforded **29** as a light yellow oil (126 mg, 82%): ^{1}H NMR ($CDCl_3$, 400 Mz) δ 7.18-7.10 (m, 4H), 2.36 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 137.7, 134.5, 130.3 (2C), 128.5, 125.7 (2C), 21.4; IR (film) ν_{max} 2923, 2848, 2192, 2138, 2896, 1653, 1636, 1502,1456, 1324, 1113, 1101, 928, 815 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for C_8H_7NS, 149.0299; found, 149.0286.

1-(Isothiocyanatomethyl)-2-methylbenzene (30): To a solution of 2-methylbenzylamine (100 µL, 0.81 mmol) in dry CH_2Cl_2 (12.0 mL) at rt was added **26** (289 mg, 1.24 mmol). The reaction was stirred for 17 h and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **30** as a yellow oil (130 mg, 99%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.32-7.23 (m, 4H), 4.70 (s, 2H), 2.36 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 136.0, 132.5, 132.0, 130.9, 128.9, 128.1, 126.7, 47.3, 19.0; IR (film) ν_{max} 3067, 3023, 2924, 2858, 2168, 2087, 1695, 1652, 1606, 1493, 1461, 1437 1340 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for C_9H_9NS, 163.0456; found, 163.0476.

1-(Isothiocyanatomethyl)-3-methylbenzene (31): To a solution of 3-methylbenzylamine (98 µL, 0.81 mmol) in dry CH_2Cl_2 (12.0 mL) at rt was added **26** (289 mg, 1.16 mmol). The reaction was stirred for 17 h and the solvent was concentrated. Flash chromatography (silica gel, 25:1 hexanes:EtOAc) afforded **31** as a colorless oil (129 mg, 98%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.29 (t, *J* = 7.8 Hz, 1H), 7.19-7.10 (m, 3H), 4.69 (s, 2H), 2.39 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 139.0, 134.3, 132.1, 129.3, 129.1, 127.8, 124.1, 48.3, 21.6; IR (film) ν_{max} 3026, 2960, 2922, 2854, 2165, 2094, 1610.18, 1492, 1439, 1338, 1260, 1095, 1019 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for C_9H_9NS, 163.0456; found, 163.0450.

1-(Isothiocyanatomethyl)-4-methylbenzene (32):[43] To a solution of 4-methylbenzylamine (100 µL, 0.70 mmol) in dry CH_2Cl_2 (10.5 mL) at rt was added **26** (250 mg, 1.08 mmol). The reaction was stirred for 17 h and the solvent was concentrated. Flash chromatography (silica gel, hexanes) afforded **32** as a colorless oil (41 mg, 29%): ^{1}H NMR ($CDCl_3$): δ 7.22 (s, 4H), 4.67 (s, 2H), 2.39 (s, 3H); ^{13}C NMR ($CDCl_3$): δ 138.3, 131.9, 131.3, 129.7 (2C), 127.0 (2C), 48.6, 21.3; IR (film) ν_{max} 3050, 3025, 2922, 2857, 2173, 2092, 1616, 1516, 1438, 1414, 1379, 1344, 1308, 1281, 1265, 1240, 1201, 1183, 1121, 1040, 1021 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for C_9H_9NS, 163.0456; found, 163.0470.

1-(Isothiocyanato)-2-(trifluoromethyl)benzene (33): To a solution of 2-(trifluoromethyl)aniline (100 µL, 0.80 mmol) in dry CH_2Cl_2 (12.0 mL) at rt was added **26** (273 mg, 1.17 mmol). The reaction was stirred for 72 h and the solvent was concentrated. Flash chromatography (silica gel, 25:1 hexanes:EtOAc) afforded **33** as a colorless oil (123 mg, 76%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.67 (d, *J* = 7.8 Hz, 1H), 7.56 (td, *J* = 8.2, 0.9 Hz, 1H), 7.42-7.35 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.0, 133.2, 129.5, 128.5, 127.1 (q, *J* = 4.8 Hz), 127.0, 125.9 (q, *J* = 31.6 Hz), 122.9 (q, *J* = 273.2 Hz); IR (film) ν_{max} 2958, 2926, 2855, 2092, 1603, 1586, 1493, 1460, 1453, 1319, 1269, 1175, 1133, 1115, 1062, 1037, 942 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_8H_4F_3NS$, 203.0017; found, 203.0027.

1-(Isothiocyanato)-3-(trifluoromethyl)benzene (34):[40] To a solution of 3-(trifluoromethyl)aniline (95 µL, 0.88 mmol) in dry CH_2Cl_2 (11.1 mL) at rt was added **26** (264 mg, 1.14 mmol). The reaction was stirred for 40 h and the solvent was concentrated. Flash chromatography (silica gel, hexanes) afforded **34** as a colorless oil (70 mg, 39%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.56-7.47 (m, 3H), 7.41 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.3, 132.6, 132.5 (q, *J* = 33.6 Hz), 130.5, 129.1, 124.0 (q, *J* = 3.8 Hz), 123.3 (q, *J* = 272.2 Hz), 122.9 (q, *J* = 3.8 Hz); IR (film) ν_{max} 3446, 2962, 2926, 2843, 2199, 2046, 1700, 1635, 1614, 1590, 1558, 1488, 1448, 1331, 1233, 1175, 1131, 1093, 1065 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_8H_4F_3NS$, 203.0017; found, 203.0006.

1-(Isothiocyanato)-4-(trifluoromethyl)benzene (35):[40] To a solution of 4-(trifluoromethyl)aniline (100 µL, 0.80 mmol) in dry CH_2Cl_2 (11.1 mL) at rt was added **26** (267 mg, 1.14 mmol). The reaction was stirred for 41 h and the solvent was concentrated. Flash chromatography (silica gel, 25:1 hexanes:EtOAc) afforded **35** as a colorless solid (149 mg, 91%): m.p. 40.1–42.6 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.63 (d, *J* = 8.2 Hz, 2H) , 7.33 (d, *J* = 8.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.5, 135.2, 129.3, (q, *J* = 32.6 Hz),127.0 (q, *J* = 3.8 Hz, 2C), 126.2 (2C), 123.8 (q, *J* = 272.2 Hz); IR (film) ν_{max} 2958, 2924, 2856, 2361, 2196, 2121, 1611, 1505, 1412, 1327, 1154, 1123, 1105, 1064, 931 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_8H_4F_3NS$, 203.0017; found, 203.0015.

1-(Isothiocyanatomethyl)-2-(trifluoromethyl)benzene (36): To a solution of 2-(trifluoromethyl)benzylamine (100 µL, 0.72 mmol) in dry CH_2Cl_2 (11.2 mL) at rt was added **26** (267 mg, 1.14 mmol). The reaction was stirred for 17 h and the solvent was concentrated. Flash chromatography (silica gel, hexanes) afforded **36** as a colorless oil (153 mg, 99%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.70 (d, *J* = 7.3 Hz, 1H), 7.67-7.61 (m, 2H), 7.51-7.45 (m, 1H), 4.97 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 134.0, 132.8, 132.7, 129.2, 128.7, 127.6 (q, *J* = 30.7 Hz), 126.5 (q, *J* = 5.6 Hz), 124.1 (q, *J* = 274.1 Hz), 45.9; IR (film) ν_{max} 3074, 2926, 2855, 2184, 2090, 1609, 1587, 1498, 1457, 1440, 1353, 1314, 1171, 1121, 1060, 1039, 948 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_9H_6F_3NS$, 217.0173; found, 217.0177.

1-(Isothiocyanatomethyl)-3-(trifluoromethyl)benzene (37): To a solution of 3-(trifluoromethyl)benzylamine (180 µL, 1.24 mmol) in dry CH_2Cl_2 (12.0 mL) at rt was added **26** (289 mg, 1.24 mmol). The reaction was stirred for 16 h and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **37** as a yellow oil (142 mg, 53%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.66-7.60 (m, 1H), 7.59-7.53 (m, 3H), 4.81 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 135.5, 134.1, 131.6 (q, *J* = 32.6 Hz), 130.3, 129.8, 125.5 (q, *J* = 3.8 Hz), 123.9 (q, *J* = 272 Hz), 123.9 (q, *J* = 3.8 Hz), 48.3; IR (film) ν_{max} 3068, 2926, 2855, 2176, 2095, 1618, 1600, 1496, 1453, 1439, 1350, 1328, 1274, 1196, 1167, 1126, 1074 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_9H_6F_3NS$, 217.0173; found, 217.0174.

1-(Isothiocyanatomethyl)-4-(trifluoromethyl)benzene (38): To a solution of 4-(trifluoromethyl)benzylamine (120 µL, 0.84 mmol) in dry CH_2Cl_2 (12.0 mL) at rt was added **26** (288 mg, 1.24 mmol). The reaction was stirred for 17 h and the solvent was concentrated. Flash chromatography (silica gel, 25:1 hexanes:EtOAc) afforded **38** as a colorless oil (159 mg, 87%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.67 (d, *J* = 8.2 Hz, 2H), 7.46 (d, *J* = 8.2 Hz, 2H), 4.81 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.4, 133.9, 130.9 (q, *J* = 32.6 Hz), 127.3 (2C), 126.2 (q, *J* = 3.8 Hz, 2C), 124.0 (q, *J* = 272.2 Hz), 48.4; IR (film) ν_{max} 2927, 2855, 2187, 2096, 1622, 1437, 1420, 1326, 1239, 1167, 1126, 1067, 1019, 945, 819 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_9H_6F_3NS$, 217.0173; found, 217.0170.

1-isothiocyanato-2-nitrobenzene (39): To a solution of 2-nitroaniline (298 mg, 2.16 mmol) in dry CH_2Cl_2 (59.0 mL) at rt was added **26** (769 mg, 3.31 mmol). The reaction was stirred for 7 days and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **39** as a near-colorless solid (152 mg, 39%): m.p. 70.8–71.6 °C; ^{1}H NMR ($CDCl_3$, 400MHz) δ 8.10 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.64 (td, *J* = 7.8, 1.4 Hz, 1H), 7.47-7.39 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 145.6, 143.8, 139.3, 134.6, 129.4, 127.4, 126.1; IR (film) ν_{max} 3054, 2987, 2306, 1607, 1422, 1265, 896, 739, 705 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_7H_4N_2O_2S$, 179.9993; found, 179.9995.

1-Isothiocyanato-3-nitrobenzene (40):[42] To a solution of 3-nitroaniline (321 mg, 2.32 mmol) in dry CH_2Cl_2 (60.0 mL) at rt was added **26** (766 mg, 3.30 mmol). The reaction was stirred for 47 h and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **40** as a pale yellow solid (411 mg, 98%): m.p. 57.2–58.0 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.14 (dt, *J* = 7.3, 2.3 Hz, 1H), 8.08 (m, 1H), 7.60-7.53 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 149.0, 139.9, 133.5, 131.7, 130.7, 122.0, 120.9; IR (film) ν_{max} 3234, 3055, 1596, 1532, 1444, 1354, 1288, 1265, 1213, 1146, 1054, 897, 825 ,799, 736, 679 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_7H_4N_2O_2S$, 179.9993; found, 180.0002.

1-Isothiocyanato-4-nitrobenzene (41):[44] To a solution of 4-nitroaniline (299 mg, 2.16 mmol) in dry CH_2Cl_2 (60.0 mL) at rt was added **26** (761 mg, 3.27 mmol). The reaction was stirred for 65 h and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **41** as a pale yellow solid (389 mg, 99%): m.p. 110.1–111.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) 8.26 (m, 2H), 7.36 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.0, 140.5, 138.1, 126.6 (2C), 125.5 (2C); IR (film) ν_{max} 3233, 3054, 2361, 2337, 1596, 1558, 1507, 1495, 1457, 1364, 1308, 1266, 1213, 1137, 1041, 896, 843, 737, 706, 667 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_7H_4N_2O_2S$, 179.9993; found, 179.9995.

1-(Isothiocyanatomethyl)-2-nitrobenzene (42): To a solution of 2-nitrobenzylamine hydrochloride (304 mg, 1.61 mmol) in dry CH_2Cl_2 (23.0 mL) at rt was added **26** (1.098 g, 4.36 mmol) and *N*,*N*-diisopropylethylamine (270 µL, 1.55 mmol). The reaction was stirred for 17 h and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **42** as a yellow solid (212 mg, 64%): m.p. 69.3–70.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.21 (d, *J* = 8.2 Hz, 1H), 7.81-7.73

(m, 2H), 7.57 (m, 1H), 5.29 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.7, 135.0, 134.7, 130.7, 129.5, 129.3, 125.7, 47.3; IR (film) ν_{max} 3054, 2987, 2305, 2094, 1529, 1421, 1351, 1265, 896, 738, 705 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_8H_6N_2O_2S$, 194.1050; found, 194.0144.

1-(Isothiocyanatomethyl)-3-nitrobenzene (43): To a solution of 3-nitrobenzylamine hydrochloride (298 mg, 1.58 mmol) in dry CH_2Cl_2 (23.0 mL) at rt was added **26** (1.097 g, 4.72 mmol) and *N*,*N*-diisopropylethylamine (280 µL, 1.61 mmol). The reaction was stirred for 16 h and the solvent was concentrated. Flash chromatography (silica gel, 5:1 hexanes:EtOAc) afforded **43** as a pale yellow solid (299 mg, 97%): m.p. 78.8–79.6 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.26-8.20 (m, 2H), 7.71 (d, *J* = 8.2 Hz, 1H), 7.62 (t, *J* = 7.8 Hz, 1H), 4.87 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.7, 136.6, 135.1, 132.9, 130.4, 123.6, 122.1, 48.2; IR (film) ν_{max} 3395, 3054, 2987, 2360, 2341, 1534, 1516, 1422, 1393, 1352, 1265, 1204, 1147, 989, 896, 739, 705, 668, 446 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_8H_6N_2O_2S$, 194.1050; found, 194.0141.

1-(Isothiocyanatomethyl)-4-nitrobenzene (44): 4-Nitrobenzylamine hydrochloride (301 mg, 1.596 mmol) was dissolved in dry CH_2Cl_2 (23.0 mL) at rt. *N*,*N*-Diisopropylethylamine (0.270 mL, 1.633 mmol) was added, followed by **26** (1.0811 g, 4.654 mmol). The reaction mixture was stirred for 68 h, followed by solvent removal in vacuo. The residue was purified by flash chromatography (silica gel, 5:1 hexanes:EtOAc) to afford **44** as a yellow oil (202 mg, 65%): ^{1}H NMR ($CDCl_3$, 400MHz): δ 8.28 (m, 2H), 7.52 (m, 2H), 4.88 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 147.9, 141.4, 134.9, 127.6 (2C), 124.3 (2C), 48.1; IR (film) ν_{max} 3403, 3054, 2987, 2361, 1653, 1521, 1421, 1350, 1265, 896, 739, 705, 449 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_8H_6N_2O_2S$, 194.1050; found, 194.0147.

4-(Methylthio)phenylmethanamine hydrochloride (52): To a solution of hydroxylamine hydrochloride (944 mg, 13.58 mmol), pyridine (2.45 mL, 30.9 mmol) and EtOH (25.0 mL) was slowly added 4-(methylthio)benzaldehyde (0.87 mL, 6.54 mmol). The reaction was heated to reflux for 4 h and the solvents were concentrated. The residue was dissolved in a solution of $LiBH_4$ in dry THF (2.0 M, 30 mL, 60.00 mmol) and was heated to reflux for 17 h. The reaction was diluted with EtOAc (100 mL) and aqueous HCl (6M, 100 mL). The aqueous phase was extracted with EtOAc (3 x 100 mL), adjusted to pH 13, and extracted with CH_2Cl_2 (3 x 100 mL). The combined organic layers were concentrated, the residue was dissolved in dry MeOH, and dry, gaseous HCl was passed through for 5 min. The solvent was concentrated and the residue was recrystallized from EtOAc:hexanes to afford **52** as a pale colorless solid. (608 mg, 49%): m.p. > 260 °C; ^{1}H NMR (CD_3OD, 400 Mz) δ 7.37 (m, 2H), 7.32 (m, 2H), 4.06 (s, 2H), 2.49 (s, 3H); ^{13}C NMR (CD_3OD, 100 MHz) δ 142.0, 130.7, 130.5 (2C), 127.5 (2C), 43.9, 15.2; IR (film) ν_{max} 3424, 3005, 2581, 2037, 2037, 1594, 1496, 1479, 1463, 1434, 1409, 1384, 1094, 901, 821, 793, 525 cm^{-1}; HRMS (ESI+) m/z: $[M - Cl]^+$ calcd for $C_8H_{12}NS$, 154.0690; found, 154.0697.

1-Isothiocyanato-2-(methylthio)benzene (53): To a solution of 2-(methylthio)aniline (0.42 mL, 3.35 mmol) in dry CH_2Cl_2 (48.0 mL) at rt was added **26** (1.16 g, 4.99 mmol). The reaction was stirred at rt for 67 h and the solvent was concentrated. Flash chromatography (silica gel, 20:1 hexanes:EtOAc) afforded **53** as a light yellow oil (598 mg, 99%): ^{1}H NMR ($CDCl_3$, 400MHz): δ 7.28-7.19 (m, 3H), 7.13 (ddd, *J* = 7.8, 6.8, 1.8, 1H), 2.50 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 137.7, 136.9, 129.4, 127.8, 126.6, 126.4, 125.9, 15.6; IR (film) ν_{max} 3061, 2986, 2920, 2850, 2165, 2071, 1579, 1567, 1464. 1319, 1283, 1235, 1163, 1130, 1076, 1036, 967, 955, 930, 851, 747, 731, 707, 668, 658, 529, 419, 412 cm^{-1}; HRMS (EI+) *m/z:* $[M]^+$ calcd for $C_8H_7NS_2$, 181.0020; found, 181.0004.

1-Isothiocyanato-3-(methylthio)benzene (54):[45] To a solution of 3-(methylthio)aniline (0.42 mL, 3.37 mmol) in dry CH_2Cl_2 (48.0 mL) at rt was added **26** (1.17 g, 5.02 mmol). The reaction was stirred at rt for 67 h and the solvent was concentrated. Flash chromatography (silica gel, 10:1 hexanes:EtOAc) afforded **54** as a yellow oil (585 mg, 96%): ^{1}H NMR ($CDCl_3$, 400MHz): δ 7.25 (t, *J* = 7.8, 1H), 7.14 (ddd, *J* = 7.8, 1.8, 0.9, 1H), 7.07 (t, *J* = 1.8, 1H), 6.98 (ddd, *J* = 7.8, 1.8, 0.9, 1H), 2.49 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 141.0, 136.0, 132.0, 129.8, 125.2, 123.0, 122.2, 15.6; IR (film) ν_{max} 3059, 2985, 2919, 2851, 2195, 2106, 1934, 1582, 1554, 1472, 1434, 1423, 1415, 1272, 1096, 1078, 995, 957, 856, 775, 751, 719, 677, 657, 557, 540, 532, 523, 511, 502, 480, 467, 446, 437, 434, 429, 423, 418, 403 cm^{-1}; HRMS (EI+) *m/z:* $[M]^+$ calcd for $C_8H_7NS_2$, 181.0020; found, 181.0006.

1-Isothiocyanato-4-(methylthio)benzene (55):[46] To a solution of 4-(methylthio)aniline (0.41 mL, 3.30 mmol) in dry CH_2Cl_2 (48.0 mL) at rt was added **26** (1.15 g, 4.96 mmol). The reaction was stirred at rt for 41 h and the solvent was concentrated. Flash chromatography (silica gel, 20:1 hexanes:EtOAc) afforded **55** as a light yellow oil (598 mg, 99%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.20 (m, 2H), 7.16 (m, 2H), 2.49 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 138.6, 135.3, 128.0, 127.2 (2C), 126.3 (2C), 15.9; IR (film) ν_{max} 2918, 2179, 2089, 1734, 1699, 1684, 1653, 1635, 1558, 1540, 1506, 1486, 1465, 1457, 1436, 1420, 1404, 1094, 928, 816, 496, 484, 474, 466, 458, 446, 436, 421, 411 cm^{-1}; HRMS (EI+) *m/z:* $[M]^+$ calcd for $C_8H_7NS_2$,181.0020 ; found, 181.0005.

1-(Isothiocyanatomethyl)-2-(methylthio)benzene (56): Hydroxylamine hydrochloride (1.826 g, 27.6 mmol) was dissolved in ethanol (95%, 50.0 mL) and pyridine (4.70 mL, 58.1 mmol). 2-(Methylthio)benzaldehyde (1.748 mL, 13.5 mmol) was added and the reaction was heated to reflux for 16 h. After concentration, $LiBH_4$ (2.0 M in THF, 30 mL, 60.0 mmol) was added and the reaction was heated to reflux for an additional 5 h. The residue was partitioned between aqueous HCl (6M, 100 mL) and EtOAc (100 mL) and the aqueous phase was extracted and washed with EtOAc (3 x 100 mL). The aqueous phase was adjusted to pH 12 with aqueous NaOH (6M) and extracted with CH_2Cl_2 (3 x 100 mL). The organic layers were concentrated, the residue was dissolved in dry CH_2Cl_2 (85 mL) and **26** (746 mg, 3.25 mmol) was added. The reaction was stirred for 90 h and the solvent was concentrated. Flash chromatography (silica gel, 12:1 hexanes:EtOAc) afforded **56** as a green-yellow oil (236 mg, 27% over three steps): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.40-7.29 (m, 3H), 7.23 (td, *J* = 7.3, 1.4 Hz, 1H), 4.82 (s, 2H), 2.51 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 137.1, 132.7, 132.4, 129.3, 128.1, 127.2, 125.9, 47.1, 16.6; IR (film) ν_{max} 3421, 3053, 2986, 2926, 2361, 2339, 2092, 1653, 1471, 1437, 1341, 1265, 1046, 896, 739, 705, 668 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_9H_9NS_2$, 195.0176; found, 195.0167.

1-(Isothiocyanatomethyl)-3-(methylthio)benzene (57): To a solution of 3-(thiomethoxy)benzylamine hydrochloride (948 mg, 5.00 mmol) and *N*,*N*-diisopropylethylamine (955 µL, 5.05 mmol) in dry CH_2Cl_2 (75 mL) was added **26** (2.45 g, 10.53 mmol). After 18 h, the reaction was concentrated. Flash chromatography (silica gel, 12:1 hexanes:EtOAC) afforded **57** as a light orange oil (913 mg, 94%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.30 (t, *J* = 7.8 Hz, 1H), 7.23-7.16 (m, 2H), 7.06 (d, *J* = 7.3 Hz, 1H), 4.68 (s, 2H), 2.49 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 139.8, 135.1, 132.8, 129.4, 126.3, 124.6, 123.4, 48.6, 15.7; IR (film) ν_{max} 2953, 2921, 2852, 2170, 2085, 1576, 1559, 1473, 1437, 1335, 1207, 1088, 968, 862, 775, 669 cm^{-1}; HRMS (EI+) *m/z:* $[M]^+$ calcd for $C_9H_9NS_2$, 195.0176; found, 195.0171.

1-(Isothiocyanatomethyl)-4-(methylthio)benzene (58): To a solution of **52** (340 mg, 1.79 mmol) in dry CH_2Cl_2 (57.0 mL) at rt was added **26** (662 mg, 2.85 mmol) and *N*,*N*-diisopropylethylamine (0.33 mL, 1.89 mmol). The reaction was stirred for 18 h and the solvent was concentrated. Flash chromatography (silica gel, 10:1 hexanes:EtOAc) afforded **58** as a light yellow oil (343 mg, 98%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.29-7.22 (m, 4H), 4.67 (s, 2H), 2.50 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 139.3, 132.6, 131.1, 127.6 (2C), 127.0 (2C); IR (film) ν_{max} 3053, 2986, 2925, 2305, 2175, 2094, 1602, 1496, 1438, 1406, 1346, 1265, 1095, 1016, 896, 801, 739, 705 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_9H_9NS_2$, 195.0176; found, 195.0167.

1-Isothiocyanato-2-(methylsulfinyl)benzene (59): To a solution of **53** (257 mg, 1.42 mmol) in dry CH_2Cl_2 (4.30 mL) was slowly added *m*CPBA (70%, 353 mg, 1.43 mmol). The reaction was stirred at rt for 4 h and was

diluted with CH_2Cl_2 (70 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (70 mL), saturated aqueous sodium chloride (70 mL), dried ($NaSO_4$), and was concentrated to afford the mixture of sulfoxide and sulfone. Flash chromatography (silica gel, 3:1 hexanes:EtOAc → 1:2 hexanes:EtOAc) afforded **59** as an off-colorless crystalline solid (208 mg, 75%): m.p. 76.0–77.8 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.91 (m, 1H), 7.49 (m, 1H), 7.34 (m, 1H), 2.18 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 142.0, 141.0, 132.2, 128.5, 128.2, 127.8, 124.9, 42.6; IR (film) ν_{max} 3072, 2996, 2922, 2853, 2167, 2060, 1582, 1572, 1466, 1439, 1414, 1293, 1127, 1076, 1047, 936, 760, 714, 532, 515, 496, 467, 457, 443, 434, 428, 418, 404 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_8H_8NOS_2$, 198.0047; found, 198.0065.

1-Isothiocyanato-2-(methylsulfonyl)benzene (65): The purification of **59** also provided **65** as a colorless solid (52 mg, 17%): m.p. 92.8–93.4 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.05 (dd, *J* = 7.8, 1.4, 1H), 7.64 (td, *J* = 7.8, 1.8, 1H), 7.45 (m, 2H), 3.22 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 141.3, 135.1, 134.5, 130.7, 129.9, 129.5, 127.5, 43.5; IR (film) ν_{max} 3090, 3069, 3024, 3008, 2955, 2925, 2853, 2177, 2099, 1874, 1737, 1727, 1584, 1571, 1469, 1443, 1409, 1316, 1271, 1244, 1212, 1152, 1127, 1070, 1036, 955, 937, 875, 777, 759, 716, 654, 545, 536, 502, 475, 465, 454, 445, 431, 421, 413, 405 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_8H_8NO_2S_2$, 213.9996; found, 213.9988.

1-Isothiocyanato-3-(methylsulfinyl)benzene (60): To a solution of **54** (254 mg, 1.40 mmol) in dry CH_2Cl_2 (4.20 mL) was slowly added *m*CPBA (70%, 351 mg, 1.42 mmol). The reaction was stirred at rt for 2 h and was diluted with CH_2Cl_2 (70 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (70 mL), saturated aqueous sodium chloride (70 mL), dried ($NaSO_4$), and was concentrated to afford the mixture of sulfoxide and sulfone. Flash chromatography (silica gel, 3:1 hexanes:EtOAc → 1:2 hexanes:EtOAc) afforded **60** as a yellow oil (194 mg, 70%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.57-7.48 (m, 3H), 7.33 (dt, *J* = 6.9, 2.3, 1H), 2.75 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.1, 138.3, 133.2, 130.7, 128.1, 122.1, 121.1, 44.2; IR (film) ν_{max} 3055, 2996, 2923, 2196, 2104, 1586, 1574, 1545, 1536, 1532, 1474, 1421, 1415, 1299, 1279, 1248, 1085, 1079, 1050, 996, 960, 789, 680, 478, 470, 466, 448, 444, 436, 420, 415, 403 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_8H_8NOS_2$, 198.0047; found, 198.0060.

1-Isothiocyanato-3-(methylsulfonyl)benzene (66):[45] The purification of **60** also provided **66** as a light yellow solid (37 mg, 13%): m.p. 75.2–77.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.84 (dt, *J* = 7.8, 1.8 Hz, 1H), 7.82 (t, *J* = 1.8 Hz, 1H), 7.59 (t, *J* = 8.2 Hz, 1H), 7.49 (ddd, *J* = 8.2, 2.3, 0.9 Hz, 1H), 3.09 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 142.4, 139.4, 133.4, 131.0, 130.7, 125.8, 124.9, 44.6; IR (film) ν_{max} 2955, 2923, 2853, 2092, 2072, 2022, 2012, 1985, 1963 ,1314, 1297, 1143, 1088, 1075, 970, 774, 731, 676, 575, 531, 517, 508, 493, 481, 458, 445, 440, 421, 412, 406 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_8H_8NO_2S_2$, 213.9996; found, 213.9989.

1-Isothiocyanato-4-(methylsulfinyl)benzene (61): To a solution of **55** (256 mg, 1.41 mmol) in dry CH_2Cl_2 (4.20 mL) was slowly added *m*CPBA (70%, 358 mg, 1.45 mmol). The reaction was stirred at rt for 2 h and was diluted with CH_2Cl_2 (70 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (70 mL), saturated aqueous sodium chloride (70 mL), dried ($NaSO_4$), and was concentrated to afford the mixture of sulfoxide and sulfone. Flash chromatography (silica gel, 3:1 hexanes:EtOAc → 1:2 hexanes:EtOAc) afforded **61** as a light yellow solid (171 mg, 62%): m.p. 74.7–77.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.65 (m, 2H), 7.37 (m, 2H), 2.73 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 144.5, 138.1, 134.4, 126.7 (2C), 125.1 (2C), 44.1; IR (film) ν_{max} 3080, 3058, 3024, 2994, 2957, 2923, 2853, 2361, 2338, 2180, 2094, 1734, 1717, 1700, 1684, 1653, 1587, 1559, 1540, 1506, 1488, 1466, 1457, 1418, 1402, 1293, 1250, 1087, 1051, 1013, 955, 931, 831, 727, 676, 668, 516, 484, 472, 453, 440, 434, 417, 401 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_8H_8NOS_2$, 198.0047; found, 198.0032.

1-Isothiocyanato-4-(methylsulfonyl)benzene (67):[45] The purification of **61** also provided **67** as a colorless solid (91 mg, 30%): m.p. 133.7–134.4 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.95 (m, 2H), 7.40 (m, 2H), 3.07 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 139.8, 138.9, 137.2, 129.4 (2C), 126.7 (2C), 44.6; IR (film) ν_{max} 3092, 3067, 3008, 3021, 2926, 2192, 2079, 1653, 1587, 1576, 1559, 1540, 1506, 1301, 1284, 1172, 1142, 1085, 931, 832, 777, 727, 668, 531, 450, 441, 434, 430, 418, 414, 404 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_8H_8NO_2S_2$, 213.9996; found, 213.9979.

1-(Isothiocyanatomethyl)-2-(methylsulfinyl)benzene (62): To a solution of **56** (174 mg, 0.89 mmol) in dry CH_2Cl_2 (2.80 mL) was slowly added *m*CPBA (70%, 230 mg, 0.93 mmol). The reaction was stirred at rt for 2.5 h and was diluted with CH_2Cl_2 (35 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (35 mL), saturated aqueous sodium chloride (35 mL), dried ($NaSO_4$), and was concentrated to afford the mixture of sulfoxide and sulfone. Flash chromatography (silica gel, 1:3 hexanes:EtOAc) afforded **62** as a yellow green oil (145 mg, 77%): ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.05 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.64 (td, *J* = 7.3, 1.4 Hz, 1H), 7.58 (td, *J* = 7.3, 1.4 Hz, 1H), 7.47 (d, *J* = 7.8 Hz, 1H), 4.90 (s, 2H), 2.81 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 144.5, 132.4, 132.1, 131.6, 130.7, 129.4, 124.7, 45.6, 43.8; IR (film) ν_{max} 3054, 2987, 2360, 2340, 1653, 1559, 1540, 1507, 1420, 1265, 896, 740, 705, 668 cm^{-1}; HRMS (EI+) *m/z*: $[M + H]^+$ calcd for $C_9H_{10}NOS_2$, 212.0204; found, 212.0217.

1-(Isothiocyanatomethyl)-3-(methylsulfinyl)benzene (63): To a solution of **57** (789 mg, 4.04 mmol) dissolved in dry CH_2Cl_2 (15.0 mL) was slowly added *m*CPBA (70%, 1.64 g, 6.65 mmol). The reaction was stirred at rt for 2 h and was diluted with CH_2Cl_2 (50 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (2 x 70 mL), saturated aqueous sodium chloride (70 mL), dried ($NaSO_4$), and was concentrated. Flash chromatography (silica gel, 5:1:1 hexanes:CH_2Cl_2:EtOAc → 1:1 CH_2Cl_2:EtOAc) afforded **63** as a brown oil (156 mg, 18%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.16 (s, 1H), 7.60-7.52 (m, 2H), 7.46 (m, 1H), 4.80 (s, 2H), 2.74 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.0, 136.3, 134.0, 130.2, 129.6, 123.7, 122.0, 48.5, 44.2; IR (film) ν_{max} 3455, 2997, 2923, 2853, 2178, 2095, 1599, 1476, 1429, 1342, 1084, 1049, 997, 957, 788, 710, 687 cm^{-1}; HRMS (EI+) *m/z:* $[M + H]^+$ calcd for $C_9H_{10}NOS_2$, 212.0204; found, 212.0199.

1-(Isothiocyanatomethyl)-3-(methylsulfonyl)benzene (69): The purification of **63** also provided **69** as a colorless solid (52 mg, 17%): m.p. 67.7–68.0 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.92 (dt, *J* = 6.4, 1.8 Hz, 1H), 7.88 (s, 1H), 7.66-7.59 (m, 2H), 4.83 (s, 2H), 3.07 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 141.6, 136.4, 134.7, 132.2, 130.4, 127.6, 126.0, 48.3, 44.7; IR (film) ν_{max} 3061, 3013, 2924, 2852, 2178, 2066, 1479, 1431, 1318, 1212, 1144, 1086, 962, 866, 759, 708 cm^{-1}; HRMS (EI+) *m/z:* $[M]^+$ calcd for $C_9H_9NO_2S_2$, 227.0075; found, 227.0089.

1-(Isothiocyanatomethyl)-4-(methylsulfinyl)benzene (64): To a solution of **58** (125 mg, 0.64 mmol) in dry CH_2Cl_2 (1.70 mL) was slowly added *m*CPBA (70%, 158 mg, 0.64 mmol). The reaction was stirred at rt for 2 h and was diluted with CH_2Cl_2 (35 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (70 mL), saturated aqueous sodium chloride (70 mL), dried ($NaSO_4$), and was concentrated to afford the mixture of sulfoxide and sulfone. Flash chromatography (silica gel, 1:1 hexanes:EtOAc → 1:1 CH_2Cl_2) afforded **64** as a light brown oil (35.9 mg, 24.9%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69 (m, 2H), 7.50 (m, 2H), 4.81 (s, 2H), 2.74 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 140.7, 140.5, 134.4, 128.3, 127.8, 48.3, 44.6; IR (film) ν_{max} 3420, 3055, 2927, 2854, 2361, 2337, 2188, 2096, 1636, 1456, 1436, 1410, 1317, 1265, 1091, 1091, 1018, 958, 896, 811, 739, 705, 668, 651 cm^{-1}; HRMS (EI+) *m/z*: $[M + H]^+$ calcd for $C_9H_{10}NOS_2$, 212.0204; found, 212.0206.

1-(Isothiocyanatomethyl)-4-(methylsulfonyl)benzene (70): The purification of **64** also provided **70** as a green oil (92 mg, 68.6 %): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.00 (d, *J* = 8.2 Hz, 2H), 7.55 (d, *J* = 8.2 Hz, 2H), 4.86 (s, 2H), 3.08 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 140.8, 140.6, 134.6,

128.4 (2C), 127.9 (2C); IR (film) ν_{max} 346, 3053, 2985, 2360, 2339, 2182, 2098, 1496, 1407, 1338, 1265, 1087, 1051, 1015, 956, 896, 807, 738, 704 cm^{-1}; HRMS (EI+) *m/z*: $[M + H]^+$ calcd for $C_9H_{10}NO_2S_2$, 228.0153; found, 228.0157.

1-(Isothiocyanatomethyl)-2-(methylsulfonyl)benzene (68): To a solution of **56** (64.4 mg, 0.31 mmol) in dry CH_2Cl_2 (6.10 mL) was slowly added *m*CPBA (70%, 150 mg, 0.61 mmol). The reaction was stirred at rt for 7 h and was diluted with CH_2Cl_2 (30 mL). The organic layer was washed with saturated aqueous sodium bicarbonate (70 mL), saturated aqueous sodium chloride (70 mL), dried ($NaSO_4$), and was concentrated. Flash chromatography (silica gel, 3:1 hexanes:EtOAc) to afford **68** as a near-colorless solid (30 mg, 43 %): m.p. 95.5–95.7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.08 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.74 (td, *J* = 7.8, 1.4 Hz, 2H), 7.67 (m, 1H), 7.60 (td, *J* = 7.8, 1.4 Hz, 1H), 5.30 (s, 2H), 3.14 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 137.9, 134.8, 134.7, 133.8, 130.4, 130.3, 129.7, 46.5, 45.2; IR (film) ν_{max} 2927, 2855, 2254, 2090, 1599, 1468, 1380, 1346, 1316, 1156, 1096, 907, 733, 651, 449 cm^{-1}; HRMS (EI+) *m/z*: $[M]^+$ calcd for $C_9H_9NO_2S_2$, 227.0075; found, 227.0090.

MTS antiproliferation assay: Human MCF-7 breast cancer cells were maintained in a 1:1 mixture of Advanced DMEM/F12 (Gibco) supplemented with L-glutamine (2 mM), streptomycin (500 µg/mL), penicillin (100 units/mL), and 10% FBS. Cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO_2), seeded (2000/well, 100 µL) in 96-well clear, flat-bottomed plates, and allowed to attach overnight. For each trial, nine, two-fold serial dilutions of ITC in DMSO (1 µL of 100x stock, final concentration range = 200 to 0.78 µm) were added in triplicate, and cells were returned to the incubator for 24 h or 72 h. At the specified time, the number of viable cells was determined using an MTS/PMS cell proliferation kit (Promega #PR-G5430) following the manufacturer's instructions. The percent viability for each well was determined relative to cells incubated with vehicle (1% DMSO). Where applicable, data was fit with the nonlinear function describing log(inhibitor) vs. response (variable slope, 4 parameters) using GraphPad Prism 6.0, allowing determination of GI_{50} values.

ARE induction assay: MCF-7-ARE cells stably transfected with the pGL3-promoter vector (Promega) containing eight copies of the antioxidant response element (ARE, 5′-GTGACAAAGCA-3′) were utilized as previously described.[30] MCF-7-ARE cells were maintained in high glucose (25 mM) DMEM supplemented with 10% FBS, streptomycin (50 µg/mL), penicillin (50 units/mL), and G418 (400 µg/mL). Cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO_2), seeded (2000/well, 100 µL) in 96-well clear, flat-bottomed plates, and allowed to attach overnight. For each trial, six, two-fold serial dilutions of ITC in DMSO (1 µL of 100x stock, final concentration range = 12.5 to 0.39 µm) were added in triplicate, and cells were returned to the incubator for 24 h or 72 h. Cells were lysed in passive lysis buffer (Promega) containing protease inhibitors (1:1000, Sigma-Aldrich). Lysate was used as substrate in the Bright-Glo Luciferase Assay System (Promega, #E2610), following the manufacturer's instructions. RLU data was normalized for protein content, determined by the Pierce BCA method (Thermo Fisher Scientific, #PI23221), and reported relative to cells incubated with vehicle (1% DMSO).

Quantitative real-time PCR (qPCR) analysis: MCF-7-ARE cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO_2), seeded (40,000/dish, 10 mL) in 10 cm Corning polystyrene dishes, and allowed to attach overnight. For each trial, six, two-fold serial dilutions of ITC in DMSO (100 µL of 100x stock, final concentration range = 12.5 to 0.39 µm) were added in triplicate, and cells were returned to the incubator for 48 h. Cells were trypsinized, washed with PBS, and lysed prior to RNA extraction using the Maxwell 16 Total RNA Purification Kit (Promega), following the manufacturer's instructions. cDNA was synthesized from samples with an RNA integrity number of 8.0 or greater. Primers and probes for qPCR were designed to only amplify genomic DNA with the following obtained from Thermo Fisher Scientific: NADPH dehydrogenase, quinone 1 (NQO1; Hs01045994_m1), glutathione *S*-transferase α1 (GSTα1; Hs00275575_m1), and heme oxygenase 1 (HMOX1; Hs01110250_m1). qPCR primers and probes were designed using Beacon Designer 7.91 (Premier Biosoftware) for thioredoxin reductase 1 (TXNRD1, nm003330.2; forward 5'-GCTTCAGCATGTCATGTG-3', reverse 5'-CTCTGTTTCACAAACACAAC-3', probe [6~FAM]CCAATTCCGAGAGCGTTCCTTC[BHQ1a~6FAM]) and glyceradehyde-3-phosphate dehydrogenase (GAPDH, nm002046.4; forward 5'-CATCCATGACAACTTTGGTA-3', reverse 5'-CCATCCACAGTCTTCTGG-3', probe [6~FAM]ACCACAGTCCATGCCATCACT[BHQ1a~6FAM]). Primers (900 nM) and probes (250 nM) were diluted in 2X Absolute Blue master mix (Thermo Fisher Scientific) and assayed as previously reported [47]. Standard MIQE guidelines were followed, including: internal primer validation through mass normalization, assessment of genomic DNA contamination, and assay efficiency.[48]

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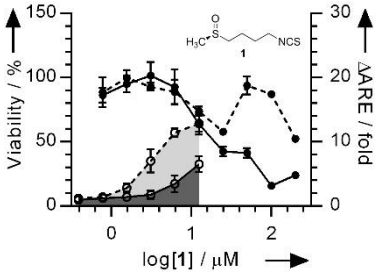

Dose-response anticancer properties of L-sulforaphane (L-SFN, **1**) against MCF-7 cells after 24 h and 72 h incubation. Plots depict data acquired via antiproliferation and ARE-induction assays; the area below ARE-induction data is shaded to improve clarity (t = 24 h, light gray; t = 72 h, dark gray).