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Quinone Induced Activation of Keap1/Nrf2 Signaling by Aspirin Prodrugs Masquerading as Nitric Oxide

Tareisha Dunlap, **Sujeewa C. Piyankarage**, **Gihani T. Wijewickrama**, **Samer Abdul-Hay**, **Michael Vanni**, **Vladislav Litosh**, **Jia Luo**, and **Gregory R. J. Thatcher***
Department of Medicinal Chemistry & Pharmacognosy, University of Illinois College of Pharmacy, University of Illinois at Chicago, 833 S Wood St, Chicago, IL 60612

Abstract

The promising therapeutic potential of the NO-donating hybrid aspirin prodrugs (NO-ASA), includes induction of chemopreventive mechanisms, and has been reported in almost 100 publications. One example, NCX-4040 (*p*NO-ASA), is bioactivated by esterase to a quinone methide (QM) electrophile. In cell cultures, *p*NO-ASA and QM-donating X-ASA prodrugs that cannot release NO rapidly depleted intracellular GSH and caused DNA damage; however, induction of Nrf2 signaling elicited cellular defense mechanisms including upregulation of NAD(P)H:quinone oxidoreductase-1 (NQO1) and glutamate-cysteine ligase (GCL). In HepG2 cells, the "NO-specific" 4,5-diaminofluorescein reporter, DAF-DA, responded to NO-ASA and X-ASA, with QM-induced oxidative stress masquerading as NO. LC-MS/MS analysis demonstrated efficient alkylation of Cys residues of proteins including glutathione-S-transferase-P1 (GST-P1) and Kelch-like ECH-associated protein 1 (Keap1). Evidence was obtained for alkylation of Keap1 Cys residues associated with Nrf2 translocation to the nucleus, nuclear translocation of Nrf2, activation of antioxidant response element (ARE), and upregulation of cytoprotective target genes. At least in cell culture, *p*NO-ASA acts as a QM-donor, bioactivated by cellular esterase activity to release salicylates, NO_3^-, and an electrophilic QM. Finally, two novel aspirin prodrugs were synthesized, both potent activators of ARE, designed to release only the QM and salicylates on bioactivation. Current interest in electrophilic drugs acting via Nrf2 signaling suggests that QM-donating hybrid drugs can be designed as informative chemical probes in drug discovery.

Keywords

Keap1; Nrf2; hybrid drugs; antioxidant response element; quinone methide; nitric oxide; NO-ASA; NO-NSAID

Introduction

Signaling via the NF-E2-related (Nrf2) transcription factor has provided a target for natural and synthetic small molecules intended to act as primary chemopreventive agents, of use in healthy individuals, and as secondary agents of use in individuals with increased risk of cancer. Many putative cell targets have been proposed for inhibition or prevention of carcinogenesis, including those associated with proliferation, inflammation, and formation of genotoxic metabolites. Nrf2 has been proposed to elicit a trio of responses. The initial response, via upregulation of cytoprotective and detoxicant enzymes, such as NAD(P)H: quinone oxidoreductase (NQO1), is mediated largely by antioxidant response element

*Correspondence: Gregory Thatcher, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, IL, 60612; thatcher@uic.edu.

(ARE) activation. Secondary and tertiary responses involve repair of DNA, proteosomal and lysosomal housekeeping, and ultimately cell regeneration.[1, 2] Induction of Nrf2 signaling requires translocation of Nrf2 to the nucleus, binding to ARE, followed by transcription of cytoprotective genes. The best understood process involves modification by oxidative and electrophilic molecules of the cysteine-rich sensor protein Keap1 that in concert with other complexed proteins controls Nrf2 ubiquitination and degradation.[3, 4]

Quinones represent an important class of oxidative and electrophilic molecules found in natural products that have provided a rich source of chemopreventive agents and also in metabolites of both endogenous compounds and xenobiotics. It has been known for some time that compounds that possess weak carcinogenic activity have the potential to protect against carcinogenesis,[5] and that this protection results in part from induction of enzymes involved in conjugative metabolism and other pathways that protect a cell from carcinogenic electrophiles and free radicals.[6] This approach represents a tight-rope act since chemopreventive inducers of Nrf2 are likely to be cellular oxidants and electrophiles themselves, possibly with weak carcinogenic activity. Furthermore, upregulation of Nrf2,[7] and its downstream target genes, NQO1[8] and glutathione-S-transferase-P1 (GST-P1)[9] is observed in various tumors, compatible with activation of Nrf2 signaling in cancer cells mediating cytoprotection and resistance to chemo- and radiotherapy.[10] The balance of effects and hence safety of Nrf2-inducing agents will be varied and intimately linked with their chemical reactivity, since oxidative and electrophilic compounds have potential to modify other proteins and signaling pathways, for example NFκB,[11, 12] and epigenetic regulators.[13]

The safety profile of chemopreventive agents must be high enough to allow chronic use in atrisk populations of otherwise healthy individuals. The use of non-steroidal anti-inflammatory drugs (NSAIDs) and in particular aspirin (ASA) in cancer chemoprevention is supported by epidemiology, but contra-indicated by gastrotoxicity.[14-16] NO-NSAID hybrid drugs were designed to supply NO to attenuate NSAID gastrotoxicity, which has been largely borne out in animal models and clinical trials.[17, 18] NO-NSAIDs, including NCX-4016 (*m*NO-ASA) and the isomeric NCX-4040 (*p*NO-ASA) have been reported to act as Nrf2-inducers,[19, 20] through "*S*-nitrosylation" of Keap1.[19] Both this reaction and electrophilic *S*-nitrosation of Keap1[21] leading to oxidative disulfide formation[22] have both been proposed as mechanisms of Nrf2 induction. Recently, extensive use of the "NO-specific" DAF probes has been used to reassert that the biological activity of *p*NO-ASA derives from NO release.[23]

An alternative mechanism for Nrf2 induction by *p*NO-ASA is via an electrophilic quinone, since cellular bioactivation of *p*NO-ASA yields a quinone methide (QM).[20, 24] Analogues of NO-ASA that are bioactivated to QM, but do not contain an NO group (X-ASA), have been shown to mirror the biological activity of NO-ASA.[20] *In vitro* and *in vivo* studies on ASA derivatives suggest strong potential for clinical use in cancer.[25-31] However, a large scale phase 2 clinical trial of NCX-4016 in colorectal cancer (CRC) chemoprevention was halted because of genotoxicity concerns.[32] The selection of NO-ASA or X-ASA derivatives for the clinic demands a better understanding of the contribution of NO and QM to either beneficial or harmful biological activity. Comparison of NO-ASA and X-ASA analogues showed that QM-forming compounds in cell culture covalently modified Keap1, activated ARE, upregulated cytoprotective target genes, induced translocation of Nrf2 to the nucleus, and activated an oxidatively sensitive "NO-specific" fluorescent reporter (Scheme 1). The central importance of QM in NO-ASA chemopreventive actions led to the design of a novel ASA derivative that on bioactivation yields only salicylic acid, ASA and QM.

Experimental Procedures

Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. X-ASA derivatives were synthesized as described in the literature, [20, 27] and the synthesis of $o(ASA)_2$ and $p(ASA)_2$ is described below. The Comet Assay Kit was purchased from Trevigen Inc. (Gaithersburg, MD). Taqman gene expression assay master mix and primers with FAM/MGB probes were purchased from Applied Biosystems (Foster City, CA). RNeasy mini kit was purchased from Qiagen (Valencia, CA). Nrf2 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluorescein conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). VECTASHIELD mounting media with DAPI was purchased from Vector Laboratories, Inc. (Burlingame, CA). GST-P1 and GST-P1(C101A) were expressed from E. coli as previously described.[33, 34]

Synthesis of $o(ASA)_2$ and $p(ASA)_2$—Under a positive pressure of nitrogen and free of moisture, a solution of either the 2- or 4-hydroxymethylphenol ester of 2-acetyloxybenzoic acid (500 mg, 1.75 mmol), 4-dimethylaminopyridine (21 mg, 0.18 mmol) and triethylamine (513 mg, 5.25 mmol) in tetrahydrofuran (10 mL) was cooled to 0 °C, and 2-chlorocarbonylphenyl acetate (2.00 mmol) was added dropwise over a period of 10 min. The resulting solution was stirred at 0-5 °C for 4 h. The reaction mixture was diluted with ethyl acetate (100 mL) and the diluted solution was washed with brine (50 mL × 3), dried over sodium sulfate, evaporated to dryness, and the residue was purified by column chromatography on silica gel.

2-{[(2-acetoxybenzoyl)oxy}methyl]phenyl 2-acetoxybenzoate [$o(ASA)_2$]—Purified using AcOEt/hexane (30:70) as eluent to afford 188 mg (24%) of the desired product as a white solid. ^{1}H NMR ($CDCl_3$): δ 8.22 (d, 1 H, *J* = 7.8 Hz, Ar), 8.00 (d, 1 H, *J* = 7.8 Hz, Ar), 7.42–7.65 (m, 4 H, Ar), 7.24–7.37 (m, 4 H, Ar), 7.17 (d, 1 H, *J* = 8.1 Hz, Ar), 7.08 (d, 1 H, *J* = 8.1 Hz, Ar), 5.33 (s, 2 H, OCH_2), 2.85 (s, 3 H, CH_3), 2.16 (s, 3 H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 169.73, 164.11, 162.66, 151.43, 150.84, 149.11, 134.87, 134.05, 132.22, 132.06, 130.53, 129.97, 128.12, 126.60, 126.33, 126.09, 124.14, 123.89, 123.00, 122.85, 122.13, 62.11, 21.04, 20.83. HRMS ($M+NH_4^+$) for $C_{25}H_{24}NO_8$ calc'd: 466.1496; observed: 466.1498.

4-({[(2-acetoxybenzoyl)oxy}methyl]phenyl 2-acetoxybenzoate [$p(ASA)_2$]—Purified using AcOEt/hexane (20:80) as eluent to afford 250 mg (32%) of the desired product as an oil. ^{1}H NMR ($CDCl_3$): δ 8.23 (dd, 1 H, J = 8.0, 1.6 Hz, Ar), 8.07 (dd, 1 H, J = 8.0, 1.6 Hz, Ar), 7.65 (t, 1 H, J = 7.8 Hz, Ar), 7.57 (t, 1 H, J = 7.8 Hz, Ar), 7.50 (d, 2 H, J = 8.0 Hz, Ar), 7.40 (t, 1 H, J = 7.6 Hz, Ar), 7.32 (t, 1 H, J = 6.4 Hz, Ar), 7.19 (m, 3 H, Ar), 7.10 (dd, J = 8.0, 1.2 Hz, 1 H, Ar), 5.32 (s, 2 H, OCH_2), 2.31 (s, 3 H, CH_3), 2.19 (s, 3 H, CH_3). ^{1}C NMR ($CDCl_3$) δ: 169.83, 164.41, 162.98, 151.32, 150.84, 150.70, 134.83, 134.18, 133.63, 132.33, 132.08, 129.96, 126.34, 126.20, 124.17, 124.01, 123.20, 122.51, 122.13, 66.43, 21.13, 20.94. HRMS ($M+NH_4^+$) for $C_{25}H_{24}NO_8$ calc'd: 466.1496; observed: 466.1491.

Cell cultures

Human Hepatoma HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). Hepa 1c1c7 murine Hepatoma cells were supplied by Dr. J.P.Whitlock, Jr. (Stanford University, Stanford, CA) and were cultured in α-MEM with 1% penicillin-streptomycin and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). HepG2 cells stably transfected with ARE-luciferase reporter gene were kindly provided by Dr. A.N.

Kong (Rutgers University, Piscataway, NJ) and cultured in F-12 medium with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids and insulin (0.2 mg/mL).[35]

GSH Levels—The total cellular glutathione (GSH + GSSG) level in HepG2 cells was measured by an enzymatic recycling procedure with some modifications as described previously.[20]

Total RNA isolation and qPCR—HepG2 cells at a density of 4×10^6 cells/mL were plated in 100 mm culture plates and treated with DMSO vehicle and compounds for 4 h. Total RNA was extracted from cell pellets using the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed using oligo$(dT)_{12\text{-}18}$ (500 ug/mL), dNTP (10 mM), and DEPC H_2O, which was added to equal a volume of 12 μL. The mixture was heated at 65°C for 5 min and thereafter quickly chilled on ice. The resulting solution was collected by centrifugation and added to DTT (0.1 M) and 4 μL first-strand buffer (40 units/μL), giving a total volume of 19 μL. The solution was heated at 42°C for 2 min before 1 μL of Superscript II RT (200 units) was added. The reaction was incubated at 42°C for an additional 90 min. The resulting cDNA (2 μL) was used for real-time PCR quantification (7500 Real-Time PCR System). Taqman gene expression master mix, containing AmpliTaq Gold® DNA polymerase, and a GCLC primer with FAM/MGB probe (Applied Biosystems, Carlsbad, CA) was added to MicroAmp® Optical 96-well reaction plate. Real-time quantitative PCR consisted of one cycle of 50°C for 2 min and 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The fluorescence signal was measured during the last 30 sec of the annealing/extension phase. Data was analyzed using comparative C_T ($\Delta\ \Delta C_T$) method, using actin as the endogenous control. The results are expressed as fold-difference in reference to actin control.

Nrf2 translocation—HepG2 cells at a density of 5×10^4 cells/mL were plated in 8-well Nunc™ chamber slides coated with poly-d-lysine. Cells were treated with compounds for 4 h, 18 h, or 24 h and incubated at 37°C. Media was removed and the cells were fixed with 4% paraformaldehyde for 10 min. Cells were then washed with PBS and 0.2% Triton X-100. Triton X-100 solution was removed after 10 min and slides were washed for an additional 10 min with PBS. Blocking was done with 10% normal goat serum added to each sample and placed on a shaker for 2 h. The cells were incubated with rabbit polyclonal anti-Nrf2 antibody overnight. Cells were incubated with fluorescein conjugated anti-rabbit IgG secondary antibody for 1 h and 4′-6-diamidino-2-phenylindole (DAPI) was added to the cells to detect nuclear staining. Imaging was performed with a Zeiss LSM 510 laser-scanning confocal microscope with the detector gain adjusted to eliminate the background autofluorescence. The fluorescence signal from Nrf2 was monitored with a 488 nm argon/krypton laser and a 530 nm band pass filter. The DAPI nuclear staining signal was monitored with a 345 nm UV laser and 420 nm band pass filter. Images were analyzed using the analysis tool provided in the Zeiss biophysical software package and ImageJ.

NQO1 activity assay—For cultured Hepa 1c1c7 liver cells, the induction of NQO1 activity was assayed as described previously with minor modifications.[36, 37]

DNA damage in Hepa 1c1c7 cells using Single-Cell Gel Electrophoresis Assay (Comet assay)—This assay was performed according to the manufacturer's recommendations (Trevigen, Inc. Gaithersburg, MD) as described previously.[20]

ARE-Luciferase Reporter Assay—HepG2-ARE-Luc cells were plated in 6-well plates at a density of 1×10^5 and luciferase activity, normalized to protein concentration, measured

as described previously.[20] *Confocal imaging with DAF-DA*: HepG2 cells were plated in 8-well Nunc™ chamber slides coated with poly-d-lysine. Cells were treated with *m*Br-ASA (25 μM), *p*Br-ASA (25 μM), *m*NO-ASA (25 μM), *p*NO-ASA (25 μM) for 24 h or spermine NONOate (25 μM) for 20 min. In addition, cells were treated with *p*Br-ASA for 4 h after pretreatment for 30 min with L-NAME (5 μM). After treatment cells were labeled with 10 μM DAF-DA for 30 min and the nucleus was stained with Hoechst (10 mg/mL).

GST-P1 and Keap1 reactions with pNO-ASA or pBr-ASA—For the analysis of alkylation of GST-P1 and Keap1 Cys residues, GST-P1 or Keap1 (15 μM) was incubated with 30 μM substrate (*p*NO-ASA/*p*Br-ASA) in the presence of porcine liver esterase (PLE, 30 μg/mL) in phosphate buffer (40 mM, pH 7.4) for 30 min at 37 °C. The unreacted thiols were then blocked by incubating the protein samples with NEM (20 mM) in the presence of 5% SDS for 30 min at 55 °C with vortexing at 5 min intervals. Prepared samples were run on SDS gel, and the separated protein bands were stained with Coomasie Brilliant Blue. Finally, bands containing GST-P1 or Keap1, determined by visual inspection, were excised, destained, and digested using trypsin or Lys-C enzymes according to the manufacturer's (Thermo Pierce, IL) instructions. The amino acid residues on either side of Cys101 in GST-P1, Arg100 and Lys102, are cleavage sites for trypsin digestion, therefore, Lys-C digestion of GST-P1 was used to obtain a detectable peptide containing Cys101 for these studies.

QM modification of Cys47 of GST in the presence of free Cys—Mutant GST-P1 (4 μM; C101A) was incubated with *p*NO-ASA (25 μM) in the presence of PLE (30 μg/mL) and cysteine (1, 10, 100, or 500 μM) in NH_4HCO_3 (40 mM, pH 7.4) for 60 min at 37 °C. The unreacted protein thiols were labeled with NEM (1 mM) in the presence of 5% SDS (as described above) and, the GST protein bands separated by SDS gel electrophoresis were excised and digested with Trypsin (Thermo Pierce, IL). Similar experiments were performed without added Cys, but using carbonate buffer and alkaline solution to provide basic conditions: 7<pH<12 (data not shown).

Mass spectroscopic analysis of digested proteins—The digested protein samples were analyzed with an Agilent 6310 ion trap mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled to Agilent 1100 series HPLC system with ESI. The samples were separated using a Hypersil BSD reversed-phase C18 column (30 × 2.1 mm, 3μm, Thermo Scientific) with gradient 90/10 (v/v) water/methanol, 0.1% formic acid and acetonitrile, 0.1% formic acid mobile phase at 300 uL/min flow rate. The positive mode electrospray ionization method was utilized for all the LC-MS/MS analyses. For the quantitative analysis of QM modification of GST in the presence of free Cys, the peak area for the QM-modified Cys47 containing peptide fragment (residue 45-54: ASCLYGQLPK) was measured and normalized against the peak area of the adjacent peptide fragment (residue 55-70: FQDGDLTLYQSNTILR) to quantitatively estimate the extent of QM modification of Cys47 with respect to cysteine concentration.

Protein digests were also analyzed using a hybrid linear ion trap FT-ICR mass spectrometer (LTQ-FT-ICR, Thermo Electron Corp., Bremen, Germany) equipped with a nanospray ESI source and nano-HPLC with autosampler (Dionex, Sunnyvale, CA). The trapping cartridge and the nano-column used for separation were Zorbax 300 SB-C18 (5 × 0.3 mm, 5 μm, Agilent Technologies) and Zorbax 300 SB-C18 (150 mm × 75 μm, 3.5 μm, Agilent Technologies), respectively. The separations were carried with gradient elution (250 nL/min; 5% acetonitrile, 0.1 % formic acid to 95% acetonitrile, 0.1% formic acid). The RAW files were converted to mzXML files and they were searched by the MassMatrix (http://www.massmatrix.net) search engine against the UniProt human database with NEM and QM as the variable modifications. The area of the QM-modified peptide peak was divided by the combined peak areas of the NEM- and QM-modified peptide peaks to estimate the

percentage QM modifications. For these quantitative estimations the ionization properties of a given modified peptide are assumed to be similar, which given the small and very similar change in peptide mass caused by QM and NEM modification is reasonable.

Results & Discussion

The chemistry of NO-ASA and X-ASA derivatives

The NO-ASA isomers, NCX-4016 (*m*NO-ASA) and NCX-4040 (*p*NO-ASA), are benzyl nitrate prodrugs that link ASA to a nitrooxy (ONO_2) group using a hydroxybenzyl alcohol linker (Fig. 1). Bioactivation by non-specific esterase liberates ASA, salicylic acid and hydroxybenzyl nitrate (HBN); NCX-4015 being the HBN product derived from NCX-4016. Hydroxybenzyl derivatives had been designed by others, previously, to liberate an electrophilic QM at an enzyme active site,[38, 39] although the connection was not noted in reports on NO-ASA. In addition, a prodrug strategy, developed for anti-infective and anticancer agents in the 1990's, is known to generate a QM as a by-product.[40] It was only noted in 2007 that *p*NO-ASA was bioactivated to a QM that likely contributed to biological activity.[20, 24] Nevertheless, reference to NO as a mediator of the biological activity of *p*NO-ASA continues. There is clearly a need both to better understand the contribution of QM to the activity of *p*NO-ASA and to explore the effects of QM release on cellular function.

The cellular effects of QMs have been most closely associated with toxicity. Indeed, the report from Hulsman et al., of QM involvement in *p*NO-ASA function, highlighted cytotoxicity that was proposed to underlie antitumor properties.[24] In the same year, Dunlap et al. reported that QM generated from *p*NO-ASA, at sub-toxic doses, was also able to activate cell defense mechanisms via ARE and upregulation of NQO1.[20] The strategy in both Hulsman and Dunlap studies was to replace the nitrate (NO_3^-) of *p*NO-ASA with an equally good leaving group, X^-, in X-ASA (Fig. 1); thus, these *p*X-ASA analogues can be bioactivated to release QM, but obviously not to release NO.

Incubation of *p*NO-ASA (100 μM) with esterase in the presence of GSH has been shown to result in quantitative generation of NO_3^- (97 μM) after only 10 min; whereas NO_2^- was only detected in 0.2% yield.[20] Therefore, *p*NO-ASA will act as a NO_3^- donor, unless reductive metabolism to NO occurs rapidly. Recently, both NO_3^- and NO_2^-, present at relatively high concentrations in tissues and plasma, have been shown to have biological activity associated with conversion to NO under hypoxic conditions,[41] therefore, it is possible that NO_3^- under these conditions contributes to biological activity *in vivo*. Similarly, intracellular esterase action on X-ASA will yield quantitative production of leaving group X group (e.g. Br^-, $MeSO_3^-$) (Fig. 1).

The fall and rise of GSH

The QM generated by NO-ASA and X-ASA is a highly reactive electrophile and Michael acceptor: the half-life for uncatalyzed addition of water is only 200 ms.[42, 43] This QM is readily trapped by GSH and is predicted to modify proteins (Fig. 1). Depletion of total GSH by trapping of the QM in a GSH conjugate was measured in HepG2 cells after treatment with *p*NO-ASA and *p*Br-ASA analogues and was observed to be rapid and significant: more than 25% of total intracellular GSH was lost after only 10 min incubation; and depletion was reported to be concentration dependent.[20] In contrast, depletion of GSH by *p*Ms-ASA was not significant in HepG2 cells.[20] In this paper, an extended study of intracellular GSH levels in response to X-ASA treatment demonstrated that not only did GSH concentration recover to initial levels, but GSH increased significantly above the original intracellular concentration with *p*Ms-ASA and *p*Br-ASA (Fig. 2a). In the case of *p*Br-ASA, cells recovered within 4 h (Fig. 2a). γ-Glutamylcysteine ligase (GCL) is important in conjugative

metabolism and detoxification and is the rate-limiting enzyme in GSH synthesis. Levels of mRNA for the catalytic subunit, GCLC, were measured by real-time quantitative PCR in HepG2 cells after incubation with drugs for 4 h, showing significant upregulation for QM-forming X-ASA, *p*Br-ASA, and *p*NO-ASA (Fig. 2b). The observations for *p*Ms-ASA and *m*NO-ASA are compatible with the hypotheses previously offered[20], that *m*NO-ASA is able to act as an electrophile through slower S_N2 reactions, and that *p*Ms-ASA is bioactivated more slowly to QM, resulting in a lower rate of reaction with cellular nucleophiles (v=k[QM]) and insignificant depletion of GSH.

DNA damage

The Comet assay was used to measure DNA single strand breaks after incubation with QM-releasing X-ASA. Compatible with the observed early and rapid intracellular GSH depletion and induction of oxidative stress, concentration-dependent DNA damage was observed after 1 h incubation with QM-donors, *p*NO-ASA, and *p*Br-ASA (Fig. 2c). The attenuated bioactivation of *p*Ms-ASA generates lower QM levels and as seen in Figure 2a, no significant depletion of intracellular GSH. Accordingly, this QM-donor did not cause DNA damage at the concentrations studied. We have previously shown that *m*Br-ASA forms GSH conjugates in the absence of cellular bioactivation by simple S_N2 reaction[20], leading to significant DNA damage at the higher concentration tested. MacDonald et al. have reported that in HepG2 cells, after 24 h treatment with *p*NO-ASA, DNA damage was not observed, and indeed, that this treatment inhibited DNA damage induced by polyaromatic hydrocarbons (PAH).[25] These observations are compatible with the rapid depletion of GSH and induction of oxidative stress by intracellular bioactivation of the reactive QM-donors, *p*NO-ASA and *p*Br-ASA, which in parallel, induces a cellular stress response that upregulates protective cellular mechanisms at later time points, including GSH synthesis and GCL upregulation, and possibly DNA repair. Figure 2d posits threshold concentrations for different biological outcomes, dependent on the flux of QM produced by the slow bioactivation of *p*Ms-ASA and the rapid bioactivation of *p*NO-ASA and *p*Br-ASA, at various initial concentrations.

The most important cellular mediator of GCL upregulation is ARE, a *cis*-acting element found in the regulatory region of over 200 genes.[44] Quinone electrophiles are known to activate ARE and induce enzymes mediating conjugative metabolism, such as GCL and NQO1, via the Nrf2 pathway.[3, 37, 45] In HepG2 cells, we reported activation of ARE by QM-forming X-ASA with high efficacy and *m*Br-ASA with low efficacy,[20] and *in simile* with GSH depletion and GCL upregulation, *p*Br-ASA and *p*NO-ASA were more potent than *p*Ms-ASA. Furthermore, the time course for ARE activation by *p*Br-ASA and *p*NO-ASA was almost identical. After 4 h, *p*Ms-ASA showed a trend to increase GCLC mRNA levels above DMSO control, and at 8 h *p*Ms-ASA significantly increased GSH levels. These observations are compatible with the slower bioactivation of *p*Ms-ASA to the QM, at levels that are still above the threshold of ARE activation required for GSH synthesis by GCL.

Activation and nuclear translocation of Nrf2

Upregulation of ARE-dependent downstream target genes by Nrf2 is dependent upon translocation of Nrf2 to the nucleus, which can be mediated by the sensor protein Keap1 or by phosphorylation triggered by activation of PI3K.[46] Nuclear translocation of Nrf2 was studied by confocal fluorescence microscopy after treatment of cells with *p*NO-ASA (6.25 μM or 25 μM) for 4 h using DAPI nuclear stain and Nrf2 immunocytochemistry (Figure 3a). The well-known Nrf2 inducer, 4′-bromoflavone (4-BF), was studied as a positive control. The DMSO control images showed cytoplasmic localization of Nrf2, whereas treatment with *p*NO-ASA or 4-BF induced nuclear translocation. Kinase induction of Nrf2 translocation via PI3K activation has been reported previously for *t*-Bu-hydroquinone in

HepG2 cells.[47] In order to rule out Nrf2 translocation mediated by this kinase signaling pathway, the PI3K inhibitor LY29402 was used, however, Nrf2 translocation induced by *p*NO-ASA was not inhibited. Similar experiments were completed to visualize Nrf2 translocation induced by *p*Br-ASA treatment (6.25 μM) after 4 h and 18 h (Figure 3b), revealing similar actions to *p*NO-ASA.

NQO1 induction

Nrf2 gene knockout experiments show substantial reductions in the ability of many chemopreventive agents to induce NQO1.[48, 49] In Hepa 1c1c7 liver cells, we observed and reported that X-ASA and NO-ASA induced NQO1 at 48 h.[20] The activation of Nrf2/ARE signaling implicates the sensor protein Keap1 as a target. Pre-incubation of cells with *N*-acetylcysteine (NAC) would be predicted to trap the QM, blocking activation of Keap1. Therefore, addition of NAC should attenuate induction of NQO1 via Nrf2/ARE activation. Induction of NQO1 by the QM-donors, *p*Br-ASA, *o*Br-ASA, and *p*Ms-ASA was indeed attenuated after 10 min pre-incubation with NAC (Figs 4a-c), however, statistical significance was only observed with *p*Ms-ASA and the lowest concentration of *o*Br-ASA. This observation is compatible with the ability of NAC to effectively and competitively trap lower concentrations of QM, and the slower bioactivation of *p*Ms-ASA to QM by cellular esterases.[20] In similar experiments, we also reported that addition of a “specific” NO trap to Hepa 1c1c7 cells was ineffective in blocking NQO1 induction by *p*NO-ASA.[20]

“NO-specific” DAF probes respond to QM-donor induced oxidative stress

Wardman has reviewed and described the promiscuity of the very popular DAF probes for NO in responding to a variety of oxidants and reactive nitrogen species.[50] Given evidence for induction of oxidative stress in HepG2 cells by NO-ASA and X-ASA and the recent use of a DAF probe to provide evidence for NO as the cardinal product from NO-ASA,[23] it was important to study the response of DAF-DA in HepG2 cells. DAF probes are a subset of 1,2-diaminoarenes, which after oxidation, trap NO leading to markedly increased fluorescence; however, the fluorescence associated with oxidation and NO trapping can also result from ROS, oxidants, and other reactants, leading to fluorescent adducts. One such example has been elegantly demonstrated in the literature for a dehydroascorbic acid adduct.[51]

HepG2 cells were treated for 24 h with *p*Br-ASA, *p*NO-ASA, or the NO-donor Spe/NO, followed by incubation with DAF-DA and nuclear staining with Hoechst. The positive response of DAF-DA elicited by Spe/NO was also observed for both NO-ASA and X-ASA treatments (Fig. 5): i.e. both NO release and the oxidative stress caused by QM release appear able to activate the DAF reporter. It is, of course, conceivable that *p*Br-ASA upregulates or activates nitric oxide synthase (NOS), therefore it was necessary to pre- and co-treat cells with the NOS inhibitor L-NAME. L-NAME treatment was without effect on *p*Br-ASA induced increase in DAF fluorescence (Supplemental Figure). One further observation of note was that oxidative stress leading to activation of the DAF reporter was evident at 4 and 24 h, despite these QM-donors causing depletion of intracellular GSH, and by inference QM itself, over much shorter time scales.

Protein modification by QM-donor *p*NO-ASA

The persistence of oxidative stress reflected in DAF fluorescence at 24 h, suggests the existence of targets for QM-donors beyond GSH. We have used human GST-P1 to study protein modification by NO, quinones, and oxidation, because Cys47 (pKa 4.2) models the reactive cysteines of proteases, phosphatases, and redox sensor proteins.[52-54] Further, GST-P1 is upregulated in various cancers, plays a role in the cellular response to oxidative stress, and modulates cell differentiation, proliferation, and death mediated by JNK (c-Jun N-terminal kinase).[55-62] Cys47 and Cys101 of GST-P1 have been highlighted to play an active

role in the signaling pathways affecting cell proliferation and apoptosis.[63] Modification of these residues by QM-releasing *p*NO-ASA and *p*Br-ASA was investigated usingPLE for bioactivation. Figures 6a-b show extracted ion chromatograms of a doubly charged peptide fragment containing QM-modified Cys47-C_7H_6OH and a triply charged peptide fragment carrying Cys101-C_7H_6OH, respectively. The MS/MS of the corresponding QM-modified Cys47 and Cys101 bearing peptide fragments are as illustrated in Figures 6c and 6d, respectively. The extent of modification of both Cys47 and Cys101 by QM from X-ASA was observed to be 70% (± 5%) after incubation with *p*NO-ASA (25 μM). Quantitation was achieved using NEM to alkylate and block Cys residues unmodified by QM.

Rokita has reported that QM-modified DNA is not stable due to base-catalyzed exchange,[64] therefore the stability of QM-modified GST-P1 was studied. Incubation of GST-P1 with *p*NO-ASA and PLE at 7<pH<12 for 24 h before blocking of free thiols with NEM, revealed no significant change in the extent of modification of Cys47 and Cys101 (data not shown). Finally, QM-modification of Cys47 of GST-P1 was studied in the presence of free cysteine (1-500 μM), anticipating that cysteine would trap any QM generated from X-ASA. For this study, mutant GST-P1(C101A) was used to exclude any role of Cys101. QM modification of Cys47 by QM derived from *p*NO-ASA was indeed observed to be inhibited by the presence of a competing free thiol in a concentration dependent manner (Fig. 7).

Keap1 modification by QM-donor pNO-ASA

The activation of ARE implicates Keap1 as a target, although alternatively GSH depletion may induce Keap1 oxidation. Covalent modification or oxidation of critical Cys residues of Keap1 has been highlighted as regulating the ability of Keap1 to repress nuclear translocation of Nrf2.[10, 65, 66] Therefore, alkylation of Keap1 by the QM generated from *p*NO-ASA, in the presence of PLE, was studied using protein expressed and purified to homogenity as previously described.[67] The Cys residues of Keap1 show variable reactivity towards different electrophiles[68] and the extent of QM modification by these residues after incubation with *p*NO-ASA (25 μM) is shown in Table 1. Five Cys residues, Cys196, Cys226, Cys249, Cys273, and Cys319, from the central linker (CLR or IVR) domain of Keap1 showed QM modifications ranging from 8%-28%. The importance of Cys23 and Cys273 residues of Keap1 has been emphasized in terms of nuclear translocation of Nrf2 and activation of the Nrf2/ARE pathway.[10, 65, 69] The only QM modification outside the CLR domain was observed with Cys23 (19%, Table 1) of the N-terminus region of Keap1. This particular Cys has also been previously characterized as readily forming disulfides with Cys38.[65] Interestingly, QM modifications were not observed for the Cys residues of BTB dimerization and Kelch repeat domains. The nuclear translocation of Nrf2 observed in HepG2 cells after treatment with *p*NO-ASA and *p*Br-ASA is entirely compatible with the selective alkylation of Keap1 residues by the QM generated on esterase-mediated bioactivation.

Rationale for design of novel ASA derivatives and activation of ARE

The results indicate that the major mechanism of Nrf2 signaling by QM-releasing NO-ASA and X-ASA is through modification of Keap1 consequent to release of the bioactive QM metabolite. Of course, a contribution from Keap1 oxidation resulting from GSH depletion by the QM metabolite is also probable; however, there is no need to invoke *S*-nitrosation of Keap1 as a mechanism of Nrf2 signaling.[19] Although the DAF fluorescent probe has been previously utilized to confirm cellular NO-release from NO-ASA,[23] this probe is not “NO specific” and was activated after treatment of HepG2 cells with QM-releasing *p*Br-ASA. Therefore, at least *in vitro*, neither the NO_3^- group intended to release NO, nor the alternative leaving groups in X-ASA (Br^- and $CH_3SO_3^-$) contribute directly to biological activity. Thus, it was rational to replace these redundant leaving groups, leading to design

and synthesis of *p*(ASA)$_2$ and *o*(ASA)$_2$. These ASA derivatives are bioactivated by esterase to release only salicylates and a QM. Comparison was made to two ASA derivatives, $BrCH_2$-ASA and $NOCH_2$-ASA, that cannot release a QM, measuring activation of ARE in HepG2 cells (Fig. 8). Both *p*(ASA)$_2$ and *o*(ASA)$_2$ showed high efficacy towards activation of Nrf2/ARE signaling.

Summary

Despite reports from our and others' labs that X-ASA analogues, without the ability to release NO, mimic the activity of *p*NO-ASA in cell cultures, claims that NO is the cardinal biological mediator persist. These ASA prodrugs are bioactivated by cellular esterase activity to release ASA and an electrophilic QM that can cause rapid depletion of intracellular GSH and oxidative stress leading to DNA damage. DAF-DA, often used as a specific reporter for NO, did respond to *p*NO-ASA treatment of HepG2 cells, however, a comparable response was observed for *p*Br-ASA. Therefore, in line with our previous work where *p*NO-ASA was shown quantitatively to release NO_3^- rather than NO,[20] and the known oxidative chemistry of DAF derivatives,[50] the response of the fluorescence reporter is ascribed to oxidative stress induced by the QM metabolite that is able to masquerade as NO, thus explaining reports in the literature.

To further support the hypothesis that *p*NO-ASA acts as a QM-donor in cell cultures, it was necessary to show covalent modification of Keap1 at Cys residues associated with dissociation of the complex with Nrf2 and translocation of Nrf2 to the nucleus. This was demonstrated for *p*NO-ASA using LC-MS/MS analysis. Furthermore, QM-releasing NO-ASA and X-ASA analogues were shown to cause translocation of Nrf2, activation of ARE, and upregulation of cellular stress response products including NQO1 and GSH. Regardless of the initial rate of QM release, QM-donor NO-ASA and X-ASA analogues induced cell defense mechanisms associated with chemopreventive actions and based upon the observed QM modification of GST-P1, it is likely that other cellular targets are involved.

QM-releasing NO-ASA and X-ASA are prodrugs of aspirin that have shown actions *in vitro* and *in vivo*, which are of potential therapeutic benefit, mostly in cancer chemoprevention. We have shown herein that covalent modification of Keap1 by the electrophilic QM metabolite provides a mechanism for activation of Nrf2 signaling. Traditionally, protein modification is not seen as desirable in a drug candidate, however, electrophilic agents are in the clinic or clinical trials, notably dimethyl fumarate (DMF) in psoriasis and multiple sclerosis, and the triterpenoid bardoxolone (CDDO-Me) and derivatives in chronic kidney disease. Both CDDO and DMF alkylate Keap1 and activate signaling pathways including Nrf2.[70, 71] Further studies are warranted on QM-donor aspirins, such as *p*(ASA)$_2$ to define important cellular targets and to further delineate mechanisms that might separate therapeutic from toxic actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

1

Ala	Alanine
ARE	antioxidant response element
Arg	Arginine
ASA	acetylsalicylic acid
BCA	bicinchoninic acid
BF	4'-bromoflavone
BSA	bovine serum albumin
BTB	Broad complex, Tramtrack and Bric-à-Brac domain
CBP/P300	p300-CBP coactivator
CDDO-Me	bardoxolone methyl
CLR	central linker region
CRC	colorectal cancer
Cys	cysteine
DAF-DA	diaminofluorescein diacetate
DAPI	4′,6-diamidino-2-phenylindole
DC	DGR/CTR region
DMF	dimethyl fumarate
ESI	electrospray ionization
FAD	flavin adenine dinucleotide
FT-ICR	fourier transform ion cyclotron resonance
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
GSH	reduced glutathione
GST-P1	glutathione-S-transferase-P1
HBN	hydroxybenzyl nitrate
HPLC	high performance liquid chromatography
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1

L-NAME	L-N^G-Nitroarginine methyl ester
LTQ	linear quadrupole ion trap
Lys	Lysine
MAP	mitogen-activated protein
MS/MS	tandem mass spectrometry
MTT	thiazolyl blue tetrazolium bromide
NAC	N-acetylcysteine
NADP	β-nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NFκB	nuclear factor kappa B
NO	nitric oxide
NO-ASA	nitric oxide-donating aspirin
NO-NSAID	nitric oxide-donating NSAID
NOS	nitric oxide synthase
NTR	N-terminal region
NQO1	NAD(P)H-dependent quinone oxidoreductase
Nrf2	nuclear factor-eryhthroid 2 related factor 2
PAH	polyaromatic hydrocarbons
PI3K	phosphoinositide 3 kinase
PLE	porcine liver esterase
QM	quinone methide
ROS	reactive oxygen species
SA	salicylic acid
SDS	sodium dodecyl sulfate
sMaf	small Maf
Spe/NO	spermine NONOate
SSA	5-sulfosalicylic acid

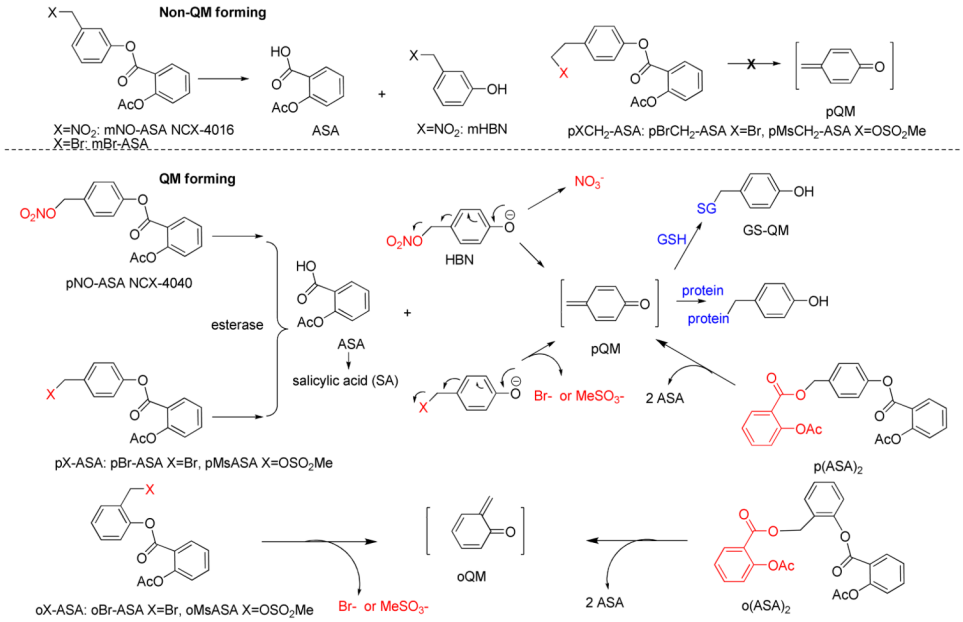


Figure 1.

Chemical structures of NO-ASA isomers, NCX-4016 (*m*NO-ASA) and NCX-4040 (*p*NO-ASA), and novel X-ASA and $(ASA)_2$ derivatives. Esterase-mediated bioactivation liberates SA, ASA, and HBN. The quinone methide is formed from *o*- and *p*- isomers, but not from *m*-isomers. The highly reactive electrophilic QM depletes GSH and modifies Cys residues of proteins including GST-P1 and Keap1.

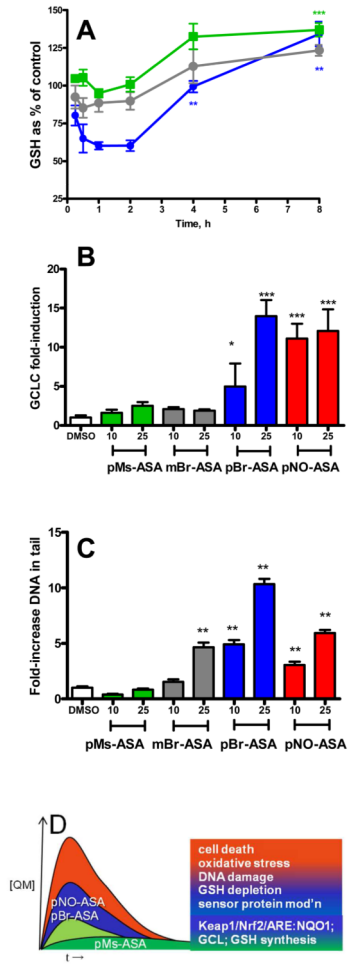


Figure 2.

Effect of NO-ASA and X-ASA analogues on intracellular GSH concentrations and DNA damage in HepG2 and Hepa 1c1c7 cells, respectively. (A) Total intracellular GSH concentrations measured after treatment of HepG2 cells with *m*Br-ASA (25 μM circle; orange), *p*Br-ASA (25 μM circle; green), or *p*Ms-ASA (25 μM square; blue), data show mean and s.e.m. analyzed by one way ANOVA with Tukey's post test relative to data at the first time point (*** $P < 0.001$; ** $P < 0.01$); (B) Expression of GCLC mRNA in HepG2 cells after 4 h treatment with *p*Ms-ASA, *m*Br-ASA, *p*Br-ASA, and *p*NO-ASA (10-25 μM). Data show mean and s.e.m. analyzed by one way ANOVA with Tukey's post test (*** $P < 0.001$) with respect to the DMSO-treated controls. (C) Single strand DNA damage, measured by Comet assay in Hepa 1c1c7 cells, after one hour incubation with *p*NO-ASA, *m*Br-ASA, *p*Br-ASA, and *p*Ms-ASA (10 μM, 25 μM). Data show mean and s.e.m: significant differences with DMSO vehicle control were analyzed (** $P < 0.01$) by ANOVA with Tukey's post test. (D) Figurative representation of the relationship between flux of QM from bioactivation and biological consequences.

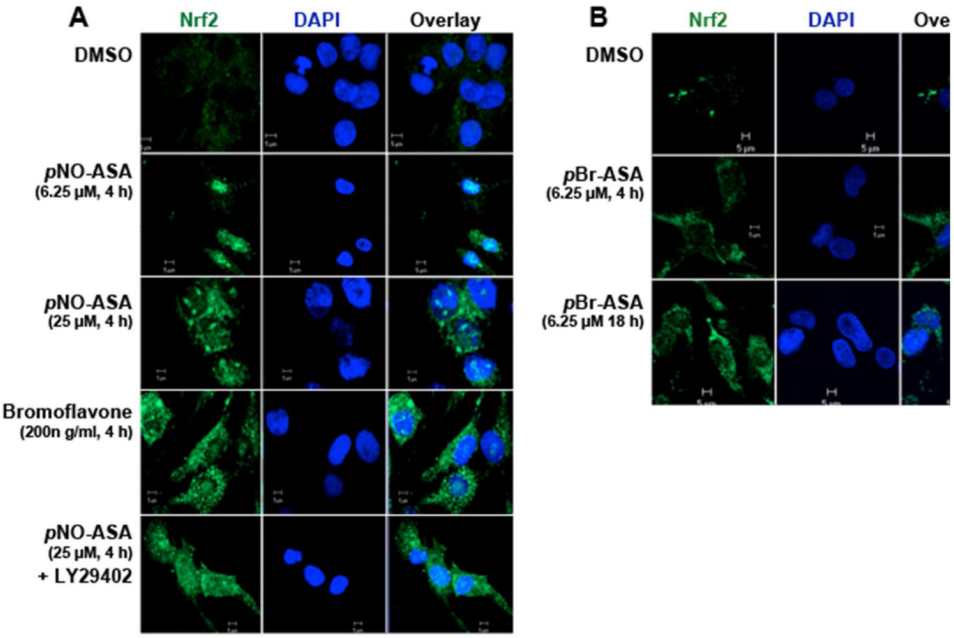


Figure 3.

QM release from X-ASA analogue, *p*Br-ASA, and *p*NO-ASA induces Nrf2 translocation in HepG2 cells. Immunofluorescence images of fluorescein Nrf2 staining and DAPI nuclear staining with overlapped images showing translocation of Nrf2 from cytoplasm to cell nucleus. HepG2 cells were treated: (A) for 4 h with DMSO, *p*NO-ASA (6.25 μM, 25 μM), 4'-bromoflavone (200 ng/mL), and *p*NO-ASA (25 μM) with LY29402; (B) DMSO control, or *p*Br-ASA (6.25 μM and 25 μM) after 4 or 18 h, respectively.

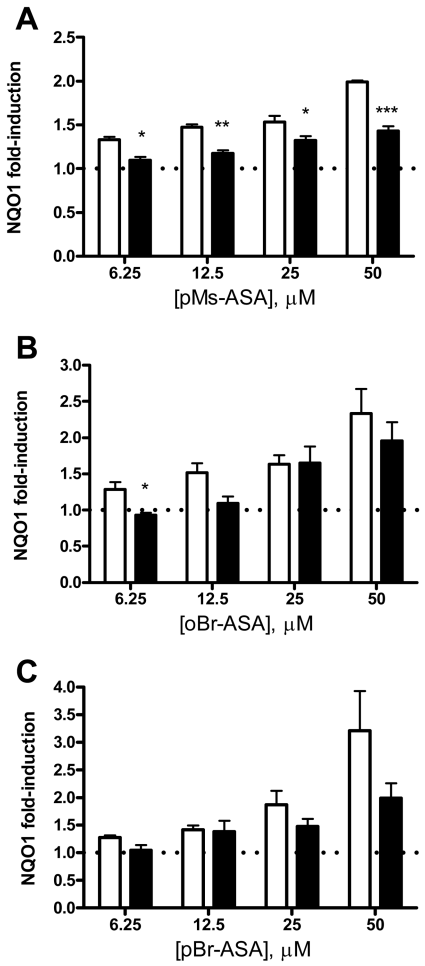


Figure 4.

Effect of NAC pretreatment on NQO1 induction by QM-releasing X-ASA. NQO1 induction in Hepa 1c1c7 cells was measured after 48 h incubation with X-ASA compounds and after 10 min pre-incubation with (solid bars) or without (open bars) addition of NAC (1 mM): (A) *p*Ms-ASA; (B) *o*Br-ASA; (C) *p*Br-ASA. Fold-induction is calculated relative to in-plate DMSO vehicle controls; for comparison 4-BF as a positive control caused approximately sevenfold induction. Data show mean and s.e.m: ** significantly different ($P < 0.05$) with respect to the DMSO-treated controls by ANOVA with Tukey's post test.

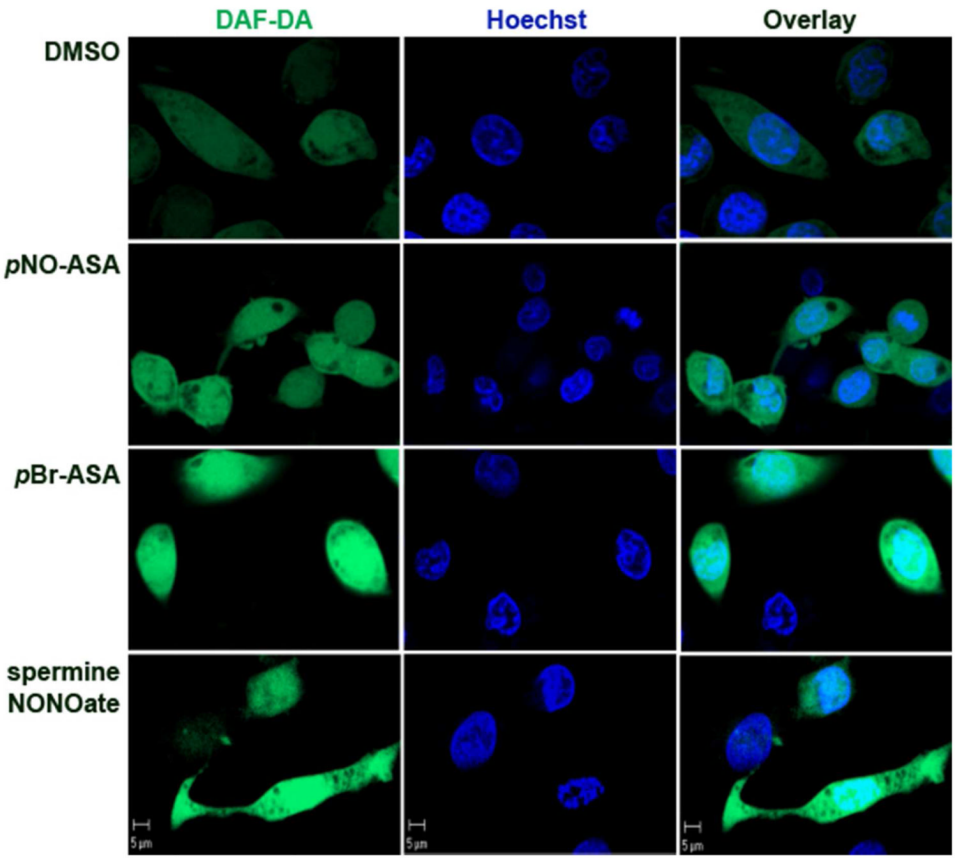


Figure 5.

The effect of X-ASA analogue, *p*Br-ASA, and *p*NO-ASA on DAF fluorescence in HepG2 cells. HepG2 cells were treated with DMSO, *p*NO-ASA (25 μM) and *p*Br-ASA (25 μM) for 24 h or spermine NONOate (25 μM) for 20 min. After treatment cells were labeled with DAF-DA and Hoechst nuclear stain. In similar experiments, the effect of NOS inhibition on *p*Br-ASA treatment showed no effect relative to *p*Br-ASA alone (Figure S1 in the Supporting Information).

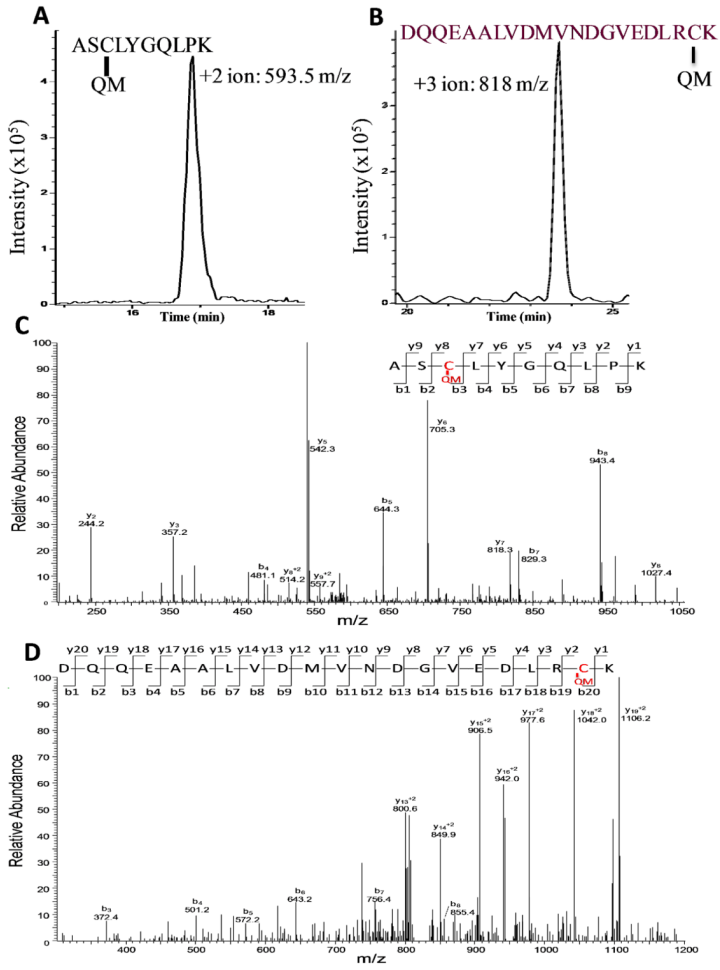


Figure 6.

LC-MS/MS analysis of GST-P1 modification by QM at Cys47 and Cys101. GST-P1 (15 μM) was treated with *p*NO-ASA or *p*Br-ASA (30 μM) for 30 min and unreacted Cys blocked with NEM (20 mM). The protein after in-gel digest was analyzed by LC-MS/MS, yielding extracted ion chromatograms of: a) QM modified Cys47 peptide, and; b) QM modified Cys101 peptide. The MS/MS spectra with b and y ion fragmentations are shown for: c) QM modified Cys47 peptide, and; d) QM modified Cys101 peptide.

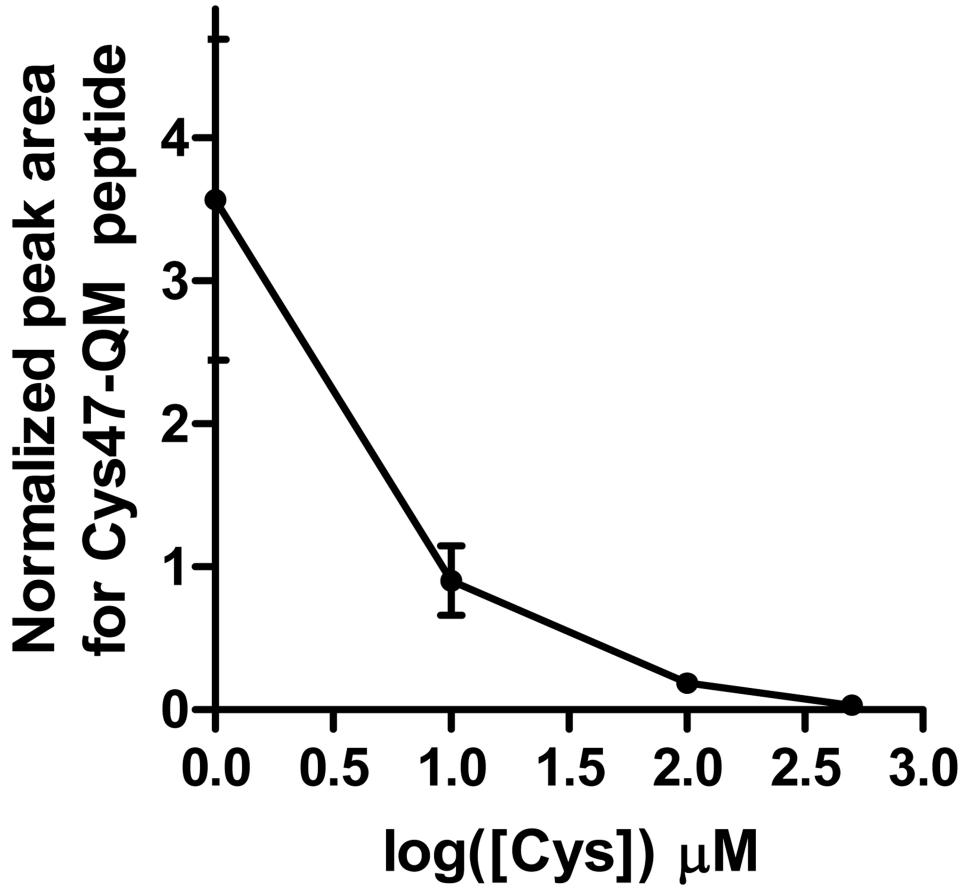


Figure 7.

Attenuated QM modification at Cys47 of mutant GST-P1(C101A) by addition of free Cys. GST-P1(C101A) (4 μM) was treated with *p*NO-ASA (25 μM) for 60 min in the presence of different Cys concentrations (1-500 μM) followed by blocking the unreacted Cys47 with NEM (20 mM). After in-gel digest, analysis by LC-MS/MS of the signal of Cys47 containing peptide (45-54: ASCLYGQLPK, 593+2) relative to the adjacent peptide peak (55-70: FQDGDLTLYQSNTILR, 943+2) yielded a relative measure of QM modification. Data shown are normalized mean and SD. (*n*=4).

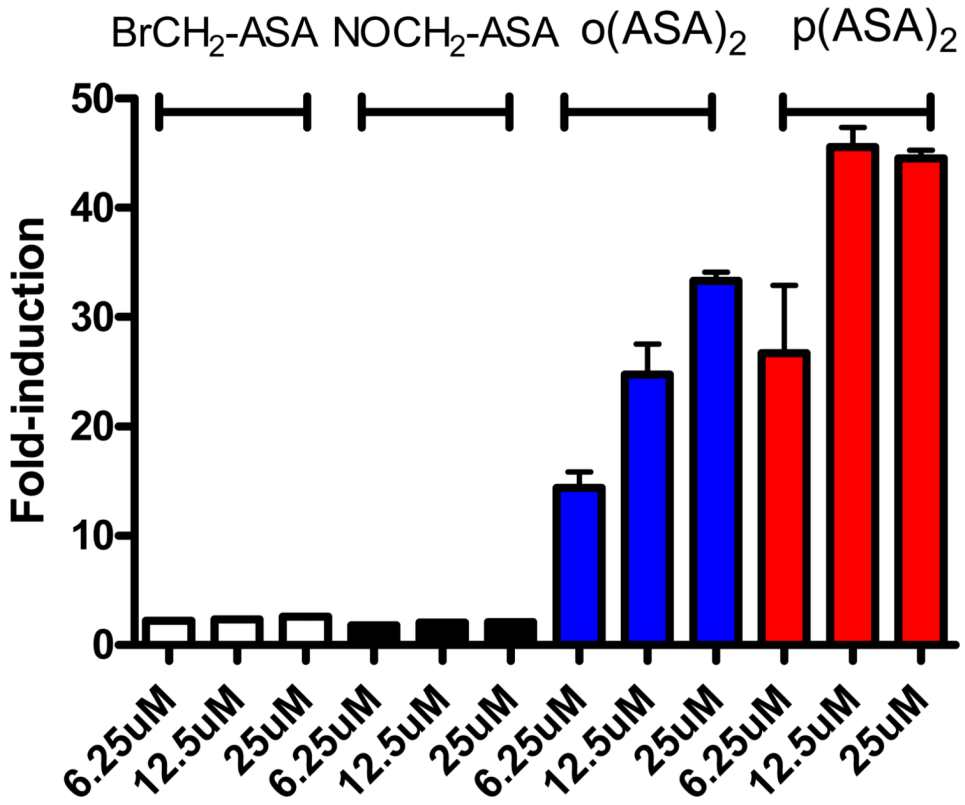
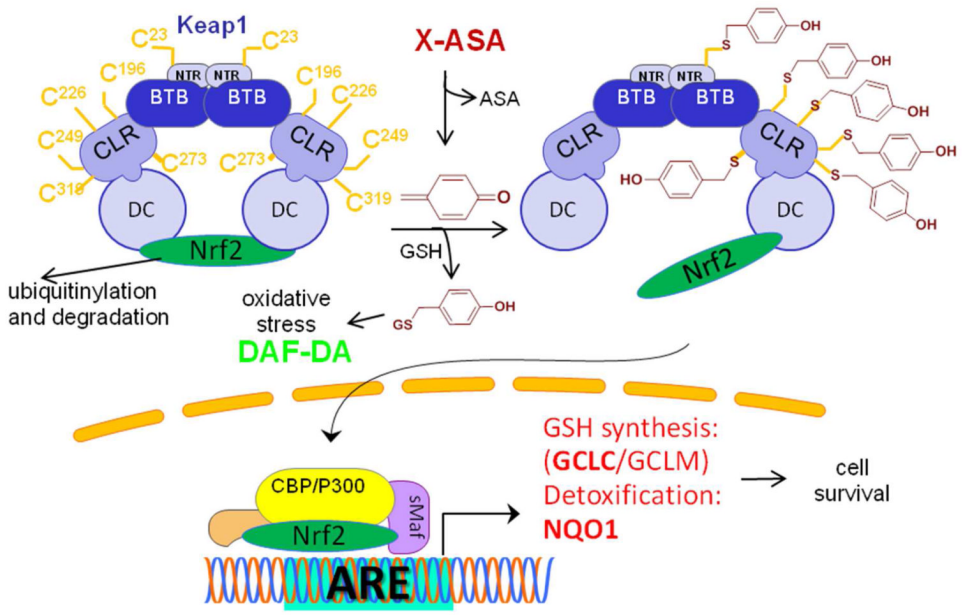


Figure 8.

Effect of novel QM releasing isomers, *o*(ASA)$_2$ and *p*(ASA)$_2$, on ARE-luciferase gene reporter induction. ASA derivatives at varying concentrations were incubated with HepG2 cells for 8 h. Results are shown as fold-induction relative to control as measured by enzyme activity and normalized to protein concentrations. Data show mean and SEM from at least triplicate measures.



Scheme 1.

Table 1

Analysis of QM and NEM modified cysteine containing peptides of Keap1[a] reacted with *p*NO-ASA.

Identified peptide fragment (starting-ending residue)	Cys	% Modification	
		QM	NEM
FLPLQSQCPEGAGDAVMYASTECK (16-39)	Cys23	19	81
	Cys38	-	100
LSQQLCDVTLQVK (72-84)	Cys77	-	100
CVLHVMNGAVMYQIDSVVR (151-169)	Cys151	-	100
ACSDFLVQQLDPSNAIGIANFAEQIGCVELHQR (170-202)	Cys171	-	100
	Cys196	14	86
QEEFFNLSHCQLVTLISR (217-234)	Cys226	28	72
CESEVFHACINWVK (241-254)	Cys241	-	100
	Cys249	14	86
CHSLTPNFLQMQLQK (273-287)	Cys273	8	92
IFEELTLHKPTQVMPCR (304-320)	Cys319	10	90
SGLAGCVVGGLLYAVGGR (363-380)	Cys368	-	100
NNSPDGNTDSSALDCYNPMTNQWSPCAPMS VPR (381-413)	Cys395	-	100
	Cys406	-	100
IGVGVIDGHIYAVGGSHGCIHHNSVER (416-442)	Cys434	-	100
SGAGVCVLHNCIYAAGGYDGQDQLNSVER (508-536)	Cys513	-	100
	Cys518	-	100
IYVLGGYDGHTFLDSVECYDPDTDTWSEVTR (566-596)	Cys583	-	100
SGVGVAVTMEPCR (602-614)	Cys613	-	100

[a] Keap1 (15 μM) was incubated with *p*NO-ASA (30 μM) for 30 minutes followed by addition of NEM (20 mM) to trap unreacted free thiols.