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Original Contribution

Brusatol provokes a rapid and transient inhibition of Nrf2 signaling and sensitizes mammalian cells to chemical toxicity—implications for therapeutic targeting of Nrf2

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ABSTRACT

The transcription factor Nrf2 regulates the basal and inducible expression of a battery of cytoprotective genes. Whereas numerous Nrf2-inducing small molecules have been reported, very few chemical inhibitors of Nrf2 have been identified to date. The quassinoid brusatol has recently been shown to inhibit Nrf2 and ameliorate chemoresistance in vitro and in vivo. Here, we show that brusatol provokes a rapid and transient depletion of Nrf2 protein, through a posttranscriptional mechanism, in mouse Hepa-1c1c7 hepatoma cells. Importantly, brusatol also inhibits Nrf2 in freshly isolated primary human hepatocytes. In keeping with its ability to inhibit Nrf2 signaling, brusatol sensitizes Hepa-1c1c7 cells to chemical stress provoked by 2,4-dinitrochlorobenzene, iodoacetamide, and *N*-acetyl-*p*-benzoquinone imine, the hepatotoxic metabolite of acetaminophen. The inhibitory effect of brusatol toward Nrf2 is shown to be independent of its repressor Keap1, the proteasomal and autophagic protein degradation systems, and protein kinase signaling pathways that are known to modulate Nrf2 activity, implying the involvement of a novel means of Nrf2 regulation. These findings substantiate brusatol as a useful experimental tool for the inhibition of Nrf2 signaling and highlight the potential for therapeutic inhibition of Nrf2 to alter the risk of adverse events by reducing the capacity of nontarget cells to buffer against chemical and oxidative insults. These data will inform a rational assessment of the risk:benefit ratio of inhibiting Nrf2 in relevant therapeutic contexts, which is essential if compounds such as brusatol are to be developed into efficacious and safe drugs.

Introduction

The redox-sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2)[2] plays a critical role in the regulation of cellular defense against chemical and oxidative stress [1]. The activity of Nrf2 is primarily governed by its physical and functional interaction with the cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1), which facilitates the ubiquitination and subsequent proteasomal degradation of Nrf2 via the Cullin 3 ubiquitin

Abbreviations: AMC, 7-amino-4-methylcoumarin; CDDO-Me, methyl-2-cyano-3,12-dioxooleana-1, 9(11)dien-28-oate; DNCB, 2,4-dinitrochlorobenzene; Gclm, glutamate–cysteine ligase regulatory subunit; HIF-1α, hypoxia-inducible factor 1α; IAA, iodoacetamide; Keap1, Kelch-like ECH-associated protein 1; NAPQI, *N*-acetyl-*p*-benzoquinone imine; NEM, *N*-ethylmaleimide; Nqo1, NAD(P)H dehydrogenase quinone 1; Nrf2, nuclear factor erythroid 2-related factor 2; RT-qPCR, real-time quantitative PCR.

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ligase complex [2,3]. Upon exposure to chemical or oxidative stresses, the ability of Keap1 to repress Nrf2 is disrupted, leading to the latter's accumulation and translocation to the nucleus, where Nrf2 induces the transcription of a battery of cytoprotective genes encoding redox-balancing proteins, phase II detoxification enzymes, and drug transporters [4]. In doing so, Nrf2 promotes the maintenance of cellular homeostasis under stress conditions. Exemplifying the above, transgenic Nrf2-knockout mice demonstrate enhanced susceptibility to various drug-induced toxicities, including acetaminophen hepatotoxicity [5,6], cisplatin nephrotoxicity [7], and bleomycin lung fibrosis [8], whereas genetic knockdown of Keap1 confers a protective phenotype [9].

Recently, a number of studies have demonstrated a link between oncogenesis and mutations in the Keap1 and/or Nrf2 genes that result in the constitutive activation of Nrf2 [10]. In keeping with its ability to protect against chemical and oxidative stress, the constitutive activation of Nrf2 has also been shown to contribute to the development of drug resistance in cancer cell lines and tissues [11]. As a result, there is a burgeoning interest in the therapeutic potential of inhibiting Nrf2 as a strategy for overcoming chemoresistance. A barrier to this goal is the paucity of small-molecule inhibitors of Nrf2 that have been described to date, whereas the consequences for nontarget cell health of inhibiting Nrf2, particularly in the context of cytotoxic cancer therapy, have yet to be fully considered.

The quassinoid brusatol, isolated from the *Brucea javanica* plant, has recently been shown to inhibit Nrf2 signaling, reduce tumor burden, and ameliorate chemoresistance in both in vitro and in vivo cancer models [12–14]. Here, using Hepa-1c1c7 hepatoma cells and freshly isolated primary human hepatocytes, we demonstrate that brusatol provokes a rapid and transient depletion of Nrf2 protein, through a posttranscriptional mechanism that is independent of Keap1, the proteasomal and autophagic protein degradation systems, and protein kinase signaling pathways that are known to regulate Nrf2. In keeping with its ability to inhibit Nrf2 signaling, we show that brusatol sensitizes Hepa-1c1c7 cells to chemical stress provoked by 2,4-dinitrochlorobenzene (DNCB), iodoacetamide (IAA), and *N*-acetyl-*p*-benzoquinone imine (NAPQI), the hepatotoxic metabolite of acetaminophen. Therefore, whereas brusatol is a valuable experimental tool for inhibiting Nrf2, the benefit:risk ratio of its therapeutic use should be considered in light of the potential for enhanced sensitivity of nontarget cells to endogenous and exogenous chemical and oxidative insults.

Materials and methods

Materials

Brusatol was extracted and purified from fractions generated from dried plant material of *Fructus Bruceae* using Diaion HP-20, Diaion HP-20ss, and Sephadex LH-20 column chromatography and purified using a C18 semipreparative HPLC column (Alltima C18 column, 10 × 250 mm, 5 μm). The structure of brusatol was confirmed by NMR using a Bruker NMR spectrometer (400 MHz) with trimethylsilane as the internal standard. The structure was further confirmed by APCI–MS using an Agilent HP 1100 series SL Trap MSD. Methyl 2-cyano-3,12-dioxooleana-1,9(11)dien-28-oate (CDDO-Me) was kindly provided by Dr. Michael Wong and Professor Paul O'Neill (Department of Chemistry, University of Liverpool). All other materials were obtained from Sigma–Aldrich (UK).

Hepa-1c1c7 cell culture

Mouse Hepa-1c1c7 hepatoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 584 mg/L L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biowest, France), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in a 5% CO_2 humidified environment.

Primary human hepatocyte isolation and culture

Liver tissue was obtained from the Liver Cell Lab at the Karolinska University Hospital (Huddinge, Sweden) or Aintree University Hospital (Liverpool, UK) by qualified medical staff, with donor informed consent following local ethical and institutional guidelines. The liver tissue used in this study was obtained from four patients (PHH1–4, see Supplementary Table S1 for details) undergoing planned liver resection for various indications. Immediately after removal from the patient, excess healthy liver parenchyma was separated from the specimen and placed in cold Eagle's minimum essential medium and transported to the laboratory on ice. Tissue dissociation and hepatocyte isolation were performed by using a two-step collagenase perfusion procedure, essentially as described previously [15]. The cells were counted and a Trypan blue exclusion test was used to calculate viability. The cell suspension was diluted to the required density in William's medium E without phenol red, supplemented with 25 mM Hepes and 2 mM L-glutamine, pH adjusted to 7.4 (modified William's medium E) supplemented with 10% FBS. Cells were seeded onto type I collagen-coated plates and cultured at 37 °C in a 5% CO_2 humidified environment. After 3 h, the medium was replaced with fresh modified William's medium E not supplemented with FBS, and the cells were cultured for a further 16 h before commencement of experiments.

Cell treatments

Cells were seeded into appropriate culture plates 24 h before the start of the experiments. All compounds were dissolved in dimethyl sulfoxide (DMSO) before addition to the cell culture medium, and the concentration of the vehicle was maintained at 0.5% (v/v) regardless of drug concentration.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer, and clarified whole-cell lysates were resolved by denaturing electrophoresis on 4–12% Novex Bis–Tris polyacrylamide gels (Life Technologies, UK). Separated proteins were transferred onto Hybond nitrocellulose membranes (GE Healthcare, UK), which were then blocked in Tris-buffered saline (TBS; pH 7.0) containing 0.1% Tween 20 and 10% nonfat milk (Bio-Rad). Blocked membranes were probed in TBS containing 0.1% Tween 20 and 2% nonfat milk supplemented with antibodies raised against Nrf2 (EP1808Y; Abcam, UK), β-actin (AC-15; Abcam), Keap1 (sc-15246; Santa Cruz Biotechnology, Germany), p62/SQSTM1 (P0067; Sigma–Aldrich), cyclin A (sc-751; Santa Cruz Biotechnology), hypoxia-inducible factor 1α (HIF-1α; 610959; BD Biosciences, UK), p53 (M7001; Dako, UK), survivin (sc-17779; Santa Cruz Biotechnology), phospho-p38 MAPK (4511S; Cell Signaling Technology), phospho-AKT (4060S; Cell Signaling Technology), phospho-ERK1/2 (4377S; Cell Signaling Technology), and phospho-SAPK (9251S; Cell Signaling Technology). Horseradish peroxidase-linked anti-rabbit (A9169; Sigma–Aldrich), anti-mouse (A9044; Abcam), and anti-goat (P0449; Dako) secondary antibodies were used as necessary. Immunoblots were visualized by enhanced chemiluminescence (PerkinElmer, UK) and exposed to Hyperfilm ECL (Amersham). Immunoreactive band volumes were quantified using TotalLab 100 software (Nonlinear Dynamics, UK) and normalized to β-actin.

Measurement of cellular ATP content

Cell viability was measured using the CellTiter-Glo luminescence assay (Promega, UK), in accordance with the manufacturer's instructions.