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Original Contribution

Cinnamaldehydes inhibit thioredoxin reductase and induce Nrf2: potential candidates for cancer therapy and chemoprevention

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ABSTRACT

Trans-cinnamaldehyde (CA) and its analogs 2-hydroxycinnamaldehyde and 2-benzoyloxycinnamaldehyde have been reported to possess antitumor activity. CA is also a known Nrf2 activator. In this study, a series of *ortho*-substituted cinnamaldehyde analogs was synthesized and screened for antiproliferative and thioredoxin reductase (TrxR)-inhibitory activities. Whereas CA was weakly cytotoxic and TrxR inhibiting, hydroxy and benzoyloxy substitutions resulted in analogs with enhanced antiproliferative activity paralleling increased potency in TrxR inactivation. A novel analog, 5-fluoro-2-hydroxycinnamaldehyde, was identified as exhibiting the strongest antitumor effect (GI_{50} 1.6 μM in HCT 116 cells) and TrxR inhibition (IC_{50} 7 μM, 1 h incubation with recombinant TrxR). CA and its 2-hydroxy- and 2-benzoyloxy-substituted analogs possessed dual TrxR-inhibitory and Nrf2-inducing effects, both attributed to an active Michael acceptor pharmacophore. At lethal concentrations, TrxR-inhibitory potencies correlated with the compounds' antiproliferative activities. The penultimate C-terminal selenocysteine residue was shown to be a possible target. Conversely, at sublethal concentrations, these agents induced an adaptive antioxidant response through Nrf2-mediated upregulation of phase II enzymes, including TrxR induction. We conclude from the results obtained that TrxR inactivation contributes at least partly to cinnamaldehyde cytotoxicity. These Michael acceptor molecules can potentially be exploited for use in different concentrations in chemotherapeutic and chemopreventive strategies.

With the uncovering of new molecular targets and a better understanding of tumor pathology, anticancer strategies aimed at taming intractable malignancies and reducing the incidence of cancer have been intensely deployed. Among these efforts, the discovery and development of novel molecules that possess ideally both a chemoprotective effect (to reduce the risk of developing cancer in healthy individuals) and an anticancer chemotherapeutic effect are pertinent. One common approach to identifying and developing new drugs involves the empirical use of natural products or their synthetic derivatives. Often, empirically developed compounds are found to act against multiple molecular targets. This is advantageous for instance in antitumor treatment when tumor cells develop resistance against specific anticancer agents over time. Plant foods such as spices, vegetables, and fruits have long caught the attention of researchers, as they comprise rich sources of components that possess potentially chemotherapeutic and/or chemopreventive activity. These natural products provide novel leads and chemical scaffolds on which chemical modifications can be applied to derive new synthetic compounds with superior anticancer properties. The drug discovery strategy therefore involves identification of components exhibiting these desirable properties, followed by employment of medicinal chemistry to derive more potent synthetic analogs and elucidate their mechanisms of action.

Redox homeostasis is critical for cell survival and growth, and under normal physiologic conditions, this is achieved by a number of cellular antioxidant systems including the two major thiol redox systems: the glutathione and thioredoxin systems. The thioredoxin system comprises thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH. TrxR catalyzes the NADPH-dependent reduction of the active-site disulfide of oxidized Trx. Trxs of all species possess a

Abbreviations: GR, glutathione reductase; GSH, glutathione; Sec, selenocysteine; Trx, thioredoxin; TrxR, thioredoxin reductase; CA, *trans*-cinnamaldehyde; CAC, cinnamic acid; SSP1, 2-methoxycinnamaldehyde; BCA, 2-benzoyloxycinnamaldehyde; HCA, 2-hydroxycinnamaldehyde; SSP2, 2-pentoxycinnamaldehyde; SSP3, 2-benzyloxycinnamaldehyde; FHCA, 5-fluoro-2-hydroxycinnamaldehyde.

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conserved redox-active dithiol/disulfide (in human Trx: Trp-Cys[32]-Gly-Pro-Cys[35]-Lys) that accounts for the oxidoreductase activity. Through thiol–disulfide exchange reactions, Trx functions as a protein disulfide reductase, which in turn contributes to its cellular functions, such as redox homeostasis through electron donation to peroxide-scavenging peroxiredoxins, redox regulation of transcription factor activity, DNA synthesis through electron donation to ribonucleotide reductase, and inhibition of apoptosis through redox-sensitive interaction with apoptosis signal-regulating kinase 1 [1,2]. In cancer, however, the biological effects of the Trx/TrxR system contribute to tumor growth and progression; overexpression of Trx and TrxR in several human cancers is reported to be associated with cancer drug resistance and poor patient prognosis [3–6]. In addition, it has been demonstrated that TrxR is essential for the carcinogenic process and invasive phenotype of cancer [7]. TrxR is a homodimeric enzyme belonging to the flavoprotein family of pyridine nucleotide–disulfide oxidoreductases, which also includes glutathione reductase (GR) [8]. Each mammalian TrxR subunit contains two distinct redox-active centers. The first is the N-terminal Cys[59]-Val-Asn-Val-Gly-Cys[64] dithiol/disulfide active site contained in the FAD-binding domain and the second consists of Gly-Cys[497]-Sec[498]-Gly residing in the 16-residue extension in the C-terminus [9]. The role of each C-terminal active site is to transfer reducing equivalents from the central redox center of the opposite subunit to the substrate [10–12]. Under physiologic conditions, the highly accessible penultimate selenocysteine (Sec) residue, as important as it is for TrxR catalytic activity, is highly nucleophilic to be targeted by electrophilic compounds. TrxR inhibition is therefore suggested to form an important basis for anticancer intervention. Indeed, a good number of natural and synthetic compounds, including several chemotherapeutic agents, have been recognized as selectively inhibiting mammalian TrxR [13–15].

In response to stress caused by exposure to electrophiles and reactive oxygen species (ROS), induction of phase II detoxification and antioxidant enzymes, such as several glutathione *S*-transferases, NAD(P)H quinone reductase, heme oxygenase 1, γ-glutamate–cysteine ligase, TrxR, Trx, and peroxiredoxin 1, offers protection against the toxic and carcinogenic effects of the electrophilic reactants [16]. Transcription factor NF-E2-related factor 2 (Nrf2) plays a pivotal role in coordinating the phase II response through its binding to and activation of the common antioxidant responsive element (ARE; consensus sequence TGACNNNGC) in the upstream 5′ flanking region of genes encoding these phase II enzymes [17]. Its indispensable role in chemoprevention is highlighted by the findings that Nrf2-deficient mice display increased susceptibility to carcinogenesis and exhibit an abrogation of phase II enzyme induction in response to chemoprotective enzyme inducers [18]. Nrf2 activity is regulated in part by actin-associated and cysteine-rich Kelch-like ECH-associated protein 1 (Keap1) through sequestering Nrf2 in the cytosol to prevent its nuclear translocation. Also, by binding to Nrf2, Keap1 regulates the Nrf2 cellular steady state by targeting it to Cullin 3-based E3 ubiquitin ligase, which then mediates Nrf2 ubiquitinylation for 26S proteasomal degradation [19,20]. Keap1 is a sensor trigger for activation of Nrf2-regulated genes; a broad spectrum of electrophiles bearing diversified structures have been found to inhibit Keap1-mediated Nrf2 degradation by causing oxidative/chemical modification of critical cysteine residues in Keap1 [21]. The inducibility of the Keap1–Nrf2–ARE signaling pathway by biologically relevant electrophiles therefore forms the basis on which the pathway can be manipulated to achieve protection of the body from chemical carcinogenesis (cancer chemoprevention).

Trans-cinnamaldehyde (CA) is a major component found in the stem bark of *Cinnamomum cassia.* It contains an α,β-unsaturated carbonyl moiety, also known as the Michael acceptor. Over the years, independent research conducted by various research groups has come to a common finding that Michael acceptor compounds are among the heterogeneous electrophiles that display chemotherapeutic and chemopreventive properties. Notably, numerous naturally occurring Michael acceptors such as curcumin as well as the flavonoids quercetin and myricetin have been found to exert antiproliferative activity through TrxR inhibition [22,23]. Furthermore, pioneering work from more than 2 decades ago reported the ability of Michael acceptors to induce cellular enzymes that were protective against carcinogenesis [24]. In particular, their inducing abilities are associated with their reactivity with sulfhydryl groups [25]. In this study, we investigated whether dietary CA and its structural derivatives could exhibit these properties. Based on the results obtained, we report that CA and its 2-hydroxy- and 2-benzoyloxy-substituted derivatives exhibit dual TrxR-inhibitory and Nrf2-inducing activities in a dose-dependent manner, which in turn resulted in a cytotoxic and cytoprotective outcome, respectively. Distinctly, these Michael acceptor molecules thus serve as potential candidates for cancer therapy and chemoprevention.

Materials and methods

Materials and cell culture

Recombinant rat TrxR was prepared as previously described, having a specific activity of 50% as determined by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay [26]. Recombinant human Trx and *Escherichia coli* Trx and TrxR were obtained from IMCO Corp. (Stockholm, Sweden). Yeast glutathione reductase, insulin, reduced glutathione (GSH), and glutathione disulfide (GSSG) were purchased from Sigma–Aldrich. Biotin-conjugated iodoacetamide (BIAM) was from Molecular Probes. Cinnamaldehyde stocks (50 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Colon carcinoma HCT 116 and mammary carcinoma MCF-7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Chemicals

CA, cinnamic acid (CAC), and 2-methoxycinnamaldehyde (SSP1) were commercially available (Sigma–Aldrich). 2-Benzoyloxycinnamaldehyde (BCA) was prepared from 2-hydroxycinnamaldehyde (HCA) in 85% yield according to a previously published method [27].

Chemical synthesis

Methyl 2-hydroxycinnamate (intermediate)

Chlorotrimethylsilane (23.4 ml, 183 mmol) was added to a solution of commercially available (*E*)-2-hydroxycinnamic acid (10.0 g, 61 mmol) in methanol (150 ml), and the reaction mixture was heated under reflux for 16 h. The reaction mixture was then concentrated in vacuo and the residue washed with excess water to give methyl 2-hydroxycinnamate as a white solid (10.5 g, 59 mmol, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (1H, d, J = 16.1 Hz, C*H*=CH), 7.50 (1H, d, J = 7.7 Hz, ArH), 7.25 (1H, t, J = 7.7 Hz, ArH), 6.95 (1H, t, J = 7.5 Hz, ArH), 6.86 (1H, d, J = 8.0 Hz, ArH), 6.64 (1H, d, J = 16.1 Hz, CH=C*H*), 6.02 (1H, s, O*H*), 3.85 (3H, s, OCH_3).

2-Pentoxycinnamaldehyde (SSP2)

To a solution of methyl 2-hydroxycinnamate (1.5 g, 8.4 mmol) in dimethylformamide (DMF) (12 ml) was added potassium carbonate (3.48 g, 25.2 mmol) and 1-iodopentane (1.26 ml, 9.6 mmol) The mixture was stirred at room temperature for 7 h and then diluted with ethyl acetate (50 ml) and washed with water (50 ml). The aqueous phase was extracted with ethyl acetate (2 × 50 m1) and the combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated in vacuo to give the intermediate methyl 3-(2-(pentoxy) phenyl) acrylate as a yellow oil (1.38 g, 5.54 mmol, 66%). Morpholine (1.68 ml, 19.3 mmol) was dissolved in dry toluene (4 ml) and the