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Biological Clues to Potent DNA-Damaging Activities in Food and Flavoring

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Abstract

Population differences in age-related diseases and cancer could stem from differences in diet. To characterize DNA strand-breaking activities in selected foods/beverages, flavorings, and some of their constituent chemicals, we used p53R cells, a cellular assay sensitive to such breaks.

Substances testing positive included reference chemicals: quinacrine (peak response, 51X) and etoposide (33X); flavonoids: EGCG (19X), curcumin (12X), apigenin (9X), and quercetin (7X); beverages: chamomile (11X), green (21X), and black tea (26X) and coffee (3 to 29X); and liquid smoke (4 to 28X). Damage occurred at dietary concentrations: etoposide near 5 µg/ml produced responses similar to a 1:1000 dilution of liquid smoke, a 1:20 dilution of coffee, and a 1:5 dilution of tea. Pyrogallol-related chemicals and tannins are present in dietary sources and individually produced strong activity: pyrogallol (30X), 3-methoxycatechol (25X), gallic acid (21X), and 1,2,4-benzenetriol (21X). From structure-activity relationships, high activities depended on specific orientations of hydroxyls on the benzene ring. Responses accompanied cellular signals characteristic of DNA breaks such as H2AX phosphorylation. Breaks were also directly detected by comet assay. Cellular toxicological effects of foods and flavorings could guide epidemiologic and experimental studies of potential disease risks from DNA strand-breaking chemicals in diets.

Keywords

DNA damage; p53; liquid smoke; tea; coffee; pyrogallol

1. Introduction

Health risks attributable to diet have been evaluated by both epidemiology and biological methods. Epidemiology, however, has poor resolution at the chemical level. For example, although screening of foods and individual chemicals for nucleotide mutagens is efficient using the Ames assay, screening for clastogens is inefficient and has employed a variety of cell- and animal-based assays.

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Among Ames-negative foods, major dietary risks include the enhanced cancer risk from the consumption of high-tannin foods (Kirby, 1960; Morton, 1992). The subcutaneous administration of the tannin fractions from several plants (including tea) produced tumors at the injection site in rats (Kapadia et al., 1976). Hot teas and their constituents were linked to elevated risks of cancer (Morton, 1987). Processed meat intake may increase the risk of colorectal cancer by 20–50% (Chan et al., 2011; Santarelli et al., 2008). Salt, liquid smoke, nitrites and nitrates are widely used in meat processing. Liquid smoke caused a many-fold increase in DNA single-strand breaks (measured as DNA eluted in alkaline conditions from a filter) in rat gastric mucosa in vivo when given orally (Ohshima et al., 1989b) and induced mutations in human lymphocytes in vitro (Braun et al., 1987). An increased number of altered pyloric glands, a neoplasia precursor, were produced in rats on a diet containing 5% liquid smoke after a single intragastric administration of N-methyl-N'-nitroso-N-nitrosoguanidine (Shichino et al., 1992). The results of the Ames test, however, were equivocal (Braun et al., 1987; Putnam et al., 1999). The genotoxic potential of liquid smoke and many other food substances thus remains largely unexamined due to the limitations of the commonly used tests.

Efficient biological assays of DNA damage could aid epidemiologic studies of foods and flavorings. We used a well characterized p53-based luciferase reporter human cell line (Cunningham et al., 2004; Gallmeier et al., 2005; Sohn et al., 2002) and confirmatory assays to investigate the genotoxic properties of selected foods and flavorings.

2. Materials and Methods

2.1. Cell Lines and Cell Culture

p53R cells were created in our laboratory (Cunningham et al., 2004; Gallmeier et al., 2005; Sohn et al., 2002). p53R, HeLa (ATCC), and AAV-293 (Stratagene) cells were grown in DMEM with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, and 20 mM HEPES. CHO AA8-Luc Tet-Off (Clontech) cells were supplemented with 100 µg/ml G418.

2.2. Substances Tested

Chemicals were from Sigma-Aldrich except as noted otherwise.

Positive controls—Quinacrine and etoposide served as positive controls for the p53R assay. Quinacrine, an intercalator and potential topoisomerase inhibitor inducing DNA strand breaks (Snyder and Arnone, 2002; Wang et al., 2005), strongly activates p53 in the p53R assay (Cunningham et al., 2004; Sohn et al., 2002). Etoposide is a potent topoisomerase inhibitor causing strand breakage and cytotoxicity, also strongly activating p53 in the assay (Sohn et al., 2002). Trichostatin A (TSA) was used to cause non-specific gene expression in CHO AA8-Luc Tet-Off cells (Cunningham et al., 2004). Hydrogen peroxide served as a positive control for the neutral comet assay.

Reference chemicals—A variety of reference chemicals were tested in the p53R assay. Among the chemotherapeutics, aminopterin and methotrexate are inhibitors of dihydrofolate reductase, fluorouracil (5-FU) forms a nucleotide analog inhibitor of thymidylate synthase, and vincristine sulfate disrupts microtubules. Cyclosporine is an immunosuppressant. Caffeine is known to inhibit ATM and ATR kinases (Sarkaria et al., 1999) and impair DNA-damage-mediated p53 induction (Kastan et al., 1991). Serine and retinoic acid were negative controls.

Foods—Among the tested items was celery extract, containing apigenin (Harnly et al., 2006). Apigenin, as with many flavonoids, is a known p53 activator (Cunningham et al.,

2004; Sohn et al., 2002). Also tested was a distillate of a peat smoke condensate (an Islay Scotch, Laphroaig); several teas: chamomile tea (Wegmans) known to contain apigenin, green tea (Twinings), black tea (Twinings Irish Breakfast), and Lapsang Souchong tea (Twinings) containing pine smoke condensates; coffees: regular and decaffeinated (purchased prepared), smokeless tobacco (Skoal Long Cut); and a non-nutritive food additive: a nitrosamines mix (Supelco).

Condiments/flavorings—Tested substances included Tabasco pepper sauce (McIlhenny), tamari soy sauce (Kikkoman), fish sauce (Polar), oyster sauce (Polar), kim chee (Jo San), black bean sauce (Kikkoman), soybean paste (Awase Miso), roaster seaweed sushinori (Nagai's), Japanese wasabi powder (Asian Gourmet), hickory smoke powder (Colorado Spice Co.), “smoke essence” powdered flavoring (Seasoning House), smoked paprikas (McCormick, Chiquilin), orange bitters (Angostura), aromatic bitters (Angostura, Peychauds), and 15 brands of liquid smoke (Wright's Hickory, Cedar House Hickory, Colgin Hickory, Colgin Mesquite, Haddon House Hickory, Long Horn Grill Mesquite, Lazy Kettles Hickory, Stubb's Hickory, Stubb's Mesquite, Heart-Loc Hickory, Figaro Mesquite, Regal Foods Texas Style, PS Seasoning and Spices Charsol Supreme, PS Seasoning and Spices Mesquite, and LEM Hickory).

Individual Flavonoids and Other Selected Food Constituents—Individual flavonoids and other selected food constituents reported in teas, coffee, and foods (Harnly et al., 2006; Ramos, 2008) were tested: apigenin (in chamomile tea), epigallocatechin-3-gallate (EGCG) (in green tea), quercetin (in black tea), genistin, genestein, glutamic acid, sulforaphane, cyanidin chloride, hesperetin, resveratrol, curcumin, p-coumaric acid, (+) α -tocopherol, and apiin (Fluka).

Individual Components of Liquid Smoke

Individual components of liquid smoke, reported multiply in literature (Fiddler et al., 1970; Fiddler et al., 1966; Guillen et al., 1995; Hruza et al., 1974; Issenber. P et al., 1971; Kim et al., 1974; Knowles et al., 1975; Lustre and Issenber. P, 1969; Meier, 2008; Ohshima et al., 1989a; Sternitzke et al., 1992), were tested as isolated chemicals. These included pyrogallol, 3-methoxy catechol, benzopyrene, acetaldehyde (supplied in ethanol), hydroquinone, p-benzoquinone, 2-methoxy-4-propyl phenol, 2-methoxy-4-vinyl phenol, eugenol, furfuryl alcohol, furfural, 5-methyl furfural, m-cresol, o-cresol, p-cresol, phenol, guaiacol, 2-methoxy-4-methyl phenol, 3, 5-dimethyl phenol, syringol (2, 6-dimethyl phenol), 2, 6-dimethoxy phenol, 4-methyl-2, 6-dimethoxy phenol, maltol, 3-methyl catechol, pyrocatechol, acetovanillone, 1,2-dimethoxy benzene, vanillin, 2-cyclopentenone, and hydroxyacetone.

Compounds Resembling Pyrogallol—To evaluate structure-activity relationships (SARs), pyrogallol-like compounds were tested: 1,2,4-benzenetriol, gallic acid, 2,3,4-trihydroxybenzoic acid, gallacetophenone, tannic acid, 3,4,5-trihydroxybenzaldehyde, phloroglucinol, resorcinol, 3-methoxyphenol, 1,2,3-cyclohexanetriol (Tokyo Chemical Industry), 3,4,5-trihydroxybenzamide, 5-methyl benzene 1,2,3-triol, and 3,4,5-trihydroxymethylbenzoate. Cupric sulfate pentahydrate (J. T. Baker) was also tested because of the reported ability of cupric ions to enhance the effects of pyrogallol (Hayakawa et al., 1997; Khan and Hadi, 1998; Stich et al., 1981).

2.3. p53R Assay

Water or medium was used as the initial diluent for each substance. Dimethyl sulfoxide (DMSO) was generally used when the compound was not water-soluble. Culture medium was used for subsequent dilutions. Substances with a high salt content such as soy sauce,

kim chee, and black bean sauce were tested over a dose range such that the final sodium concentration was maintained around or below 150 mM. For each treatment, cells were plated in triplicate and treated the following day with the chemical for 18 hours. A luciferase assay was performed (Promega Steady-Glo) using a PerkinElmer Microbeta Trilux plate reader. The mean for each triplet was plotted for each concentration tested.

2.4. Special Procedures for Liquid Smoke

To study the effect of desiccation on the ability of liquid smoke to evoke a p53 response, liquid smoke was vacuum-dried. To investigate the effect of filtration, liquid smoke was centrifuged at $500 \times g$ for 5 min and the supernatant passed through a $0.2 \mu\text{M}$ filter. To determine whether charcoal adsorption could eliminate the p53-activating property, liquid smoke was treated as described (Carter, 1978). Briefly, 420 mg of activated charcoal (Sigma-Aldrich) was added to 3 ml of undiluted or 1:10 diluted liquid smoke. The mixture was vortex-mixed 10 times for 1 s intervals, shaken overnight, and centrifuged for 20 min at $3000 \times g$. The supernatant was removed, centrifuged again for 20 min at $3000 \times g$, and the supernatant passed through a $0.2 \mu\text{M}$ filter. To adsorb liquid smoke with protein, liquid smoke (manufacturer's stock) diluted in 5% milk (from dry skim milk) was used to treat cells. To explore the effect of chloroform extraction, equal volumes of liquid smoke and chloroform were mixed by shaking vigorously and vortexing for 30 s each. The mixture was centrifuged at $3000 \times g$ for 1 min. The aqueous and chloroform fractions were each vacuum-dried. The aqueous residue was reconstituted in water, and the chloroform residue, in DMSO. To examine the effect of various heat-treatments on the p53-activating property, liquid smoke, pyrogallol solution, or 3-methoxycatechol solution was moderately heated at 80°C for 24 h, boiled at 100°C for 1 h, or slow-cooked at 225°F for 8 h on a heat block. The manufacturer's stock of liquid smoke, pyrogallol solution, or 3-methoxycatechol solution was baked in a conventional oven at 350°F for 1 h to simulate standard baking conditions. The tacky residue from cooking was restored to original volume by adding water.

2.5. Mixing Experiments

To investigate the behavior of complex mixtures in the p53R assay, several mixtures were prepared. The 1x mixture of the top eight most active chemical constituents was defined as containing 30 $\mu\text{g/ml}$ pyrogallol, 7.5 $\mu\text{g/ml}$ 3-methoxycatechol, 10 $\mu\text{g/ml}$ 1,2,4-benzenetriol, 10 $\mu\text{g/ml}$ gallic acid, 20 $\mu\text{g/ml}$ 2,3,4-trihydroxybenzoic acid, 20 $\mu\text{g/ml}$ gallacetophenone, 50 $\mu\text{g/ml}$ hydroquinone, and 50 $\mu\text{g/ml}$ p-benzoquinone; it was tested at 0.01x through 5x in the p53R assay. The 1x mixture of the top four most active chemicals was defined to contain 30 $\mu\text{g/ml}$ pyrogallol, 7.5 $\mu\text{g/ml}$ 3-methoxycatechol, 10 $\mu\text{g/ml}$ 1,2,4-benzenetriol, and 10 $\mu\text{g/ml}$ gallic acid; it was tested at 0.01x through 10x. The 1x mixture of pyrogallol and p-benzoquinone was defined to contain 30 $\mu\text{g/ml}$ pyrogallol and 50 $\mu\text{g/ml}$ p-benzoquinone at 1x; it was tested at 0.02x through 10x. The 1x mixture of Wright's Hickory and Figaro Mesquite was defined to contain 0.001x Wright's Hickory and 0.005x Figaro Mesquite at 1x; it was tested at 0.02x through 10x.

2.6. Immunoblot

Cells were lysed by rocking in a detergent (50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, and 1% v/v Triton X-100, and a protease inhibitor cocktail (Roche)) for 1 h at 4°C . The cell lysate was clarified by centrifugation. The protein concentration was determined by the DC protein assay (Bio-Rad). After adding a denaturant (2% m/v sodium dodecyl sulfate (SDS), 10% v/v glycerol, 0.002% m/v bromophenol blue, 2 mM EDTA, 50 mM Tris pH 6.8, and 1% v/v β -mercaptoethanol), samples were boiled and resolved on a 4–12% Bis-Tris gel (NuPAGE Invitrogen). After transfer to a polyvinylidene fluoride (PVDF) membrane (Pierce), blots were incubated with primary antibodies: p53 (Santa Cruz), p21 (Cell Signaling), γ -H2AX (Millipore), and GAPDH (Santa Cruz), followed by horseradish

peroxidase (HRP)-conjugated anti-mouse IgG secondary antibodies (Santa Cruz). Membranes were developed with the Immobilon substrate (Millipore), and signals recorded on film.

2.7. Neutral Comet Assay

p53R cells were treated with 0.0005x Wright's Hickory liquid smoke or 15 µg/ml pyrogallol for 30 min at 37°C. As a positive control, cells were also treated with 0.03% hydrogen peroxide for 30 min at 4°C. Cells were detached using 0.05% trypsin, diluted to 100,000 cells/ml, mixed with molten (37°C) 0.75% low melting agarose (1:10, v/v) and immediately layered onto pre-treated slides (Trevigen). Gels were incubated at 4°C in the dark for 30 min to adhere to the slides. Cells were then lysed in pre-chilled lysis buffer (2.5 M sodium chloride, 100 mM EDTA, 10 mM Tris, 1% m/v sodium lauroyl sarcosinate, and 1% v/v Triton X-100) overnight at 4°C. After a 15-min wash step in neutral TBE buffer, electrophoresis was performed in TBE at 23 V for 15 min at 4°C. Slides were then washed in water for 5 min, submerged in 70% ethanol for 5 min, and air-dried overnight. The slides were stained with SYBR Green (Molecular Probes) and imaged using a fluorescent microscope with a 10x objective and a Nikon Digital Eclipse DXM 1200 camera. The CometScore software was used to morphometrically integrate the tail moment of at least 45 randomly selected comets from each gel.

3. Results

The cytomegalovirus (CMV) promoter in CHO AA8-Luc Tet-Off cells is constitutively active, thus decrements reflected in the luciferase assay on these cells served as a read-out of toxicity due to chemical exposure. The p53 response (elevating the assay value) was superimposed on the toxicity of any given chemical exposure in the p53R assay. The typical dose-response relationship thus had a peak at a threshold value, beyond which cytotoxicity dominated. Positive controls for p53 activation are summarized in Table 1.

Among selected foods, flavorings, and constituents, the p53 activation assay gave strongly positive findings (Tables 2, 3, and 4). These included teas: chamomile tea (11X), green tea (21X), black tea (26X), and Lapsang Souchong tea (20X); coffees: regular (3 to 29X) and decaffeinated (8 to 23X); condiments/flavorings: all 15 brands of liquid smoke (4 to 28X, with a tendency for hickory products to test higher than mesquite.) and hickory smoke powder from Colorado Spice Co. having the aroma of liquid smoke (12X); celery extract (5X); and some flavonoids: apigenin (9X), EGCG (19X), quercetin (7X), and curcumin (12X). Black tea produced a strong p53 response irrespective of the method of extraction (boiling in water for 10 min versus extracting from a tea bag in hot water for 5 min). We tested different preparations of coffee from a local vendor to reflect conventional drinking practices and obtained different results from different brews. The values ranged from 3 to 29X for regular coffee and from 8 to 23X for decaffeinated coffee. We also found evidence for an aging effect: the activity diminished 10 to 15-fold with coffee storage over a one-week period. DNA-damaging activity was often seen at concentrations consumed dietarily; a 1:1000 dilution of liquid smoke, a 1:20 dilution of coffee, or a 1:5 dilution of brewed black tea produced responses similar to etoposide near 5 µg/ml.

We found weak to negative responses with smokeless tobacco boiled in water for 10 min (4X) or incubated at 37°C for 1 h in culture medium to possibly simulate oral mucosal wetting (1.8X). Some flavonoids and other constituents tested weak (numbers given) or less than 2X (values not given). These included genistin (2X), genestein (4X), glutamic acid, sulfuraphane, cyanidin chloride, hesperetin, resveratrol, p-Coumaric acid, (+) α-tocopherol, apiin, nitrosamines mix, Islay Scotch (under various conditions: desiccated and re-dissolved in water as well as directly diluted in water or medium), soy sauce, kim chee, black bean

sauce, orange and aromatic bitters, smoked paprika, and a “smoke essence” powdered flavoring not having the aroma of liquid smoke.

Due to surprisingly strong findings in teas and liquid smoke, further characterization was undertaken. We focused investigation on liquid smoke due to its known capacity to cause DNA damage in vivo, its expected paucity of p53-activating flavonoids, published lists of its constituents, and an expected analytic advantage from its extreme value in the p53R assay. The genotoxic activity in liquid smoke was confirmed using Wright’s Hickory and Cedar House Hickory by immunoblots detecting an increase in p53 protein level and γ -H2AX (Fig. 1A), indicating DNA double-strand breaks in p53R cells. H2AX phosphorylation was observed also in AAV-293 cells (Fig. 1B). Similar results were obtained after treatment with black tea (Fig. 1B). The p53 protein level was disregarded in AAV-293 cells, because the endogenous level is known to be high in these cells (Yin et al., 2011). With liquid smoke treatment, the level of γ -H2AX increased over time, as did p21, a transcriptional target of p53 (Fig. 1C). DNA double-strand breaks in p53R cells were also detected by the neutral comet assay (Fig. 2).

The p53-activating property in liquid smoke (Wright’s Hickory and Cedar House Hickory) was eliminated by standard baking conditions (350°F for 1 h). The p53-activating property in liquid smoke could not be eliminated, however, by different fractionation techniques including filtration, adsorption on charcoal or protein, chloroform extraction (the activity was retained in the aqueous fraction), desiccation (with or without alcohol), moderate heating (80°C for 24 hours), boiling (100°C for 1 hour), and slow cooking (225°F for 8 hours).

As we tested multiply reported individual components of liquid smoke in the p53R assay (Table 4), we encountered strong activity from pyrogallol (30X) and 3-methoxycatechol (25X) (Fig. 3). An additional 28 compounds known to constitute liquid smoke were negative (Table 4).

The possibility of pyrogallol being auto-luminescent was excluded by performing the luciferase assay in the absence of substrate and in the absence of cells. Concerns about non-specificity were addressed by treating CHO AA8-Luc Tet-Off cells with pyrogallol. Pyrogallol did not elevate luciferase gene expression in CHO AA8-Luc Tet-Off cells. The activity of pyrogallol was substantially reduced by standard baking conditions (350°F for 1 h), but was not eliminated by moderate heating (80°C for 24 h), boiling (100°C for 1 h), or slow cooking (225°F for 8 h). The neutral comet assay also confirmed the presence of DNA double-strand breaks after treatment of p53R cells with pyrogallol (Fig. 2). In contrast to pyrogallol, the strong activity of 3-methoxycatechol (25X) in the p53R assay was eliminated by moderate heating (80°C for 24 h), boiling (100°C for 1 h), slow cooking (225°F for 8 h) or standard baking (350°F for 1 h).

Because luciferase provides an integration of the p53 activity over time, and because p53 has oscillatory behavior (Geva-Zatorsky et al., 2006), we confirmed the p53 response to pyrogallol in p53R cells at 6, 12, and 20 h by immunoblotting for p53, p21, and γ -H2AX in a time-course experiment (Fig. 1C). Pyrogallol induced γ -H2AX in a dose- and time-dependent manner. 5 μ g/ml pyrogallol did not alter p21, but higher doses of pyrogallol activated p21 expression in a time-dependent manner. The time-course of p53 stabilization was complex. 5 μ g/ml pyrogallol gave a peak p53 protein response at 6 h. 15 μ g/ml pyrogallol gave a peak response at 12 h in a manner characteristic of DNA-damaging agents. At 30 μ g/ml, pyrogallol gave a muted response at the tested time-points. 2 μ g/ml quinacrine produced a time-dependent p53 response which was not associated with γ -H2AX induction.

This was not surprising because quinacrine is reported in some cells to activate p53 without causing significant genotoxicity (Gasparian et al., 2011; Wang et al., 2005).

Previous studies suggested that cupric ions could augment the DNA-damaging effects of pyrogallol in vitro (Hayakawa et al., 1997; Stich et al., 1981). Copper is present in the chromatin, and chemical agents can mobilize endogenous copper to induce DNA fragmentation (Burkitt et al., 1996). We treated cells with 68 μM Cu^{2+} or 272 μM Cu^{2+} in the presence of increasing concentrations of pyrogallol. Cupric ions did not amplify the activity of pyrogallol here.

We explored the behavior of complex mixtures in the p53R assay. A mixture of the top eight most active components gave 27X activity, whereas a mixture of the top four components gave 18X activity. A mixture of Wright's Hickory (strong activity) and Figaro Mesquite (weak activity) diluted the stronger activity as expected for additive interactions. A mixture of pyrogallol (strong activity) and p-benzoquinone (weak activity) also diluted the p53 response as expected. Thus, we did not find clear evidence for or against synergy (a greater-than-additive effect) among these compounds from this preliminary qualitative exploration. Additional quantitative explorations of mixed components might reveal interactions of interest.

In an effort to identify the essential structural groups (the pharmacophore) accounting for the DNA-damaging activity in pyrogallol and pyrogallol-related compounds, we screened a number of related compounds and observed the following activities in the p53R assay (Table 4 and Fig. 3): 1,2,4-benzenetriol (21X), gallic acid (21X), 2,3,4-trihydroxybenzoic acid (7.3X), gallacetophenone (3.8X), 3,4,5-trihydroxybenzamide (3.7X), tannic acid (1.4X), 3-methoxyphenol (1.3X), 3,4,5-trihydroxymethylbenzoate (1.2X), 1,2,3-cyclohexanetriol (1.1X), 3,4,5-trihydroxybenzaldehyde monohydrate (1.1X), 5-methylbenzene 1,2,3-triol (1.1X), phloroglucinol (1X), and resorcinol (1X). We concluded that the three adjacent hydroxyl groups on a benzene ring were necessary but not sufficient for strong activity among this structural family, for additional substitution on the benzene ring could impair the activity.

4. Discussion

p53-based assays emerged with the discovery of the DNA sequence bound by p53 (Kern, 1991; Kern et al., 1991) and the discovery of p53 activation by DNA strand breaks (Kastan et al., 1991). There is a long history of using p53 for qualitative assays, but few authors have adapted p53-responsive assays as quantitative screening tools (Cunningham et al., 2004; Gasparian et al., 2011; Gurova et al., 2005; Sohn et al., 2002; Wang and El-Deiry, 2003). When testing dose-response relationships, chemicals inducing DNA strand breaks produce a peak reporter activity centered at one concentration. We assume that most of the measured effects follow the predictions of Loewe additivity (Berenbaum, 1978). Effects are truncated at higher concentrations when overtaken by cellular toxicity, which reduces the ability of the reporting cells to respond or survive in culture (Cunningham et al., 2004). This theory is an imprecise simplification, as shown by the non-linearity of some dose-response curves.

It is likely that, even when using isolated chemicals, multiple mechanisms of action and of toxicity are engaged during the exposure. Threshold effects can occur, as when compensating mechanisms are overwhelmed and other forms of cellular toxicity further impair any compensating mechanism. High amounts of nucleotide excision repair would cause interactions between DNA regions undergoing repair, leading to double-strand breaks in DNA, even when cells are exposed to chemicals that do not directly induce strand breaks. Based on the simplest assumptions, the peak p53R activity, measured in a given assay setting, is expected to be a value innate to a tested chemical, being independent of the

concentration of the starting material. Active DNA-damaging chemicals, when tested in the presence of simple toxins having no particular DNA-damaging ability, could yield values lower than the peak activity of the same chemical tested alone, but would not be expected to yield a higher value.

When tested in the presence of other DNA-damaging agents, the resulting mixture's peak activity could reflect at most the peak activity of the most potent DNA-damaging agent, in the absence of any synergistic interaction. Since different DNA-damaging agents are unlikely to have the same toxicity threshold, synergistic interactions are difficult to evaluate in a complex mixture of such chemical agents and for the sake of simplicity are not considered here. These assumptions, to the extent that they hold true, permit an analytic fractionation of a complex mixture to identify at least some of the compound(s) responsible for the peak activity. The responsible compound(s) should, when tested alone, have a peak activity at least as high as did the mixture. When the mixture has a very high peak activity, the number of suitable candidate compounds will be small. In a mixture containing multiple toxic substances, the compound(s) responsible for the peak activity should be present in relatively higher concentration(s). Otherwise, the mixture's peak activity would be truncated prematurely in a dose-escalation experiment owing to the toxicity from these other, assay-negative constituents. The peak activity would be variable when tested in different settings, such as when using different measures of p53 activation (immunoblots of p53 protein, p53 mRNA transcripts, genes downstream of p53 in signal transduction of the DNA-damage response), different cell lines, and conditions preventing/stimulating drug uptake or preventing/augmenting cytotoxicity. To some extent, it is thus valuable to maintain the test situation constant when comparing the tested substances. The peak activity is expected to decrease when testing active chemicals diluted adequately with inactive chemicals.

Constituents of teas and coffees with relevant activities for a p53-based screen for clastogens include flavonoids, tannins, and pyrogallol/gallic acid subunits. Flavonoids are known to activate p53 in the p53R assay (Sohn et al., 2002). Many are reported to be topoisomerase inhibitors (Bandeled and Osherooff, 2008; Birt et al., 2001; Lopez-Lazaro et al., 2011; Lopez-Lazaro et al., 2007a; Lopez-Lazaro et al., 2007b; Markovits et al., 1989; Strick et al., 2000). Tannins and pyrogallol/gallic acid subunits are present in substantial amounts in teas and coffees (Chaturvedula and Prakash, 2011; Hussain et al., 2008; Kanwal et al., 2009; Lang et al., 2006; Muller et al., 2006). Gallic acid and EGCG are topoisomerase inhibitors reportedly mediated by the pyrogallol moiety (Lopez-Lazaro et al., 2011). Pyrogallol-like compounds in acellular and cellular conditions are known to damage DNA by activated oxygen species (Hayakawa et al., 1997; Stich et al., 1981).

From our findings and using some allowance for imprecision, we presume that apigenin ($\approx 9X$) might fully explain the activity in chamomile tea ($\approx 11X$) and in celery extract ($\approx 5X$). Aqueous standardized chamomile extract contains approximately 1.2% apigenin (Srivastava and Gupta, 2007). Celery contains 1.3 mg of apigenin per 100 g of fresh weight (Harnly et al., 2006). The future use of quantitative methods would provide more certainty in these rough attributions. Further, EGCG (19X) could largely explain the activity in green tea (21X). However, quercetin (7X) could not fully explain the activity in black tea (26X). EGCG is present in green tea but not in black tea because during black tea production, the catechins are converted to theaflavins and thearubigins (Lorenz et al., 2009). Nevertheless, black tea contains large amounts of pyrogallol and gallic acid among other complex products (Hussain et al., 2008; Kanwal et al., 2009).

Health concerns about the carcinogenic potential of liquid smoke have not been comprehensively addressed; inconclusive to weak results were obtained from the Ames test (Braun et al., 1987; Putnam et al., 1999). We found, as with teas and coffee, strong activities

causing cellular DNA-damage-responses after treatment with liquid smoke. Pyrogallol is a major constituent of liquid smoke (Issenber.P et al., 1971; Kim et al., 1974; Knowles et al., 1975; Meier, 2008; Ohshima et al., 1989a; Sternitzke et al., 1992). Pyrogallol and liquid smoke share an unusual strength of p53R activity, similar chemical stabilities upon heating, and other similar chemical properties.

Pyrogallol and flavonoid toxicities are closely related. Many flavonoids contain a catechol or pyrogallol moiety; EGCG contains both a gallic acid and a pyrogallol moiety. A structure-activity analysis of polyphenols found that flavonoids containing a pyrogallol moiety had the most cytotoxic activity, causing apoptosis (Mitsuhashi et al., 2008; Saeki et al., 2000). In our SAR study, pyrogallol-like compounds had high p53R activities dependent on specific orientations of the three hydroxyl groups on the benzene ring. They had generally reduced activity when further modified by side-groups, methylation, or polymerization. For example, pyrogallol (30X) was more potent than 3-methoxycatechol (25X) or gallic acid (21X), whereas tannic acid (1.4X) and syringol (1.2X) were both very weak. A similar trend was observed in an in vitro DNA-cleavage assay (Khan and Hadi, 1998). Likewise, the clastogenic activity of di- and tri-hydroxylated phenolics in CHO cells was also reduced by their methylation (Stich et al., 1981). Pyrogallol and tannin subunits thus suggest unifying structural features of the DNA-damaging activities in foods and flavorings. Pyrogallol and related structures are likely responsible for the strong activity in liquid smoke and perhaps some teas and coffee.

Our results are derived from a particular cell line, RKO colorectal cancer cells, the parental line for p53R cells. p53R assays reflect a chemical's intracellular accumulation and metabolism, the cellular repair functions subsequent to a chemical exposure, and any influences of the culture medium. It is interesting to note that our measured p53R activities of individual compounds do not closely match the relative levels of DNA damage measured in earlier assays produced in vitro by similar chemical panels (Hayakawa et al., 1997) or produced in vivo using the CHO cell clastogenic assay measuring visible chromosomal aberrations (Stich et al., 1981). The p53R assay might be more sensitive than these earlier assays.

Pyrogallol is a trihydroxylated phenol, all hydroxyl groups being adjacent. It is obtained by the heating (pyrolysis) of gallic acid, which is a building block for some flavonoids (such as EGCG) and for tannins. Pyrogallol is highly water-soluble, but weakly soluble in chloroform, similar to the fractionation pattern observed for the p53R-activating property of liquid smoke. It boils at a much higher temperature (309°C) than water and would not enter conventional distillates even when other smoky flavorings would, as in the distillation of Scotch. Pyrogallol is toxic to most vital organs and known to generate free radicals in cells, among other biological interactions (NIEHS, 1998; NTP, 2012). It is ingested from smoked foods, smoke condensates in food, cigarette smoke, and some ground waters (Knowles et al., 1975; Ohshima et al., 1989a). Among many uses, it is a topical anti-psoriatic medication, a component of hair dyes, and used as a photographic developer (NTP, 2012). Pyrogallol is present in tea, coffee, cigarette smoke, bread crust, roasted malt, and cocoa powder (Hussain et al., 2008; Kanwal et al., 2009; Lang et al., 2006; NTP, 2012). Pyrogallol is efficiently formed during coffee roasting from carbohydrates and amino acids (the Maillard reaction) and from quinic acid moieties (Muller et al., 2006).

High quantities of pyrogallol and similar compounds exist in substances found by us to have high activities (liquid smoke, teas, and coffee) in the p53R assay. Pyrogallol is absorbed from oral ingestion and metabolized to 2-*O*-methylpyrogallol with 6% detected in urine as such (Bakke, 1970; NTP, 2012). It can be formed from gallic acid and related polyphenols by intestinal bacteria (Daykin et al., 2005; Meselhy et al., 1997; Schantz et al., 2010; Soni,

2012; Yoshida and Yamada, 1985). Brewed black tea was reported to contain 17 nM gallic acid (Kanwal et al., 2009). Brewed green tea contained 35 nM pyrogallol and 73 nM gallic acid (Kanwal et al., 2009). Brewed coffee contained 75 nM pyrogallol (Kanwal et al., 2009). Dried green tea leaves contained pyrogallol at the concentration of 133–418 mg/g and gallic acid at 1–13 mg/g (Hussain et al., 2008). In dried black tea leaves, the concentration of pyrogallol was 60–171 mg/g, and that of gallic acid, 7–17 mg/g (Hussain et al., 2008).

DNA-breakage by phenolic compounds, including trihydroxyphenols and flavonoids, is widely reported, although the literature emphasized in vitro studies of purified reaction components and the generation of reactive oxygen species (Kawanishi et al., 1989; Kim et al., 2008; Lee et al., 1995; Said Ahmad et al., 1992; Yamada et al., 1985). Pyrogallol is also reported to induce mutations in some strains of *Salmonella* (NTP, 2012), to induce chromosomal aberrations in CHO cells (Stich et al., 1981), cultured human lymphocytes (NIEHS, 1998), and bone marrow cells in vivo in mice (NIEHS, 1998), to induce micronuclei and sister chromatid exchanges in V79 cells (Glatt et al., 1989), to induce mutations in L5178Y mouse lymphoma cells (NIEHS, 1998), and to increase the frequency of micronucleated polychromatic erythrocytes in mice (Gocke et al., 1981). Its clastogenic potential is thought to follow an order wherein trihydroxylated phenols are more active than dihydroxylated, dihydroxylated more than monohydroxylated phenols (Stich et al., 1981). Pyrogallol does not require a metal ion catalyst to produce $\cdot\text{OH}$ radicals in vitro. It is a more potent generator of hydrogen peroxide in vitro than other polyphenols (pyrogallol > 1,2,4-benzenetriol > hydroquinone > catechol) (Lee and Lin, 1994; NTP, 2012). Pyrogallol-like compounds are reported to stimulate neoplasia and other hyperproliferative lesions. 3-methoxycatechol in the drinking water induced little gastric hyperplasia, but greatly enhanced hyperplasia and papilloma formation when sodium nitrite was co-administered with 3-methoxycatechol (Hirose et al., 1990). 3-methoxycatechol also promoted esophageal carcinogenesis and fore-stomach carcinogenesis, the latter being augmented with sodium nitrite co-administration (Hirose et al., 1993). Pyrogallol and related structures thus may be a major DNA-damaging entity in the diet and upon smoke inhalation. Cancer and other diseases of aging are often seen as a product of our modern industrial age (Dunn, 2012). Dietary changes have included higher consumption of such beverages as tea and coffee, resulting in enhanced exposure to pyrogallol-like clastogenic substances. Speculatively, population differences in any resulting disease risks could reflect differences in diet, gut bacterial flora, and bowel transit time. Specific areas of interest, such as the possible protective effects of individual foods such as green tea, are not addressed directly by our study. The properties detected by p53R cells were more widely distributed among foods and do not take into account any genoprotective effects or tissue differences. Our acute exposure studies also do not directly address the clinical situation of chronic exposure nor of exposures in non-gastrointestinal tissues, where blood-borne chemicals would be at low concentrations.

Our findings have practical implications. Future studies should assess whether pyrogallol and related compounds have DNA-damaging properties in the concentrations and forms typically encountered by humans and investigate the physiology used by the gastrointestinal mucosa and other cells to limit DNA damage from pyrogallol-like activities. One wonders whether there are molecular signatures of exposure in tissues, especially in terms of mutation fixation and any effect on human tumorigenesis. It is feasible to survey whether other major food substances contain similar DNA-damaging activities. For example, one could examine naturally smoked foods for similar DNA-damaging activity. It will be helpful to determine whether the variation in p53R assay results among otherwise similar foods (such as different brands) can be explained using quantitative analysis of the candidate chemicals implicated here. If the DNA-damaging activities of liquid smoke were thought to

be deleterious, it might be possible to replace liquid smoke with other, safer, smoky substances. One might identify constituents of liquid smoke missed in earlier studies due to choice of solvents. It might also be advisable to measure the intake of pyrogallol-like substances as well as their levels in urine and serum samples in epidemiologic studies. Methods have been developed to monitor ingested exposure by measuring the amount of pyrogallol or its conjugates in urine and serum samples, and have found application as a diagnostic marker for oak toxicosis in cattle (Bakke, 1970; NTP, 2012; Tor et al., 1996).

Together with our earlier studies (Cunningham et al., 2004; Gallmeier et al., 2005; Sohn et al., 2002), especially our use of the p53R assay quantitatively on tens of thousands of compounds (Sohn et al., 2002), this report presents future directions for genotoxicity screening and further establishes the utility and informativeness of biological assays as a screening method for environmental exposures. The ToxCast Program of the U.S. Environmental Protection Agency also uses cell lines incorporating multiple potential signals. This strategy was employed semi-quantitatively and qualitatively on about three hundred compounds (Judson et al., 2010; Knight et al., 2009) and is currently being used to analyze compounds to prioritize them for further testing under the Endocrine Disruption Screening Program (Mahadevan et al., 2011). Future cellular toxicological screens of foods and flavorings could be augmented with chemical characterization, fractionation, and testing of constituent chemicals to define the major sources of DNA strand-breaking activity in food and flavoring. Pyrogallol-related compounds are likely to dominate the assay results in foods and flavorings containing them, with flavonoids providing widespread but less intense activities. Such a knowledge base may prove useful in guiding studies of epidemiologic risks of age-related diseases and cancer that would address DNA strand-breaking chemicals in diets.

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Abbreviations

γ-H2AX	phosphorylated histone protein H2AX
5-FU	fluorouracil
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
CHO	Chinese hamster ovary
CMV	cytomegalovirus
DMEM	<u>Dulbecco's</u> modified Eagle's medium
DMSO	dimethyl sulfoxide
EGCG	epigallocatechin-3-gallate
FBS	fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PVDF	polyvinylidene fluoride
SAR	structure-activity relationship

SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TBE	Tris/Borate/EDTA
TSA	trichostatin A

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Highlights

We used a cellular biological (p53R) assay to screen for DNA-damaging activity in selected foods and flavorings.

Potent DNA-damaging activity was seen in tea, coffee, and liquid smoke at concentrations consumed dietarily.

DNA double-strand breaks were confirmed by the comet assay and immunoblots reporting H2AX phosphorylation.

Pyrogallol-like substances are likely to be a major source of genotoxic activity in food and flavoring.

These findings have implications for evaluation of dietary risks in epidemiologic studies and in designing safer diets.

Fig 1. p53 and γ -H2AX protein levels after treatment with liquid smoke, black tea, and pyrogallol. (A) p53R cells were treated with Wright's Hickory liquid smoke (Ls W) or Cedar House Hickory liquid smoke (Ls C) at 0.0005x and 0.001x for 18 h. Untreated cells (U) and 2 μ g/ml quinacrine-treated cells (Q) served as a negative and a positive control for p53 induction respectively. Cellular p53 and γ -H2AX protein levels were evaluated by immunoblotting. (B) AAV-293 cells were treated with black tea (Tea B) at 0.05x and 0.2x or liquid smoke solutions (Ls W or Ls C) at 0.0005x and 0.001x for 18 h. Cellular γ -H2AX protein levels were assayed by immunoblotting. (C) p53R cells were treated with 0.001x Ls W, various concentrations of pyrogallol, or 2 μ g/ml quinacrine for 6, 12, or 20 h. Cellular p53, p21, and γ -H2AX levels were assessed by immunoblotting.

Fig 2. Neutral comet assay after treatment with liquid smoke and pyrogallol. The neutral comet assay was performed on p53R cells treated with 0.0005x Wright's Hickory liquid smoke (Ls W) or 15 $\mu\text{g/ml}$ pyrogallol. At least 45 cells were analyzed per sample and the mean tail moment of the cell population was calculated using CometScore. (A) Representative pictures of cells. (B) Mean tail moment for each treatment with error bars representing the standard error of the mean (SEM) for the cell population.

A

B

Fig 3. p53 reporter activation after treatment with (A) pyrogallol, (B) 3-methoxycatechol, (C) gallic acid, and (D) 1,2,4-benzenetriol. p53R cells were plated in triplicate and treated with various doses of each compound for 18 h. p53 reporter activity was assessed in a luciferase assay. Luminescence was calculated relative to the untreated sample. The mean for each triplet was plotted for each concentration tested.

Table 1

Reference Chemicals

Compound ^a	Maximal response (fold increase) ^b	Concentration at maximal response
DNA intercalator/topo. inh.		
Quinacrine	51	5 μ M (2 μ g/ml)
Topoisomerase inhibitor		
Etoposide	33	10 μ M (5.9 μ g/ml)
Antimetabolite		
Aminopterin	6.4	320 μ M (140 μ g/ml)
5-fluorouracil	4.8	100 μ M (13 μ g/ml)
Antimicrotubule agent		
Vincristine sulfate	3.0	7.7 μ M (7.1 μ g/ml)
Metabolite		
Retinoic acid	1.9	100 μ M (30 μ g/ml)
Amino acid		
Serine	1.3	3.4 mM (360 μ g/ml)
ATM/ATR inhibitor		
Caffeine	1.1	3.6 mM (17 mg/ml)
Antimetabolite		
Methotrexate	1.1	20 nM (9.1 ng/ml)
Immunosuppressant		
Cyclosporin	1.0	20 nM (24.1 ng/ml)

^aCompounds were initially dissolved in DMSO except caffeine and serine, which were dissolved in culture medium. Quinacrine has multiple functions attributed.

^bRelative luciferase activity of p53R cells relative to untreated cells, tested over a wide dose range.

Table 2

Foods, flavorings

Substance ^a	Maximal response (fold increase)	Concentration at maximal response
Food/Beverage		
Coffee		
Regular	29	0.05x ^b
Decaffeinated	23	0.05x ^b
Teas		
Chamomile tea	11	1x ^c
Green tea	21	0.2x ^c
Black tea	26	0.2x ^c
Lapsang Souchong tea	20	0.02x ^c
Smokeless tobacco		
Boiling water 10 min	3.7	0.5x ^d
37°C culture medium 1h	1.8	0.5x ^e
Celery extract	5.2	0.7x ^c
Islay Scotch: Laphroaig	1.0	0.1x ^f
Condiment/flavoring		
Fish sauce	5.9	0.02x ^f
Oyster sauce	4.6	0.05x ^f
Soy sauce	1.1	0.005x ^f
Black bean sauce	1.1	0.001x ^f
Tabasco sauce	1.0	0.002x ^f
Soybean paste	2.2	0.005x ^f
Roasted seaweed	2.4	0.1x ^c
Kim Chee	1.0	0.002x ^f
Wasabi powder	2.4	200 µg/ml
Hickory smoke powder (Colorado Spice Co.)	12	500 µg/ml
Smoke "essence" (Seasoning House)	1.9	5 mg/ml
Smoked Paprika		
McCormick	1.0	200 µg/ml
Chiquilin	1.1	1 µg/ml
Bitters		
Aromatic		
Angostura	1.4	1 mg/ml
Peychaud's	1.2	100 ng/ml
Orange		
Angostura	1.3	1x ^g

^aSubstances were initially extracted or diluted in water, except for chamomile flowers (extracted in methanol), smoke essence and paprika (extracted in DMSO), and aromatic bitters (after desiccation, the tacky residue was weighed and reconstituted in water).

^b16 ounces of coffee beans were brewed in 180 ounces of water. The solution was vacuum-dried until around 90% of water evaporated. The resulting concentrated stock solution was arbitrarily designated 10x. The peak response varied between samples, ranging from 3 to 29X for regular and 8 to 23X for decaffeinated coffee.

^c2 g of substance was extracted in 50 ml of solvent by boiling for 10 minutes. The solution was filtered and vacuum-dried until around 90% of original volume evaporated. The resulting concentrated stock solution was arbitrarily designated 10x.

^d1 g of substance was extracted in 25 ml of water by boiling for 10 minutes. The solution was filtered and vacuum-dried until around 90% of original volume evaporated. The resulting concentrated stock solution was arbitrarily designated 10x.

^e1 g of substance was extracted in 25 ml of culture medium by incubating at 37°C for 1 hour. The solution was filtered and vacuum-dried until around 90% of original volume evaporated. The resulting concentrated stock solution was arbitrarily designated 10x.

^fManufacturer's product was considered 1x. Scotch was toxic above 10% (4% alcohol by volume), but was tested negative after dessication and reconstitution in water

^gManufacturer's product was vacuum-dried until all of water and ethanol evaporated to its glycerin-rich residue. The resulting concentrate was arbitrarily designated 100x.

Table 3**Brands of liquid smoke**

Brand	Ingredients^a	Max. response (fold increase)	Concentration at maximal response^b
Wright's Hickory	W Ls	28	0.001x
Haddon House Hickory	W Ls P	24	0.001x
Lazy Kettles Brand Hickory	Ls	24	0.0005x
Colgin Hickory	W Ls V Ml C Sa	21	0.003x
PS Seas. & Spices Charsol Supreme	L A	20	0.0002x
Cedar House Hickory	W Ls Ms	17	0.001x
Heart-Loc Hickory	W Ls Ml C V Su	15	0.002x
Regal Foods Texas Style	W Ls P	14	0.001x
LEM Hickory	Ls P	10	0.0002x
Colgin Mesquite	W Ms V Ml C Sp	9	0.01x
Stubb's Hickory	W Ss Ls V Hf C Sp	6	0.05x
Figaro Mesquite	W Ms V Ss Su C Sp	4	0.005x
Long Horn Grill Mesquite	W Ms V Su H	4	0.01x
PS Seasoning and Spices Mesquite	Ms P D	4	0.001x
Stubb's Mesquite	W Ss V Hf Ms C Sp	4	0.002x

^aIngredients are given in order stated on the product label. W, water. Ls, liquid smoke or hickory smoke, or smoke flavor, or smoke concentrate or condensate. Ms, mesquite smoke or smoke flavor, smoke concentrate, or condensate. V, vinegar. Ss, soy sauce or soy protein. Ml, molasses. Su, sugar. Ho, honey. P, polysorbate 80. D, dimethylpolysiloxane. Hf, high-fructose corn syrup. Sp, spices or flavoring. C, coloring agents. Su, sulfating agents. Sa, salt. A, acetic acid.

^bManufacturer's product was considered 1x.

Table 4

Chemical constituents of foods and flavorings

Chemical ^a	Maximal response (fold increase)	Concentration at maximal response ^b
Flavonoids, other		
EGCG	19	100 μ M
Curcumin	12	27 μ M
Apigenin	8.7	260 μ M
Quercetin	7.0	250 μ M
Genestein	4.1	100 μ M
Genistin	2.2	100 μ M
Hesperetin	1.8	100 μ M
Sulforaphane	1.6	10 μ M
Resveratrol	1.6	10 μ M
Cyanidin chloride	1.4	100 μ M
Glutamic acid	1.3	6.8 μ M
(+) α -tocopherol	1.1	100 μ M
Apiin	1.1	100 μ M
Nitrosamines mix	1.1	2 μ g/ml each component
p-Coumaric acid	1.0	1 μ M
Reported in liquid smoke in multiple publications		
Pyrogallol	30	15 μ g/ml
3-methoxycatechol	25	7.5 μ g/ml
Hydroquinone	3.2	50 μ g/ml
p-benzoquinone	3.1	50 μ g/ml
Eugenol	2.3	0.0002x
2-methoxy-4-propyl phenol	2.2	0.00005x
2-methoxy-4-vinyl phenol	2.2	0.00001x
Benzopyrene	2.1	5 μ g/ml
p-cresol	1.9	100 μ g/ml
2-methoxy-4-methylphenol	1.8	0.0001x
Acetaldehyde	1.6	1 mM
2,6-dimethylphenol	1.5	50 μ g/ml
3,5-dimethylphenol	1.5	50 μ g/ml
1,2-dimethoxybenzene	1.3	0.0005x
2-cyclopentenone	1.3	0.00001x
o-cresol	1.3	0.000001x
4-methyl-2,6-dimethoxyphenol	1.2	10 μ g/ml
Furfural	1.2	0.0000002x
Furfuryl alcohol	1.2	0.00001x
Guaiacol	1.2	200 μ g/ml
Syringol	1.2	100 ng/ml
3-methylcatechol	1.1	5 μ g/ml

Chemical ^a	Maximal response (fold increase)	Concentration at maximal response ^b
5-methylfurfural	1.1	0.0002x
Acetovanillone	1.1	100 ng/ml
Maltol	1.1	100 ng/ml
m-cresol	1.1	0.0001x
Hydroxyacetone	1.0	0.00001x
Phenol	1.0	100 µg/ml
Pyrocatechol	1.0	100 ng/ml
Vanillin	1.0	10 µg/ml
Similar to pyrogallol^c		
1,2,4-benzenetriol	21	10 µg/ml
Gallic acid	21	10 µg/ml
2,3,4-trihydroxybenzoic acid	7.3	20 µg/ml
Gallacetophenone	3.8	20 µg/ml
3,4,5-trihydroxybenzamide	3.7	100 µg/ml
Tannic acid	1.4	100 ng/ml
3-methoxyphenol	1.3	0.000001x
3,4,5-trihydroxymethylbenzoate	1.2	500 ng/ml
1,2,3-cyclohexanetriol	1.1	1 µg/ml
5-methylbenzene 1,2,3-triol	1.1	2 µg/ml
3,4,5-trihydroxybenzaldehyde	1.1	100 ng/ml
Phloroglucinol	1.0	500 ng/ml
Resorcinol	1.0	200 ng/ml

^a Dry chemicals were initially dissolved in DMSO, except for glutamic acid, hydroquinone, p-benzoquinone, p-cresol, guaiacol, and phenol (in water or culture medium), nitrosamines mix (in methanol), and acetaldehyde (supplied in ethanol).

^b Dilutions of a liquid chemical are given as fractions of the pure compound, which was considered 1x.

^c Not a reported constituent or not multiply reported.