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Mechanism-based in vitro screening of potential cancer chemopreventive agents

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Abstract

Identification and use of effective cancer chemopreventive agents have become an important issue in public health-related research. For identification of potential cancer chemopreventive constituents we have set up a battery of cell- and enzyme-based in vitro marker systems relevant for prevention of carcinogenesis in vivo. These systems include modulation of drug metabolism (inhibition of Cyp1A activity, induction of NAD(P)H:quinone reductase (QR) activity in Hepa1c1c7 murine hepatoma cell culture), determination of radical scavenging (DPPH scavenging) and antioxidant effects (scavenging of superoxide anion-, hydroxyl- and peroxyl-radicals), anti-inflammatory mechanisms (inhibition of lipopolysaccharide (LPS)-mediated nitric oxide (NO) generation by inducible NO synthase (iNOS) in Raw 264.7 murine macrophages, cyclooxygenase-1 (Cox-1) inhibition), and anti-tumor promoting activities (inhibition of phorbol ester-induced ornithine decarboxylase (ODC) activity in 308 murine keratinocytes). We have tested a series of known chemopreventive substances belonging to several structural classes as reference compounds for the identification of novel chemopreventive agents or mechanisms. These include organosulfur compounds (phenethylisothiocyanate (PEITC), diallylsulfide, diallyldisulfide), terpenes (limonene, perillyl alcohol, oleanolic acid, 18-β-glycyrrhetinic acid), short-chain fatty acids (sodium butyrate), indoles (indole-3-carbinol), isoflavonoids (quercetin, silymarin, genistein), catechins ((−)-epigallocatechin gallate (EGCG)), simple phenols (ellagic acid, resveratrol, piceatannol, curcumin), pharmaceutical agents (piroxicam, acetylsalicylic acid, tamoxifen), and vitamins/derivatives (ascorbic acid, Trolox). We confirmed known chemopreventive mechanisms of these compounds. Additionally, we could demonstrate the usefulness of our approach by identification of hitherto unknown mechanisms of selected agents. As an example, we detected anti-inflammatory properties of PEITC, based on NF-κB-mediated inhibition of NO production. Further, PEITC inhibited phorbol ester-induced superoxide anion radical production in granulocytes, and ODC induction in the 308 cell line. These mechanisms might contribute to the chemopreventive potential of PEITC.

Keywords: Cancer chemoprevention; Bioassay systems; Mechanisms; PEITC; NF-κB; iNOS

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1. Introduction

Advances in our understanding of the carcinogenic process at the cellular and molecular level made over the past few decades have led to the development of a promising new approach to cancer prevention, termed "chemoprevention" [1]. Chemoprevention aims to halt or reverse the development and progression of pre-cancerous cells through use of non-cytotoxic nutrients and/or pharmacological agents during the time period between tumor initiation and malignancy [2]. Carcinogenesis can be regarded as an accumulation of genetic or biochemical cell damage which offers possibilities to interrupt this process at various steps during the initiation, promotion or progression stage [3,4].

Modulation of enzymes involved in metabolic activation and excretion of carcinogens is one of the best investigated mechanisms of chemopreventive agents [5]. Phase 1 enzymes (cytochromes P450) activate xenobiotics by addition of functional groups which render these compounds more water-soluble. Although phase 1 functionalization might be required for complete detoxification, induction of phase 1 enzymes might increase the risk to produce ultimate carcinogens capable of reacting with DNA and initiating carcinogenesis. Phase 2 enzymes conjugate the activated compounds to endogenous ligands like glutathione (GSH), glucuronic-, acetic-, or sulfuric acid, thus enhancing their excretion in form of these conjugates. Generally, inhibition of phase 1 enzymes concomitantly with induction of phase 2 enzymes is considered a logical strategy in chemoprevention, which is especially beneficial in early stages of carcinogenesis. Consequently, for the identification of modulators of carcinogen metabolism, we have selected inhibition of cytochrome P450 1A (Cyp1A) activity as a representative for carcinogen-activating enzymes, and NAD(P)H:quinone reductase (QR) activity as a model for phase 2 enzyme induction. Beside reactive carcinogen metabolites, reactive oxygen species (ROS) play an important role in tumor initiation. In a healthy organism ROS levels are controlled by endogenous mechanisms including GSH levels and enzymes like catalase or superoxide dismutase. Insufficient oxygen consumption in mitochondria during fat metabolism (lipid peroxidation) might result in the overproduction of ROS. In addition, manifestation of oxidative stress by immune diseases, chronic inflammation or infections disturbs the intracellular homeostasis of prooxidants and antioxidants. Elevated ROS levels can initiate DNA damage, for example the formation of oxidized DNA bases, but are also involved in tumor promotion, and might ultimately lead to carcinogenesis [6]. In addition to the generation of oxidative stress, chronic inflammation and infections stimulate the inducible form of nitric oxide (NO) synthase (iNOS) and consequently, enhance the generation of NO, which forms peroxynitrite by reaction with superoxide anion radicals. Generally, NO is an important signaling molecule that physiologically acts as a vasorelaxant and modulator of neurotransmission and is involved in the immune defense against pathogens and certain tumor cells. Long-term elevated levels of NO, however, have been linked to early steps in carcinogenesis via nitrosative desamination of DNA bases and DNA adduct formation [7]. Excessive production of prostaglandins (PGs), i.e. hormone-like endogenous mediators of inflammation, from arachidonic acid by cyclooxygenase-1 (Cox-1) and the inducible form Cox-2 is thought to be a causative factor of cellular injury and may ultimately lead to carcinogenesis. Since PG levels and the expression of Cox-2 are often elevated in tumor tissue in comparison to normal tissue, and PGs enhance cell proliferation and stimulate the formation of new blood vessels (angiogenesis) and tumor invasiveness, inhibitors of the arachidonic acid cascade including non-steroidal anti-inflammatory drugs (NSAIDs) like Aspirin® or selective Cox-2 inhibitions (e.g. celecoxib) are regarded as promising chemopreventive agents and inhibitors of tumor promotion [8]. Ornithine decarboxylase (ODC) catalyses the decarboxylation of ornithine to putrescine, which is further converted to higher polyamines essential for duplication of DNA. This pathway is the only source of putrescine in mammalian cells, and ODC is thus regarded as a key enzyme in polyamine biosynthesis. Although ODC and the resulting polyamines are necessary for cellular proliferation, induction of ODC is involved in tumor promotion and cell transformation, and cultured tumor cells often contain high levels of ODC. Therefore, ODC is considered an attractive target in both chemotherapy and chemoprevention [9].

Based on these considerations, we have set up a broad spectrum of cell- and enzyme-based in vitro

assays using these markers relevant for measuring inhibition of carcinogenesis in vivo. To demonstrate the feasibility of our approach, i.e. the use of optimized in vitro test systems with selected targets as a means to identify potential chemopreventive agents, we have selected a series of 22 known chemopreventive agents from various structural classes. The spectrum of compounds included organosulfur compounds like phenethylisothiocyanate (PEITC) derived from watercress, as well as diallylsulfide and diallyldisulfide from *Allium* species (garlic, onion); terpenes, including monoterpenes like limonene and perillyl alcohol, which are found in essential oils from lavender, mint and citrus fruits, oleanolic acid, a triterpenoid acid which occurs in rosemary and other plants, and 18-β-glycyrrhetinic acid from licorice root (*Glyccyrrhiza glabra* L.); short-chain fatty acids like butyric acid (used as sodium salt), which represents a fermentation product of dietary fiber; indole-3-carbinol, which derives from a precursor glucosinolate in cruciferous vegetables like Chinese cabbage; flavonoids including quercetin, which is found in high quantities in onions, silymarin, a mixture of three flavonoids silybin, silydianin, and silychristin, from the seeds of the herb milk thistle (*Silybum marianum*), and the isoflavonoid genistein from soy beans (*Glycine max* (L.) Merr.); catechins like (−)-epigallocatechin gallate (EGCG) from green tea; simple phenols including ellagic acid found in various fruit and nuts, resveratrol from grape skin, its metabolite piceatannol, a tyrosine kinase inhibitor, and curcumin from turmeric and spices (Curry); pharmaceutical agents, including the NSAIDs piroxicam and acetylsalicylic acid (Aspirin®) and the breast cancer therapeutic agent tamoxifen; and finally two vitamins/derivatives, ascorbic acid (Vitamin C) and Trolox, a water soluble Vitamin E analog.

These compounds were tested to deduce activity profiles in our bioassay systems. Most interestingly, PEITC was identified as an inhibitor of iNOS induction, and we identified transcription factor NF-κB DNA binding as a target. Further, PEITC was found to prevent TPA-mediated production of superoxide anion radicals and ODC induction. These novel findings underline the usefulness of broad-spectrum in vitro testing to elucidate mechanisms of chemopreventive agents.

2. Materials and methods

2.1. Chemicals

All cell culture media and supplements were obtained from GIBCO BRL Life Technologies (Eggenstein, Germany). Fetal bovine serum was from Greiner Labortechnik GmbH (Frickenhausen, Germany). L-(1-^{14}C)-Ornithine (56 mCi/mmol, 100 μCi/ml) was purchased from Biotrend (Cologne, Germany). β-Phycoerythrin, calcein AM, 3-cyano-7-ethoxycoumarin (CEC), and 3-cyano-7-hydroxycoumarin (CHC) were purchased from Molecular Probes (Mobitec, Göttingen, Germany). PEITC (CAS no. 2257-9-2), diallyldisulfide (CAS no. 2179-57-9), diallylsulfide (CAS no. 592-88-1), limonene (CAS no. 138-86-3), L(−)-perillylalcohol (CAS no. 536-59-4), oleanolic acid (CAS no. 508-02-1), 18-β-glycyrrhetinic acid (CAS no. 471-53-4), sodium butyrate (CAS no. 156-54-7), indole-3-carbinol (CAS no. 700-06-1), quercetin (CAS no. 117-39-5), silymarin (CAS no. 22888-70-6), genistein (CAS no. 446-72-0), (−)-EGCG (CAS no. 989-51-5), ellagic acid (CAS no. 476-66-4), resveratrol (CAS no. 501-36-0), piceatannol (CAS no. 10083-24-6), curcumin (CAS no. 458-37-7), piroxicam (CAS no. 36322-90-4), acetylsalicylic acid (CAS no. 50-78-2), tamoxifen (CAS no. 10540-29-1), ascorbic acid (CAS no. 50-81-7), Trolox (CAS no. 53188-07-1) and all other chemicals were purchased from Sigma (Deisenhofen, Germany).

2.2. Inhibition of Cyp1A activity

Cyp1A activity was determined by measuring the rate of dealkylation of 3-cyano-7-ethoxycoumarin (CEC) to the fluorescent 3-cyano-7-hydroxycoumarin (CHC) based on a previously reported method [10]. Homogenates from cultured H4IIE rat hepatoma cells induced with 10 μM β-naphthoflavone (β-NF) were used as a source of Cyp1A activity. Cells were harvested after 38 h by scraping in 1 ml per plate buffer P (200 mM potassium phosphate buffer, pH 7.4, containing 10 mM $MgCl_2$) and snap frozen in liquid nitrogen. Serial two-fold dilutions of test compounds (10 μl) in 10% DMSO were added to each well of a 96-well plate. A freshly prepared reaction mixture (100 μl), consisting of 5.2 μl 50 mM $NADP^+$ in water, 4.4 μl 150 mM glucose-6-phosphate in water, 0.5 U

glucose-6-phosphate dehydrogenase, 0.1 μl 10 mM CEC (diluted from 10 mM stock solution in DMSO) and 90.25 μl buffer P, was then added. The reaction was started by addition of 90 μl cell homogenate, passed once through a 27 gauge injection needle and diluted with buffer P to a final protein concentration of 50–100 μg/ml. The rate of CEC conversion was measured for 40 min at 37 °C in a Cytofluor 4000 microplate fluorescence reader (PE Applied Biosystems, excitation wavelength Ex 408/20 nm, emission wavelength Em 460/40 nm). Inhibition of enzymatic activity was calculated in comparison with a CHC standard curve. α-Naphthoflavone, a known Cyp1A inhibitor with an IC_{50} value of 0.011 ± 0.001 μM ($n = 4$) was employed as a positive control.

2.3. Determination of QR activity in mouse hepatoma cell culture

QR activity was determined by measuring the NADPH-dependent menadiol-mediated reduction of 3 - (4,5 -dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan as described previously [11]. Protein was measured by crystal violet staining of an identical set of test plates. Induction of QR activity was calculated from the ratio of specific enzymatic activities of compound-treated cells in comparison with a solvent control. CD values (concentration required to double the specific enzyme activity in μM) were generated from the results of eight serial two-fold dilutions tested in duplicate. β-NF with a CD value of 0.033 ± 0.004 μM ($n = 3$) was used as a positive control.

2.4. Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

Radical scavenging potential was determined photometrically by reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals in a microplate format at 515 nm. Dilutions of test compounds (in 100% DMSO) were treated with a solution of 100 μM DPPH in ethanol for 30 min at 37 °C. Scavenging potential was compared with a solvent control (0% radical scavenging) and Vitamin C (250 μM final concentration, 100% radical scavenging, used as a blank), and the half-maximal scavenging concentration SC_{50} was generated (modified from [12]).

2.5. Inhibition of superoxide anion radical formation by xanthine/xanthine oxidase (X/XO assay)

Superoxide anion radicals were generated by oxidation of 50 μM hypoxanthine to uric acid by 12 mU xanthine oxidase (XO). Scavenging was quantified via the rate of reduction of 25 μM nitroblue tetrazolium (NBT) to a dark-blue formazan measured at 550 nm (modified from [13] and adjusted to a 96-well microplate format). Alternatively, the yellow tetrazolium salt XTT, which forms an orange formazan dye, was used. Under these conditions, the amount of XO was reduced to 3 mU and measurements were performed at 480 nm. V_{max} values were computed, and the half-maximal scavenging concentration SC_{50} was generated from the data obtained with five serial two-fold dilutions of inhibitors tested in duplicate. Superoxide dismutase (SOD, 30 U/ml) was used as a control. To exclude a direct inhibitory effect on XO, formation of uric acid was monitored directly at 290 nm under identical conditions as described above, but without addition of NBT or XTT. In the reaction mixture, 50 μM hypoxanthine was replaced by 100 μM xanthine. Allopurinol, a known inhibitor of XO, was used as a positive control with an IC_{50} value of 6.3 ± 3.7 μM ($n = 3$).

2.6. Inhibition of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide anion radical generation in differentiated HL-60 cells (HL-60 assay)

TPA-induced superoxide anion radical formation was detected in differentiated HL-60 human promyelocytic leukemia cells by photometric determination of cytochrome *c* reduction [14]. Cultured HL-60 cells were treated with 1.3% DMSO to induce granulocyte differentiation. After four days, cells were harvested by centrifugation and washed twice with Hanks balanced salt solution, pH 7.8, containing 30 mM Hepes (HHBSS). A total of 2×10^5 cells per well (100 μl) were pre-incubated with test compounds (25 μl, in 10% DMSO) for 5 min prior to addition of 75 μl cytochrome *c* solution in HHBSS (5 mg/ml, 1.25 mg/ml final concentration). SOD solution (25 μl) (600 U/ml in HHBSS, 12 U per well final concentration) was used as a positive control, all other wells obtained 25 μl HHBSS. Superoxide anion radical formation

was started by addition of 25 μl TPA (0.55 mg/ml in HHBSS, 55 ng/ml final concentration). After an incubation period of 30 min at 37 °C, the reaction was stopped by chilling the plates on ice for 15 min. The plates were centrifuged, and cytochrome *c* reduction was determined in supernatants at 550 nm using a microplate reader (Spectramax 340, Molecular Devices). The cell pellet was washed twice with PBS, and cell viability was measured fluorimetrically by enzymatic hydrolysis of the fluorogenic esterase substrate calcein AM (250 nM in PBS, 100 μl per well) at 37 °C in a Cytofluor 4000 microplate fluorescence reader (excitation wavelength Ex 485/20 nm, emission wavelength Em 530/25 nm). Using this method, we could avoid unspecific effects of reducing test compounds which falsify commonly used viability assays based on MTT or XTT bioreduction. The reaction was linear for at least 30 min. IC_{50} values (half-maximal inhibitory concentration of TPA-induced superoxide burst) were generated from the results of eight serial dilutions of inhibitors tested in duplicate. Only inhibitor concentrations resulting in >50% cell viability were considered to calculate scavenging potential.

2.7. Measurement of oxygen radical absorbance capacity (ORAC)

Peroxyl- ($ORAC_{ROO}$) or hydroxyl- ($ORAC_{OH}$) radical absorbance capacity of test compounds was tested in a modified ORAC assay [15] adapted to a 96-well plate format (Gamal-Eldeen et al., in preparation). β-Phycoerythrin (β-PE) was used as a redox-sensitive fluorescent indicator protein, 2,2′,-azobis-(2-amidinopropane) dihydrochloride (AAPH) as a peroxyl-radical generator and H_2O_2–$CuSO_4$ as a hydroxyl-radical generator. Reaction mixtures contained 170 μl 75 mM sodium potassium phosphate buffer, pH 7.0 (phosphate buffer), 10 μl 9.43 nM β-PE in phosphate buffer and 10 μl of the inhibitor solution (20 μM in 10% DMSO, 1 μM final concentration) or 10% DMSO as a negative control, respectively. For the $ORAC_{ROO}$ assay, the reaction was initiated by addition of 10 μl 320 mM AAPH in phosphate buffer, whereas for the $ORAC_{OH}$ assay, 10 μl of a 1:1 mixture of H_2O_2 (12%) and $CuSO_4$ (0.36 mM in phosphate buffer) were added. The decline of β-PE fluorescence was measured at 37 °C for 100 min until completion using a Cytofluor 4000 fluorescent microplate reader (excitation wavelength Ex 530/25 nm, emission wavelength Em 585/30 nm). Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of β-PE produced by 1 μM Trolox, a water soluble Vitamin E analog. Scavenging capacities >1 ORAC unit were considered as positive.

2.8. Inhibition of LPS-mediated inducible nitric oxide synthase (iNOS) induction in murine macrophages

Inhibition of lipopolysaccharide-mediated iNOS induction in Raw 264.7 murine macrophages was determined via the Griess reaction as described previously [16]. Cells were plated at a density of 1×10^5 cells per well in DMEM in 96-well plates. After a pre-incubation period of 24 h, the medium was changed. Inhibitors (10 μl in 10% DMSO, eight serial two-fold dilutions) were added, and iNOS was induced by addition of 20 μl LPS solution (500 ng/ml in serum-free DMEM). After 24 h, iNOS activity was determined via the quantification of nitrite levels in 100 μl of cell culture supernatants according to the Griess reaction and compared to a nitrite standard curve. To determine cytotoxic effects of test compounds, cell numbers were estimated by sulforhodamin B staining. Generally, compounds were tested at non-toxic concentrations (cell staining >50% of LPS-treated control cells).

2.9. Inhibition of cyclooxygenase (Cox) activity

Cox activity was measured at 37 °C by monitoring oxygen consumption during conversion of arachidonic acid to prostaglandins in a 1.0 ml incubation cell of an Oxygen Electrode Unit (Hansatech DW, based on a Clark-type O_2 electrode) ([17], with modifications). The reaction mixture, containing 0.1 M sodium potassium phosphate buffer, pH 7.4, 1 mM hydroquinone, 0.01 mM hemin and approximately 0.2 U Cox-1 in 100 μl microsome fraction derived from ram seminal vesicles as a crude source of Cox-1 (specific activity 0.2-1 U/mg protein), was incubated with 10 μl DMSO (negative control) or inhibitor solution (10 mM in DMSO), respectively, for 90 s. The reaction was started by addition of 2 μl 50 mM arachidonic acid in ethanol (100 μM final concentration), and oxygen consumption was monitored for 20 s. For calculation,

the rate of O_2 consumption was compared to a DMSO control (100% activity).

2.10. Determination of TPA-induced ornithine decarboxylase (ODC) activity in cultured mouse 308 cells

Culture of mouse 308 cells, treatment of cells with inhibitors added in serial dilutions in DMSO (0.5% final DMSO concentration), and determination of ODC activity were performed as described previously [18]. Protein content of cell-lysates using bovine serum albumin as a standard was measured according to Bradford and used to calculate ODC specific activity (pmol $^{14}CO_2$/mg protein/h). Results were expressed as a percentage in comparison with a control sample treated with DMSO and TPA. IC_{50} values were generated from the results of at least five serial dilutions of inhibitors tested in duplicate.

3. Results and discussion

A series of 22 selected cancer chemopreventive compounds was tested in a spectrum of ten in vitro bioassay systems with endpoints indicative of chemopreventive potential in vivo. Two test systems (inhibition of Cyp1A activity, induction of QR activity in Hepa1c1c7 murine hepatoma cell culture) were selected to identify modulators of carcinogen metabolism (results summarized in Table 1), five endpoints (scavenging of DPPH, superoxide anion-, hydroxyl- and peroxyl-radicals) dealt with radical-scavenging and antioxidant activities (results in Table 2) and three test systems (inhibition of LPS-mediated iNOS induction in Raw 264.7 murine macrophages, Cox-1 inhibition, inhibition of TPA-induced ODC activity in 308 cells) were chosen for the identification of anti-inflammatory and anti-tumor promoting mechanisms (summary in Table 3).

Table 1
Effects of chemopreventive reference compounds on carcinogen-metabolizing enzymes

Compound	NAD(P)H:quinone reductase (QR) induction		Cyp1A inhibition
	CD (μM)[a]	IC_{50} (μM)[b]	IC_{50} (μM)[b]
Acetylsalicylic acid	>50 (1.1)[c]	>50	>5 (11)[d]
Ascorbic acid (Vitamin C)	>50 (1.1)	>50	>5 (7)
Curcumin	2.7	9.7	0.74
Diallyldisulfide	>50 (1.5)	>50	>5 (15)
Diallylsulfide	>50 (1.2)	>50	>5 (0)
(−)-EGCG	>50 (1.4)	>50	>5 (32)
Ellagic acid	>12.9 (1.4)	12.9	>5 (4)
Genistein	16.2	23.9	0.2
18-β-Glycyrrhetinic acid	>41.9 (1.6)	41.9	>5 (2)
Indole-3-carbinol	37.8	>50	>5 (11)
Limonene	>50	>50	>5 (0)
Oleanolic acid	>50 (1.1)	>50	>5 (24)
PEITC	0.9	3.2	>5 (15)
L(−)-Perillylalcohol	>50	>50	>5 (13)
Piceatannol	>21.1 (1.4)	21.1	>5 (13)
Piroxicam	>50 (1.8)	>50	>5 (14)
Quercetin	2.6	14.0	0.02
Resveratrol	23.8	29.4	0.23
Silymarin (μg/ml)	3.6	12.0	0.42
Sodium butyrate	>50 (1.1)	>50	>5 (10)
Tamoxifen	>5.9 (1.9)	5.9	3.9
Trolox	>50 (1.4)	>50	>5 (0)

[a] CD: concentration required to double the specific activity of QR.
[b] IC_{50}: half-maximal inhibitory concentration.
[c] Values in parentheses indicate the maximum fold induction at the indicated concentration.
[d] Values in parentheses indicate the percentage of inhibition at the indicated concentration.

Table 2
Summary of radical-scavenging and antioxidant effects of reference compounds

Compound	DPPH	X/XO	HL-60	ORAC	
	SC_{50} (μM)[a]	SC_{50} (μM)[a]	IC_{50} (μM)	ROO (units)[b]	OH (units)[b]
Acetylsalicylic acid	>250 (3)[c]	>100 (0)[c]	>100 (35)[c]	0.9	2.2
Ascorbic acid	8.5	>100 (29)	>100 (0)	2.0	3.4
Curcumin	20.7	54.9	12.5[d]	2.1	1.7
Diallyldisulfide	>250 (4)	>100 (0)	>100 (12)	0.4	2.8
Diallylsulfide	>250 (1)	>100 (0)	>100 (22)	1.0	3.1
(−)-EGCG	3.4	4.0	>100 (8)	3.4	5.1
Ellagic acid	8.4	37.3	>100 (20)	2.4	5.1
Genistein	>250 (2)	>100 (18)	>100 (20)	1.9	2.8
18-β-Glycyrrhetinic acid	>250 (2)	>100 (1)	>100 (33)	0.9	5.2
Indole-3-carbinol	>250 (1)	>100 (0)	>100 (7)	2.6	3.4
Limonene	>250 (4)	>100 (0)	>100 (2)	0.9	2.6
Oleanolic acid	>250 (5)	>100 (0)	42.4	1.7	2.8
PEITC	>250 (1)	>100 (0)	3.5	0.4	2.5
L(−)-Perillylalcohol	>250 (3)	>100 (0)	>100 (12)	2.3	2.6
Piceatannol	11.8	3.1[e]	>100 (0)	3.8	1.7
Piroxicam	138.2	>100 (16)	>100 (27)	2.0	2.7
Quercetin	7.7	34.2	>100 (0)	2.7	3.4
Resveratrol	82.0	>100 (50)	>100 (29)	2.1	3.2
Silymarin (μg/ml)	33.8	24.0	>40 (15)	0.0	4.4
Sodium butyrate	>250 (0)	>100 (0)	>100 (0)	1.1	2.8
Tamoxifen	>250 (0)	>100 (0)	7.8[d]	N.D.	3.0
Trolox	9.7	>100 (11)	>100 (35)	1.0	1.0

N.D.: not determined.
[a] SC_{50}: half-maximal scavenging concentration.
[b] 1 ORAC unit equals the net protection of β-PE produced by 1 μM Trolox.
[c] Numbers in parentheses indicate the percentage of scavenging or inhibition at the indicated concentrations.
[d] Curcumin and tamoxifen inhibited cell viability with IC_{50} values of 49.8 and 22.6 μM, respectively.
[e] Piceatannol inhibited XO activity with an IC_{50} value of 19.4 μM.

Curcumin, quercetin and resveratrol were rated as the most potent chemopreventive agents, with 9, 8, and 7 out of 10 positive results within the concentration limits set for each assay, followed by piceatannol and silymarin, with 6 results within the limits. Due to the overrepresentation of ROS included as targets in the test panel, antioxidants were generally more potent than compounds lacking multiple antioxidant properties, e.g. limonene, 18-β-glycyrrhetinic acid, diallylsulfide, diallyldisulfide or acetylsalicylic acid.

We also utilized these in vitro bioassays as a valuable tool for the identification of novel potential chemopreventive agents. As an example, liverworts (Hepaticae) are a unique source of bibenzyl compounds [19], which display some structural similarity with resveratrol. Simple synthetic analogs of lunularic acid, a main component of liverworts, were detected as potent bifunctional inducers of drug-metabolizing enzymes, and CD values as a measure for induction of QR activity in Hepa1c1c7 cells were as low as 0.03 μM ([20], Gerhäuser et al., in preparation). We further identified xanthohumol (XN), a prenylated chalcone from hop (*Humulus lupulus* L.) as an interesting novel lead structure for chemoprevention with an exceptional broad spectrum of inhibitory mechanisms at the initiation, promotion and progression stage of carcinogenesis. Consistent with previous reports, XN potently inhibited Cyp1A activity and induced QR. Moreover, XN was able to scavenge ROS, including hydroxyl- and peroxyl-radicals, and to inhibit superoxide anion radical and nitric oxide production. It demonstrated anti-inflammatory properties by inhibition of Cox-1 and -2 activity and proved to be anti-estrogenic without possessing intrinsic

Table 3
Summary of anti-inflammatory and anti-tumor promoting effects of reference compounds

Compound	Inhibition of iNOS induction		Cox-1 inhibition	Inhibition of ODC induction
	A-IC_{50} (μM)[a]	T-IC_{50} (μM)[b]	IC_{50} (μM)	IC_{50} (μM)
Acetylsalicylic acid	>50 (8)[c]	>50	>100 (49)[c]	>10 (0)[c]
Ascorbic acid	>50 (0)	>50	>100 (70)	>10 (4)
Curcumin	5.0	25.0	>100 (45)	3.7
Diallyldisulfide	>50 (30)	>50	>100 (0)	>10 (19)
Diallylsulfide	>50 (21)	>50	>100 (12)	>10 (0)
(−)-EGCG	>50 (32)	>50	21.1	>10 (0)
Ellagic acid	>50 (16)	>50	>100 (0)	>10 (3)
Genistein	>50 (34)	>50	>100 (11)	>10 (0)
18-β-Glycyrrhetinic acid	>50 (0)	>50	>100 (6)	>10 (22)
Indole-3-carbinol	>50 (16)	>50	>100 (11)	>10 (0)
Limonene	>50 (46)	>50	>100 (9)	>10 (0)
Oleanolic acid	>50 (24)	>50	>100 (20)	>10 (0)
PEITC	5.0	>50	>100 (21)	2.7[d]
L(−)-Perillylalcohol	>50 (35)	>50	>100 (0)	>10 (0)
Piceatannol	17.2	>50	81.4	>10 (48)
Piroxicam	>50 (23)	>50	0.35	>10 (0)
Quercetin	19.8	>50	>100 (47)	1.3
Resveratrol	31.7	>50	1.56	>10 (0)
Silymarin (μg/ml)	>20 (29)	>20	36.9	>4 (5)
Sodium butyrate	>50 (0)	>50	>100 (2)	N.D.
Tamoxifen	>10.3 (3)	10.3	54.9	4.8
Trolox	>50 (2)	>50	>100 (24)	>10 (0)

N.D.: not determined.
[a] A-IC_{50}: half-maximal inhibitory concentration of nitrite production (activity).
[b] T-IC_{50}: half-maximal inhibitory concentration of cell viability (toxicity).
[c] Numbers in parentheses indicate the percentage of inhibition at the indicated concentration.
[d] PEITC concomitantly displayed cytotoxic effects with an IC_{50} value of 9.5 μM.

estrogenic potential. Anti-proliferative mechanisms of XN to prevent carcinogenesis in the progression phase included inhibition of DNA synthesis, and induction of cell cycle arrest in S-phase, apoptosis and cell differentiation (data not shown). Importantly, XN at nanomolar concentrations prevented carcinogen-induced pre-neoplastic lesions in mouse mammary gland organ culture [21].

In addition to identification of novel candidate chemopreventive agents, in vitro models are important for the elucidation of their mechanism of action. To this end, we discovered several additional mechanisms which might contribute to the chemopreventive potential of PEITC. PEITC has been demonstrated to inhibit rodent lung carcinogenesis mainly by modifying carcinogen metabolism via inhibition of phase 1 enzymes and/or induction of phase 2 enzymes [22]. These activities could be confirmed in our experiments, and we determined a CD value for the induction of QR activity of 0.9 μM with an IC_{50} value for the inhibition of cell growth of 3.2 μM (Table 1). The ratio between the half-maximal cytotoxic concentration (IC_{50}) and the effective dose (CD value), previously defined as chemopreventive index (CI) [11], was calculated as 3.6, i.e. the margin between activity and toxicity was rather narrow. This trend was also observed with other active inducers of QR, e.g. quercetin (CI = 5.4), silymarin (CI = 3.3), genistein (CI = 1.5), resveratrol (CI = 1.2), and curcumin (CI = 3.6), indicating that these compounds might stimulate their own 'detoxification'. PEITC was inactive in inhibiting Cyp1A activity at concentrations below 5 μM. We selected this concentration limit, since the Cyp1A assay utilizing CEC as a substrate is about 10-fold more sensitive than the commonly used EROD assay.

With respect to antioxidant effects, PEITC inhibited TPA-induced superoxide burst in differentiated HL-60 cells (Table 2). The compound was devoid activity in the X/XO system, where superoxide anion radicals are generated by the oxidation of hypoxanthine to uric acid. We therefore concluded that PEITC inhibits the signal transduction cascade resulting in the generation of superoxide anion radicals after stimulation of granulocytes by TPA, rather than acting as a superoxide anion radical scavenger [23]. PEITC was about 2.5-fold more potent that Trolox in scavenging hydroxyl-radicals in the $ORAC_{OH}$ assay. Other reference compounds displayed $ORAC_{OH}$ units of 1.7 (curcumin) to >5 (18-β-glycyrrhetinic acid, EGCG, ellagic acid), indicating potent hydroxyl-radical scavenging capacity. On the other hand, the capacity to scavenge peroxyl radicals was generally lower (Table 2).

Interestingly, PEITC was as effective as curcumin in inhibiting LPS-mediated induction of NO production (Table 3). When Raw 264.7 murine macrophages were incubated with PEITC in a concentration range of 0.8–50 μM, nitrite levels in cell culture supernatants were reduced with an IC_{50} value of 5.0 μM, without inhibitory effects on cell growth (Fig. 1A). We have recently described anti-inflammatory mechanisms of sulforaphane, an isothiocyanate which is derived from a glucosinolate precursor in broccoli, and identified transcription factor NF-κB as a molecular target [16]. Accordingly, we analyzed the potential of PEITC to inhibit NF-κB DNA binding in electrophoretic mobility shift assay (EMSA) analyses. As indicated in Fig. 1B, treatment of Raw 264.7 murine macrophages with 20 μM PEITC reduced LPS-induced DNA binding of NF-κB to its consensus sequence in the iNOS promoter region, providing evidence that this mechanism might also be relevant for additional isothiocyanate chemopreventive agents. As an anti-tumor promoting mechanism, we analyzed TPA-mediated induction of ODC activity in 308 murine keratinocytes. In agreement with its potential to prevent TPA-mediated induction of superoxide anion radical production in granulocytes, PEITC also inhibited the induction of ODC by TPA with an IC_{50} value of 2.7 μM. We observed a similar pattern of activities with curcumin and tamoxifen. This might be an indication for a common target of these chemopreventive compounds in the signaling cascade induced by TPA, e.g. protein kinase C or the MAP kinase pathway [23].

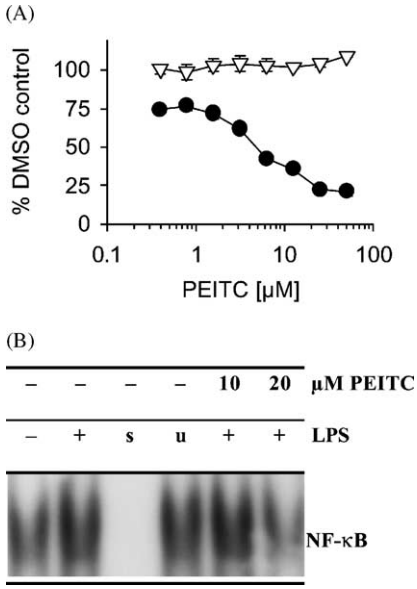

Fig. 1. (A) Inhibition of LPS-induced generation of NO by PEITC. Raw 264.7 murine macrophages were treated with PEITC or DMSO and stimulated with LPS for 24 h. Nitrite, as a measure of NO production (●) was determined in cell culture supernatants. Effects of PEITC on cell growth were measured by SRB staining (▽). (B) Electrophoretic mobility shift assay of NF-κB DNA-binding capacity. Raw 264.7 murine macrophages were treated with DMSO (−) or PEITC (as indicated) and stimulated with LPS (+) for 45 min before nuclear protein was isolated. DNA-binding was analyzed using a specific ^{32}P-labeled oligonucleotide probe for NF-κB. Specificity was demonstrated by co-incubation with a 25-fold excess of unlabeled specific (s) or unspecific probe (u) for competition.

Using the described approach, we have demonstrated that in vitro bioassays offer fast (within days), sensitive and cost effective identification and evaluation of lead compounds for the development of effective chemopreventive agents and the elucidation of their mechanism of action. This will allow the optimal use of most promising compounds for in vivo application.

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