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Anti-/pro-oxidant effects of phenolic compounds in cells: are colchicine metabolites chain-breaking antioxidants?

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Abstract

Effective scavenging of reactive radicals and low reactivity of generated secondary antioxidant radicals towards vital intracellular components are two critical requirements for a chain-breaking antioxidant. Tubulin-binding properties aside, colchicine metabolites remain largely untested for other possible biological activities, including antioxidant activity. Mourelle et al. [Life Sci. 45 (1989) 891] proposed that colchiceine (EIN) acts as an antioxidant and protective agent against lipid peroxidation in a rat model of liver injury. Since EIN as well as two other colchicine metabolites, 2-demethylcolchicine (2DM) and 3-demethylcolchicine (3DM), possess a hydroxy-group on their carbon ring that could participate in radical scavenging, we tested whether they can act as chain-breaking antioxidants. Using our fluorescence-HPLC assay with metabolically incorporated oxidation-sensitive *cis*-parinaric acid (PnA) we studied the effects of colchicine metabolites on peroxidation of different classes of membrane phospholipids in HL-60 cells. None of the colchicine metabolites in concentrations ranging from 10^{-6} to 10^{-4} M was able to protect phospholipids against peroxidation induced by either azo-initiators of peroxyl radicals or via myeloperoxidase (MPO)-catalyzed reactions in the presence of hydrogen peroxide. However, the metabolites did exhibit dose-dependent depletion of glutathione, resembling the behavior of etoposide, a hindered phenol with antioxidant properties against lipid peroxidation. Electron spin resonance (ESR) experiments demonstrated that in a catalytic system containing horseradish peroxidase (HRP)/H_2O_2, colchicine metabolites undergo one-electron oxidation to form phenoxyl radicals that, in turn, cause ESR-detectable ascorbate radicals by oxidation of ascorbate. Phenoxyl radicals of colchicine metabolites formed through MPO-catalyzed H_2O_2-dependent reactions in HL-60 cells and via HRP/H_2O_2 in model systems can also oxidize GSH. Thus, colchicine metabolites possess the prerequisites of many antioxidants, i.e. a nucleophilic hydroxy-group on a carbon ring and the ability to scavenge reactive radicals and form a secondary radical. However, the latter retain high reactivity towards critical biomolecules in cells such as lipids, thiols, ascorbate,

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thereby, rendering colchicine metabolites effective radical scavengers but not effective chain-breaking antioxidants.

Keywords: Lipid oxidation; Antioxidant; Radical scavenger; Colchicine metabolites; Parinaric acid; Etoposide

1. Introduction

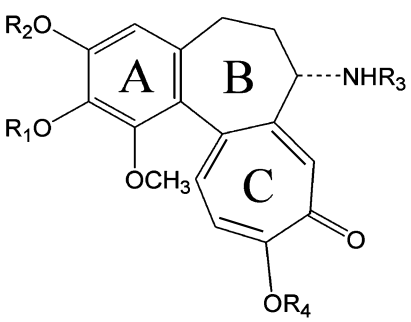
Life-times of reactive free radical intermediates such as hydroxyl, alkoxyl, and peroxyl radicals are too short to render their effective control by antioxidant enzymatic mechanisms and suggest that additional specialized systems are required for the regulation of these free radical species. Indeed, there is a unique network of small antioxidants that includes both lipid- and water-soluble molecules. Although these antioxidants are located in separate cell compartments they are engaged in close and effective redox interactions with each other providing for antioxidant recycling. It is tempting to assume that supplementation with exogenous natural antioxidants or pharmacologic use of synthetic low molecular weight antioxidants can offer relatively simple and effective ways to control oxidative stress. These assumptions, however, should be based on detailed knowledge of antioxidant mechanisms and pathways essential for uninterrupted, physiologically justified antioxidant protection in specific intracellular environments: several critical requirements should be fulfilled in order to create an effective new antioxidant molecule. These include, but are not limited to (i) effective radical scavenging; (ii) low reactivity of secondary antioxidant radicals towards vital intracellular components; (iii) low level of one-electron enzymatic metabolism of antioxidants; (iv) lack of interference with endogenous antioxidant network resulting in wasteful consumption of their resources; and (v) no inhibition of vital oxidative signaling pathways (e.g. phosphatidylserine (PS)-dependent recognition of apoptotic cells).

In this work, we studied anti-/pro-oxidant properties of colchicine and its metabolically and pharmacologically relevant homologs as compared with several other phenolic radical scavengers.

Colchicine, an alkaloid traditionally extracted from *Colchicum autumnale* (meadow saffron), remains a common choice in treatment of acute gout arthritis. Colchicine's popularity rose with results obtained in studies of prophylaxis of familial Mediterranean fever (FMF). It has been used recently for other indications including primary biliary cirrhosis (PBC), alcohol-induced cirrhosis, and Behcet's syndrome (Ben-Chetrit and Levy, 1998, and references therein). Clinical trials of colchicine in chronic liver disease, however, yielded mixed results (Kaplan et al., 1999; Kershenobich et al., 1988; Warnes et al., 1987). Despite controversies, the alkaloid is actively investigated for different biologic effects in association with liver injury. It was tested in acetaminophen (Muriel et al., 1993) and carbontetrachloride (CCl_4) (Martinez et al., 1995) models of acute liver damage. In spite of its apparent affinity for a lipid environment and its effects on membranes (Mons et al., 2000), the proposed antioxidant effect of colchicine was discounted by a recent study that re-examined the animal model in connection with liver cirrhosis (Das et al., 2000). Thus, most of the colchicine's biological effects can be ascribed to its tubulin-binding ability (Ben-Chetrit and Levy, 1998). Even though colchicine metabolites and any artificial derivatives are subjects of tubulin-binding tests (cf. De Vincenzo et al., 1999), other possible biologic properties of these substances, including antioxidant activity, remain untested.

In humans, colchicine's metabolism involves demethylation mediated by cytochrome P450 (CYP) 3A4 isoform, yielding 2-demethylcolchicine (2DM) and 3-demethylcolchicine (3DM) (Tateishi et al., 1997; Fig. 1). Although not confirmed to occur in humans, 10-demethylcolchicine, also called colchiceine (EIN), is produced in rodents by the same CYP homolog (Schonharting et al., 1974). EIN is the metabolite suggested to have beneficial effects in a biochemical model of liver

injury (Rodriguez et al., 1998) and was shown to prevent acute and chronic CCl_4 injury in rats (Mourelle et al., 1989; Nava-Ocampo et al., 1997). In an earlier study, EIN was shown to prevent lipid peroxidation-the centerpiece of CCl_4-induced injury. Protection against lipid peroxidation also was observed in a rat model of biliary obstruction in which it was compared with vitamin E (Muriel and Suarez, 1994). High concentrations of EIN were also shown to protect reduced glutathione (GSH) against oxidation by atmospheric oxygen and to prevent oxygen-induced inactivation of enzymes with catalytically essential sulfhydryl groups (Schnell et al., 1976). While the pharmacologically relevant concentrations of EIN used in the in vivo experiments (10 and 60 μg/day per rat) suggest that EIN acts as an effective antioxidant (Mourelle et al., 1989; Muriel and Suarez, 1994), direct evidence for EIN radical scavenging activity and protection against lipid peroxidation is lacking. The two other natural metabolites, 2 and 3DM, have not been tested as antioxidants. Each contains an electron-donating hydroxy-group on the phenolic ring (Fig. 1) which is essential for radical scavenging activity and which can be converted to a respective phenoxyl radical upon interaction with the reactive radicals.



Substance	R_1	R_2	R_3	R_4
Colchicine	CH_3	CH_3	$COCH_3$	CH_3
2-Demethylcolchicine	H	CH_3	$COCH_3$	CH_3
3-Demethylcolchicine	CH_3	H	$COCH_3$	CH_3
Colchiceine	CH_3	CH_3	$COCH_3$	H
N-Deacetylcolchiceine	CH_3	CH_3	H	H

Fig. 1. Chemical structures of colchicine and its metabolites.

In this study, we determined the antioxidant activities of three colchicine metabolites-2DM, 3DM, and EIN on lipid peroxidation using cell culture models. *N*-deacetylcolchiceine (NDE), a derivative of EIN (Fig. 1), was also tested in our assay systems. We investigated the reactivity of the radicals generated from colchicine homologs towards GSH in cells and in model systems. We also used electron spin resonance (ESR)-detectable ascorbate radical formation as evidence for one-electron conversions of colchicine homologs. For comparison, we conducted studies of several other phenolic compounds such as phenol, etoposide, and a vitamin E-homolog, 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC).

2. Methods

2.1. Chemicals

Colchicine, 2DM, and NDE were prepared at the Institute of Medical Chemistry and Biochemistry, Palacky University, Olomouc, Czech Republic. The purity of the compounds was verified by HPLC and exceeded 98%. 3DM and EIN were kindly provided by Dr. Norbert Hedbecker (MADAUS AG, Cologne, Germany). *cis*-Parinaric acid (PnA) was obtained from Molecular Probes, Inc. (Eugene, OR). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 6-hydroxy-2,2,5,7,8-pentamethylchromane (PMC) were supplied by Wako Chemicals USA, Inc. (Richmond, VA). 2,2′-Azobis(2,4-dimethylaminovaleronitrile) (AMVN) was purchased from Polysciences, Inc. (Warrington, PA). ThioGlo-1™, a maleimide-based reagent was purchased from Covalent Associates, Inc. (Woburn, MA). Etoposide (VP-16), hydrogen peroxide (H_2O_2), reduced glutathione (GSH), diethylenetriaminepentaacetic acid (DTPA), and 3-amino-1,2,4-triazole (3-AT) were purchased from Sigma (St. Louis, MO). All other chemicals, reagents, and enzymes were obtained from various sources and were of the highest quality commercially available.

2.2. Cell culture

Human promyelocytic leukemia HL-60 cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere. Cells collected for experiments were from passages 25 to 40 and their density was approximately 1×10^6 cells per ml. Cell viability was determined by light microscopy using the Trypan blue exclusion test.

2.2.1. Assay for phospholipid peroxidation in HL-60 cells: metabolic incorporation of cis-PnA into cell phospholipids

The assay procedure followed previously described protocol by Ritov et al. (1996), with minor adjustments. Cells from maintenance cultures were washed once with serum free phenol red-free RPMI 1640 medium and then incubated for 2 h at a density of 2×10^6 cells per ml with the complex of PnA with human serum albumin (hSA) in serum free phenol red-free RPMI medium at 37 °C. The final concentration of PnA in the suspension was 2 μg/ml. After incubation, cells were washed once with L1210 buffer (115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 10 mM glucose, and 25 mM HEPES, pH 7.4) containing 0.5 mg/ml of fatty acid-free hSA and then once with L1210 buffer lacking hSA. Cells were counted and their density was adjusted to 1×10^6 cells per ml.

2.3. Lipid peroxidation experiments

Two milliliter of cell suspension (1×10^6 cells per ml) were transferred to glass tubes with screw-caps and were pre-incubated for 15 min with one of the following agents: PMC, phenol, etoposide, 2DM, 3DM, COL, EIN, or NDE. Concentration of colchicine and its metabolites varied from 10^{-6} to 10^{-4} M. Control samples were treated with the vehicle DMSO. Pre-incubated cells were exposed to three different oxidants: a lipid-soluble azo-initiator of peroxyl radicals, AMVN (0.5 mM for 2 h at 37 °C), a water-soluble azo-initiators, AAPH (50 mM for 30 min at 37 °C), and H_2O_2 (25 μM, added every 15 min during 1 h incubation at 37 °C).

2.4. Extraction of cell lipids

Total lipid extracts were obtained by a modified Folch procedure (Ritov et al., 1996): 2 ml of methanol containing 0.5 mM butylated hydroxy toluene (BHT) was added to the cell suspension followed by 4 ml of chloroform. The mixture was kept on ice in the dark for 1 h or in a −20 °C freezer overnight after which 0.1 M NaCl (1 ml) was added and the mixture was vigorously vortexed. The lower phase (chloroform) was collected by Pasteur pipette, transferred into a clean tube and then dried under nitrogen. The resulting lipid extract was dissolved in 250 μl of 2-propanol:hexane mixture 4:3 (v/v).

2.5. HPLC analysis of cell lipids

Lipid extracts were separated by HPLC using ammonium acetate gradient (Geurts van Kessel et al., 1977). Samples were injected into a Supelcosil LC-Si column (5 μm, 4.6 mm × 250 mm) equilibrated with a 9:1 (v/v) mixture of mobile phase A [57:43:1 (v/v/v) 2-propanol:hexane:H_2O] and mobile phase B [57:43:1 (v/v/v) 2-propanol:hexane:40 mM ammonium acetate, apparent pH 6.7]. The column was eluted at 1 ml/min using the following solvent composition: linear gradient from 10 to 37% B, 0–3 min; isocratic at 37% B, 3–15 min; linear gradient to 100% B, 15–23 min; and isocratic at 100% B, 23–45 min. Separations were performed on a Shimadzu HPLC (model LC-600) equipped with an in-line configuration of Shimadzu fluorescence detector (model RF-551) and UV–vis detector (model SPD-10A). Effluent was monitored by absorbance at 205 nm for lipid detection and by fluorescence (excitation at 324 nm, emission at 420 nm) to detect PnA. All data were stored and processed using SHIMADZU EZCHROM software.

2.6. Total phosphorus determination

A modification of the method described by Chalvardjian and Rudnicki (1970) was used. Solvent from aliquots of lipid extracts was evaporated to dryness under a nitrogen stream. Fifty microliters of 70% (w/v) aqueous perchloric acid

were added to each of the dried samples, which were then incubated for 20 min at 170–180 °C. After the samples cooled, 400 μl of distilled water were added to each tube followed by 2 ml of sodium molybdate/malachite green solution [a 1:3 (v/v) solution of 4.2% (w/v) sodium molybdate in 5.0 N HCl and 0.2% (w/v) aqueous malchite green] and then 80 μl of 1.5% (v/v) Tween 20. To stabilize color, the tubes were vortexed immediately. Absorbance was measured at 660 nm and three determinations of each lipid extract sample were made.

2.7. EPR detection of ascorbate radical formation

Experiments were performed with a JEOL-RE1X ESR spectrometer (Tokyo, Japan) at room temperature in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness). The tube (approximately 8 cm in length) was filled with 60 μl of sample solution, folded into quarters, and placed in an open 3 mm (internal diameter) ESR quartz tube such that all of the sample was within the effective microwave irradiation area. Actual instrument settings are given in the figure legends. ESR signal of ascorbate radical was recorded in samples containing ascorbate (250 μM), H_2O_2 (400 μM), and horseradish peroxidase (HRP) (0.03 U/μl) in 50 mM phosphate buffer, pH 7.4 in the presence of DTPA (100 μM) at room temperature.

2.8. Fluorescent assay of GSH

The GSH content both in cells and in model systems was determined using ThioGlo-1™—a maleimide reagent, which produces a highly fluorescent product upon its reaction with SH groups (Langmuir et al., 1996).

HL-60 cells (1×10^6 cells per ml, 1 ml per well) were suspended in L1210 buffer containing 100 μM DTPA and 2.5 mM 3-amino-triazole (3-AT), seeded into 24-well culture plates, and then exposed to H_2O_2 for 60 min at 37 °C under 5% CO_2, 95% air. Every 15 min, 12.5 μM H_2O_2 was added to each well. Cells were treated with PMC, etoposide, phenol, or colchicine metabolites at the indicated concentrations 15 min before administration of H_2O_2. The ThioGlo-1™ procedure was used to measure GSH content in the cells as was previously described (Kagan et al., 1999). In brief, HL-60 cells treated with H_2O_2 and/or colchicine metabolites were harvested and lysed by freezing and thawing three times. After addition of 10 μM ThioGlo-1™ to the cell lysates, fluorescence was measured in a CytoFluor 2350 (Millipore, Bedford, MA) fluorescence microplate reader using an excitation wavelength of 360 nm (40 nm slit) and an emission wavelength of 530 nm (25 nm slit). The protein concentration of cell lysates was measured by the method of Bradford (1976).

In model cell-free systems, HRP-catalyzed oxidation (0.01 U/μl) was performed in the presence of H_2O_2 (2 mM) and GSH (14 μM) as well as a phenolic compound-colchicine metabolites (1, 10 or 100 μM), phenol (100 μM), or etoposide (100 μM)—in 50 mM phosphate buffer (pH 7.4) in the presence of DTPA (100 μM). The samples were incubated for 15 min at 37 °C and the reaction was stopped by cooling the samples. GSH content was measured as described above.

2.9. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using MINITAB software (Minitab Inc., State College, PA).

3. Results

3.1. Effect of colchicine metabolites on peroxidation of phospholipids in HL-60 cells

In our initial experiments, we studied whether colchicine and its metabolites can act as antioxidants towards intracellular phospholipids. To this end, we used an experimental system of live HL-60 cells labeled with PnA, a natural fluorescent oxidation-sensitive unsaturated fatty acid. The technique makes use of metabolic incorporation of PnA into different classes of cell membrane phospholipids, and the fact that fluorescence of PnA-labeled phospholipids proportionally decreases upon oxidation as determined by fluorescence HPLC (Ritov et al., 1996). We challenged

labeled HL-60 cells with three different oxidants: a lipid-soluble azo-initiator of peroxyl radicals, AMVN, a water-soluble azo-initiator of peroxyl radicals, AAPH, and H_2O_2.

PnA-labeled HL-60 cells were exposed to azo-initiators, AMVN (0.5 mM for 2 h) or AAPH (50 mM for 30 min), respectively, in the presence and absence of colchicine metabolites (10^{-6}–10^{-4} M). Both radical initiators generated peroxyl radicals at a constant rate (Niki, 1991) and caused significant peroxidation of different classes of phospholipids in HL-60 cells. There was no protection by either colchicine or any of its metabolites against phospholipid peroxidation induced by the initiators. Fig. 2 shows the lack of effect against AAPH-induced oxidation of three phospholipid classes of PnA-labeled phospolipids: PC, PE, and PS. In contrast, the vitamin E homolog PMC exerted substantial protection of these phospholipids against AAPH-induced oxidation. Thus, colchicine or its metabolites exert no antioxidant activity towards azo-initiator induced phospholipid peroxidation in HL-60 cells. Similar results were obtained when AMVN was used as a source of peroxyl radicals instead of AAPH (data not shown).

Since HL-60 cells contain high myeloperoxidase (MPO) activity, we were next eager to determine whether colchicine homologs were able to function as antioxidants against MPO-catalyzed peroxidation (Wagner et al., 2000). Therefore, we tested effects of these compounds on H_2O_2-induced phospholipid peroxidation in HL-60 cells. Table 1 summarizes the H_2O_2-induced oxidation in four phospholipid classes of HL-60 cells incubated in the presence or absence of 100 μM concentrations of colchicine metabolites. Under the test conditions, H_2O_2 caused a pronounced and uniform loss of approximately 2/3 in each class of PnA-labeled phospholipids. No colchicine metabolite was effective in inhibiting phospholipid peroxidation. In sharp contrast, PMC provided almost complete protection against H_2O_2-induced oxidative loss of PnA-labeled phospholipids (Table 2).

Our previous studies demonstrated that MPO-catalyzed reactions of phenolic compounds may cause either enhancement or attenuation of phospholipid peroxidation depending on the reactivity of phenoxyl radical intermediates produced. As shown in Table 2, phenol and etoposide represent typical compounds that can propagate or quench free radical oxidation of lipids, respectively. High

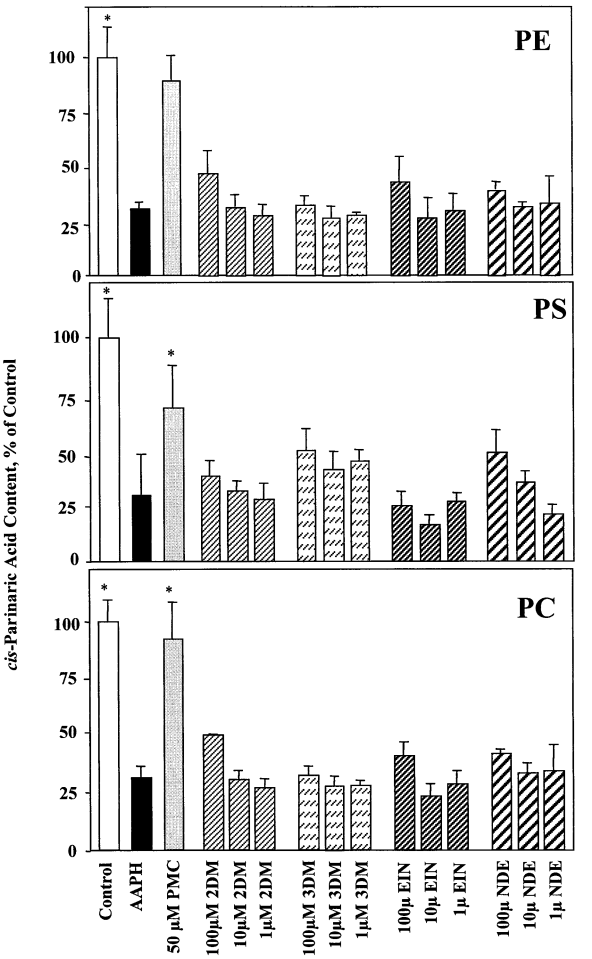


Fig. 2. Effect of colchicine metabolites on AAPH-induced lipid peroxidation in HL-60 cells. Cells (1×10^6 cells per ml) were incubated in the presence or absence (control) of 50 mM AAPH in L1210 buffer, pH 7.4 for 30 min at 37 °C under a 5% CO_2, 95% air atmosphere. Cells were treated with PMC (50 μM), or colchicine metabolites (1, 10 or 100 μM) 15 min before AAPH treatment. Oxidation of individual phospholipids: phosphatidylethanolamine (PE), PS and phosphatidylcholine (PC) was evaluated by measuring the oxidation of incorporated PnA by HPLC (see Section 2). Data are mean ± S.D. of three independent measurements. *, $P < 0.001$ vs. AAPH-treated cells.

reactivity of MPO-catalyzed phenoxyl radicals generated from phenol caused further augmentation of phospholipid peroxidation while MPO-catalyzed etoposide phenoxyl radicals with relatively low reactivity were unable to directly oxidize phospholipids-resulting in a protective effect of etoposide. Notably, colchicine metabolites caused neither protection nor enhanced oxidation of cellular lipids.

Thus, in HL-60 cells, colchicine homologs demonstrated no antioxidant activity towards phospholipid peroxidation induced by either azo-initiators or MPO-catalyzed H_2O_2-dependent reactions. GSH and other small molecular weight thiols such as cysteine and homocysteine are important natural intracellular antioxidants. The level of GSH or the ratio of reduced to oxidized GSH (GSH/GSSG) is frequently used to evaluate antioxidant status of cells and biologic fluids. Since thiols are known to react more readily with phenoxyl radicals than lipids do, we further studied effects of colchicine metabolites on the content of GSH in HL-60 cells.

3.2. Effect of colchicine metabolites on MPO/H_2O_2-induced oxidation of GSH in HL-60 cells and model systems

Exposure to H_2O_2 yielded a dramatic ($\approx 80\%$) depletion of GSH in HL-60 cells (Fig. 3). In HL-60 cells pretreated with various concentrations of colchicine metabolites 15 min before H_2O_2 administration, essentially no protection against GSH oxidation was observed at the highest concentration used (100 μM). Very weak ($\leq 15\%$) protective effects were observed for some of colchicine metabolites at lower concentrations (1 and/or 10 μM). In agreement with our previous observations, etoposide and phenol exacerbated H_2O_2-induced GSH oxidation in HL-60 cells. Finally, PMC demonstrated a strong protective effect against GSH oxidation.

We and others demonstrated that H_2O_2-dependent MPO-catalyzed generation of phenoxyl radicals from phenolic compounds may be involved in GSH oxidation in HL-60 cells. To further substantiate the engagement of MPO and phenoxyl radicals in GSH oxidation we tested the reactivity of colchicine metabolites towards GSH in a cell-free model system that includes H_2O_2/HRP. All colchicine metabolites showed concentration dependent oxidation of glutathione (Fig. 4). Whereas, at the concentration of 10^{-6} M, colchicinoids induced the same level of GSH oxidation as H_2O_2 alone, at concentrations of 10^{-4} M they showed increased GSH oxidation comparable with that displayed by etoposide. Phenol, a powerful redox cycling agent whose phenoxyl radical effectively interacts with GSH (Shvedova et al., 2000), caused greater GSH oxidation than all other substances tested.

Table 1
Effect of colchicine metabolites on oxidation of PnA-labeled phospholipids induced by hydrogen peroxide in HL-60 cells, percent of control

Additions	PI	PE	PS	PC
None	100 ± 4.7	100 ± 3.9	100 ± 10.9	100 ± 4.8
H_2O_2	31.7 ± 14.2*	38.1 ± 13.7*	38.7 ± 12.7*	35.1 ± 9.9*
2DM + H_2O_2	29.5 ± 8.4*	44.6 ± 4.5*	41.3 ± 2.7*	40.3 ± 2.5*
3DM + H_2O_2	34.7 ± 5.3*	38.9 ± 8.1*	42.2 ± 4.8*	38.3 ± 6.9*
EIN + H_2O_2	50.8 ± 4.0*	62.1 ± 1.3*	53.8 ± 3.5*	56.1 ± 3.6*
NDE + H_2O_2	40.0 ± 5.2*	36.7 ± 0.4*	59.1 ± 5.2*	36.4 ± 0.9*

PnA-loaded cells were exposed to H_2O_2 in the absence and in the presence of colchicine metabolites (100 μM) for 1 h at 37 °C. Colchicine metabolites were added 15 min prior the first addition of H_2O_2. H_2O_2 (25 μM) was added every 15 min. All data are mean ± S.D. *, $P < 0.001$ vs. control (none). PI, phosphatidylinositol; PE, phosphatidyethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; 2DM, 2-demethylcolchicine; 3DM, 3-demethylcolchicine; EIN, colchicine; NDE, *N*-deacetylcolchicine.

Table 2
Effect of etoposide, PMC and phenol on oxidation of PnA-labeled phospholipids induced by hydrogen peroxide in HL-60 cells, percent of control

Additions	PI	PE	PS	PC
None	100 ± 8.3	100 ± 12.4	100 ± 10.6	100 ± 6.6
Etoposide	108.7 ± 10.2	85.6 ± 3.3	119.9 ± 22.8	96.3 ± 2.7
PMC	87.7 ± 15.5	100.3 ± 7.8	106.4 ± 8.7	98.1 ± 13.3
Phenol	89.2 ± 8.2	75.9 ± 3.0	108.8 ± 9.1	82.6 ± 1.4
H_2O_2	26.0 ± 10.2*	23.4 ± 4.2*	42.9 ± 3.4*	27.9 ± 6.1*
PMC + H_2O_2	81.1 ± 14.4**	85.4 ± 16.3*	99.1 ± 13.9*	87.1 ± 14.8**
Etoposide + H_2O_2	71.1 ± 4.0**	62.2 ± 6.6**	90.8 ± 11.3	69.1 ± 6.1**
Phenol + H_2O_2	15.8 ± 2.0	12.6 ± 0.2**	36.7 ± 3.7	18.2 ± 1.9

PnA-loaded cells were exposed to H_2O_2 in the absence and in the presence of etoposide (50 μM), PMC (50 μM) or phenol (100 μM) for 1 h at 37 °C. Etoposide, PMC or phenol were added 15 min prior the first addition of H_2O_2. H_2O_2 (25 μM) was added every 15 min. All data are mean ± S.D. *, $P<0.001$ vs. control (none); **, $P<0.001$ vs. H_2O_2. The integration of PnA into PI, PE, PS and PC was estimated as 19.4 ± 1.6, 116.6 ± 14.5, 6.2 ± 0.7, and 372.9 ± 24.6 ng PnA per μg total lipid phosphorus, respectively. PI, phosphatidylinositol; PE, phosphatidyethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PMC, 6-hydroxy-2,2,5,7,8-PMC.

3.3. ESR evidence for the formation of phenoxyl radicals during peroxidase-catalyzed oxidation of colchicine metabolites

The above results indicate that MPO-catalyzed phenoxyl radicals of colchicine metabolites might be responsible, at least in part, for GSH oxidation in HL-60 cells. To confirm that one-electron oxidation intermediates are in fact formed by peroxidase-catalyzed reactions from colchicine metabolites we used ESR spectroscopy. Since phenoxyl radicals of colchicine homologs are expected to be too short-lived to be directly detectable by ESR, we used a different approach based on their interactions with ascorbate: one-electron oxidation of ascorbate by a phenoxyl radical produces the ascorbate radical that has a characteristic doublet ESR signal (Fig. 5, trace A). This radical is fairly stable and can be used for detecting other radicals which have a short half-life or are otherwise undetectable. An increased signal of ascorbate radical in a system containing a tested phenolic compound as well as a phenoxyl radical-generating system such as HRP/H_2O_2 indicates the formation of the phenoxyl radical via its reduction by ascorbate. If a time course of ascorbate radical generation is followed, faster depletion of ascorbate is observed with potential appearance of the tested phenoxyl radical coinciding with ascorbate disappearance (Kagan et al., 1999). Such a model system was used to verify that colchicine metabolites can undergo HRP/H_2O_2-catalyzed one-electron oxidation to their respective phenoxyl radicals by monitoring the ESR signal of ascorbate.

Colchicine was incapable of radical formation and showed the same behavior as the control sample containing ascorbate alone (Fig. 5, compare traces C and A). This was expected because colchicine lacks a hydroxy-group on its carbon skeleton that could undergo one-electron oxidation to the phenoxyl radical. On the other hand, etoposide (trace B) and EIN (trace D) displayed an increased magnitude of ascorbate radical ESR signal as compared with the signal formed by HRP/H_2O_2 from ascorbate alone indicating that phenoxyl radicals generated caused enhanced one-electron ascorbate oxidation. Similarly, increased ESR signals were observed for the remaining colchicine metabolites tested (not shown). Phenol (trace E) showed a much higher magnitude of ascorbate radical ESR signal as compared with colchicine metabolites in accordance with effective redox cycling of ascorbate by its phenoxyl radical. Thus, we conclude that colchicine metabolites are capable of forming the phenoxyl radicals that are quickly reduced by ascorbate.

3.4. Effect of colchicine metabolites on viability of HL-60 cells

We were interested in determining whether the redox effects of colchcine metabolites were realized in viable HL-60 cells. Since all colchicine derivatives display antimitotic activity and are frequently tested for that activity in cancer cell lines such as our model HL-60 cells, we assayed for cell viability under the test conditions used as estimated from Trypan blue exclusion test. The cell viability after incubations, irrespective of colchicine metabolite or radical initiator presence, was always on the order of 90%. However, blebbing and a decrease in cell size, two morphological features of the ensuing apoptotic process, were evident in 30–40% cells after 2 h incubation in the presence of 100 μM colchicine metabolites. These features became more pronounced in the presence of oxidants and likely reflected tubulin-binding effects of colchicine metabolites as well as oxidative damage.

4. Discussion

Several critical requirements should be met to design a 'perfect' new antioxidant molecule. In particular, effective scavenging of reactive radicals is a necessary but not a sufficient condition for a potent antioxidant molecule. Another critical requirement is that interaction of the candidate antioxidant molecule with reactive radicals forms an antioxidant radical that has a low reactivity towards vital intracellular components such as lipids, proteins, and nucleic acids. Thus, an effective radical scavenger is not necessarily a good antioxidant. We and others have demonstrated that several very effective radical scavengers such as etoposide or phenol proved to be only feeble antioxidants, and in addition, exerted harmful and toxic effects. Therefore, testing for antioxidant activity of molecules with pronounced pharmacologic effects should include not only evaluation of their radical scavenging effectiveness but also studies of the reactions of secondary

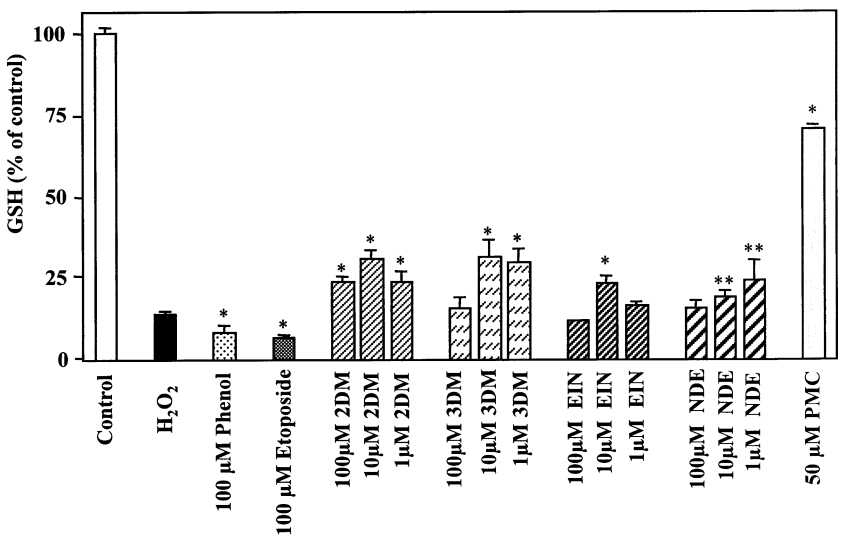


Fig. 3. Effect of colchicine metabolites on H_2O_2-induced GSH oxidation in HL-60 cells. Cells (1×10^6 cells per ml) were incubated in the presence or absence (control) of H_2O_2 in L1210 buffer, pH 7.4 containing DTPA (100 μM) and 3-AT (2.5 mM) for 60 min at 37 °C under a 5% CO_2, 95% air atmosphere. H_2O_2 (12.5 μM) was added into the buffer every 15 min. Cells were treated with PMC (50 μM), etoposide (100 μM), phenol (100 μM) or colchicine metabolites (1, 10 or 100 μM) 15 min before H_2O_2 treatment. Intracellular GSH content was measured using fluorescent probe ThioGlo-1™ as described in Section 2. The GSH content was expressed as a percentage of GSH in untreated control cells (50.43 ± 1.25 nmol/mg protein). Data are mean ± S.D. of three to seven separate experiments. Note: some error bars are too small to visualize. *, $P < 0.01$ and **, $P < 0.05$ vs. H_2O_2-treated cells.

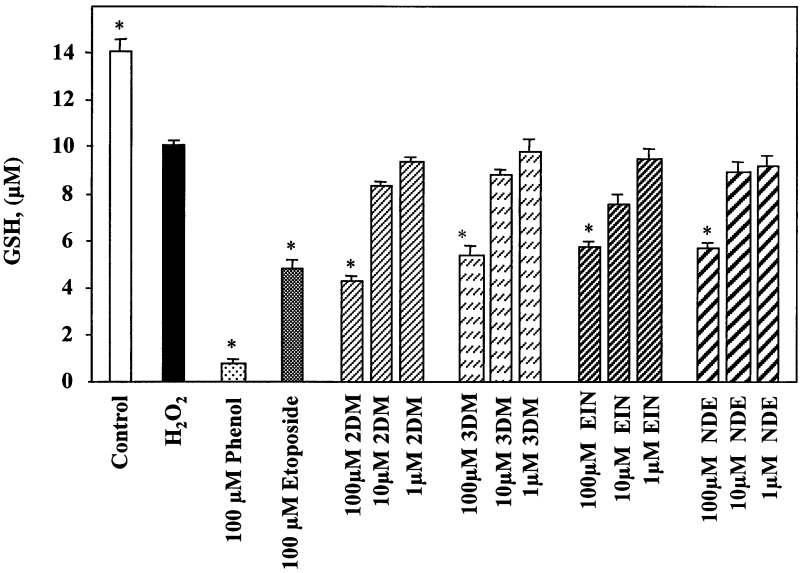


Fig. 4. Effect of colchicine metabolites on HRP-H_2O_2-catalyzed GSH oxidation. Hydrogen peroxide-induced GSH oxidation was evaluated in the presence or absence of colchicine metabolites (1, 10 or 100 μM), phenol (100 μM) or etoposide (VP-16, 100 μM). Samples containing the indicated concentration of tested substance (bar labels) or corresponding volume of DMSO were incubated for 15 min at 37 °C with H_2O_2 (2 mM) and HRP (0.01 U/μl) in 50 mM PBS buffer, pH 7.4, with DTPA (100 μM). After cooling the sample on ice, the GSH content was measured using fluorescent ThioGlo-1™ reagent (see Section 2). Data are mean ± S.D. of three separate experiments. *, $P < 0.001$ vs. H_2O_2 alone.

antioxidant radicals with biomolecules. Moreover, enzymatic one-electron metabolism of such molecules to free radical intermediates (e.g. by peroxidases) also underscores the importance of secondary reactions of antioxidant radicals.

For colchicine, radical scavenging/antioxidant activity was suggested because it offered a simple way to explain certain phenomena that had no apparent connection to its well-known antimitotic activity. It is known to be effective in situations of acute inflammation, i.e. interference with neutrophil oxidative burst, and a radical scavenging role seems to be tempting. The simple 'antioxidant explanation', however, does not seem reasonable for structural reasons: colchicine does not contain functional groups with pronounced electron-donating properties. In our experiments, colchicine failed to show radical formation in a one-electron oxidizing catalytic model system, HRP/H_2O_2 (Fig. 5, trace C). This lends further support to the lack of antioxidant properties in colchicine as has been suggested by others (Das et al., 2000).

The proposed antioxidant role of EIN in vivo cannot be dismissed as readily as that for colchicine (Mourelle et al., 1989; Muriel and Suarez, 1994). EIN antioxidant activity, however, would be suspiciously intense, considering the microgram quantities used in the experiments and accounting for possible accumulation and bio-availability effects. In vivo experiments are too complex to render a simple singular explanation. Rather, several mechanisms of protection against CCl_4-induced lipid peroxidation may be operative because the solvent requires initial activation to $CCl_3^{\bullet}$ radical by cytochrome P450 (CYP) 2E1. Inhibition of catalytic activity or protein synthesis of this CYP isoform by EIN are two distinct possibilities. On the otherhand, lipid peroxidation subsequent to biliary obstruction is not a single event or enzyme dependent feature that may be prevented by EIN. Clearly, additional in vitro evidence is necessary to verify or refute the hypothesis of colchceine as an effective chain-breaking antioxidant against lipid peroxidation.

In this work, we tested our hypothesis in an in vitro cell culture model using human promyelocytic leukemia HL-60 cells. The advantage of this cell line is that it is fairly easily maintained and gives consistent responses to metabolic incorporation of PnA into membrane phospholipids. The disadvantage is that this cell line is often used for studying prospective antitumor drugs (including colchicine) as inducers of apoptosis. Some HL-60 cells treated with colchicine metabolites undoubtedly underwent apoptosis despite the lower antimitotic activities of the metabolites in comparison with the parent drug. To minimize any artifacts caused by accumulation of apoptotic cells we used short-term incubations. Considering the precautions taken, we regard the issue of apoptosis irrelevant because our focus was on the effect of colchicine metabolites on lipid oxidation.

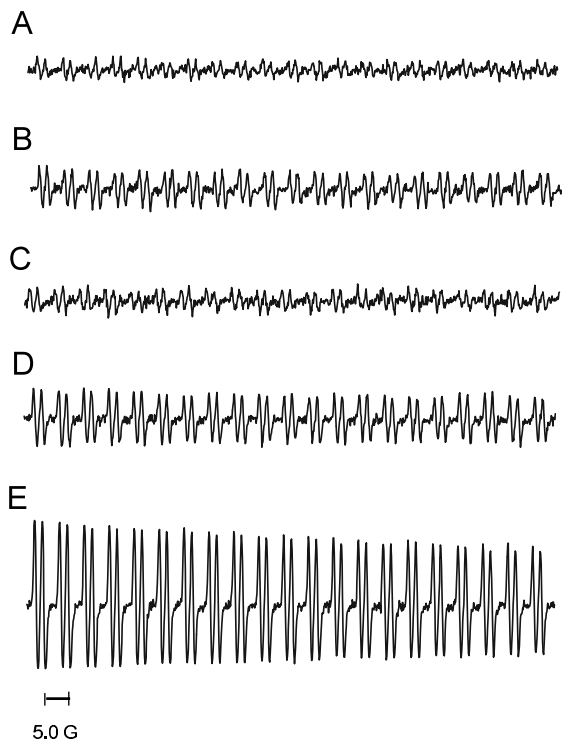


Fig. 5. Effects of colchicine metabolites, etoposide, and phenol on HRP-H_2O_2-catalyzed ascorbate radical formation. ESR signal was recorded in samples containing ascorbate (250 μM), H_2O_2 (400 μM), and HRP (0.03 U/μl) in 50 mM PBS buffer, pH 7.4, with 100 μM DTPA. The following additions were made: trace A, none; trace B, 100 μM etoposide; trace C, 100 μM colchicine; trace D, 100 μM EIN; trace E, 100 μM phenol. Instrument settings were: H_0, 335.5 Mt; sweep width, 0.25 mT; field modulation, 0.079 mT; receiver gain, 4000; time constant, 0.1 s; scan time, 8 min/360 mm; and power, 20 mW.

We used AMVN and AAPH, lipid- and water-soluble azo-initiators of peroxyl radicals, to mimic the effects of $CCl_3^{\bullet}$ radicals and induce phospholipid peroxidation in HL-60 cells. We found that both azo-initiators were very effective and caused pronounced peroxidation of different classes of membrane phospholipids. None of the colchicine metabolites tested, however, was able to render any protection against phospholipid peroxidation independently of whether it was initiated within hydrophobic membrane domains (AMVN) or more polar aqueous environments (AAPH). This may be due to: (i) the inefficiency of radical scavenging or to (ii) the high reactivity of secondary (phenoxyl) radicals formed from colchicine homologs. To gain further insight into these alternative mechanisms, we performed additional experiments in which we utilized MPO/H_2O_2-catalyzed initiation of phospholipid peroxidation.

The colchicine metabolites tested contain electron-donating hydroxy-groups that can readily undergo one-electron oxidation by MPO/H_2O_2 to their respective phenoxyl radicals. Our results clearly demonstrated that even at high concentrations (up to 100 μM), colchicine metabolites were unable to substantially inhibit MPO/H_2O_2-catalyzed phospholipid peroxidation in HL-60 cells (in contrast to a vitamin E homolog, PMC, or the phenolic antitumor drug etoposide). Neither did colchicine metabolites enhance phospholipid peroxidation- the effect readily observed in H_2O_2-treated HL-60 upon exposure to phenol. These results suggest that phenoxyl radicals formed from colchicine homologs are likely to be less reactive than the radicals generated from phenol but reactive enough to directly attack phospholipids and cause their peroxidation.

To obtain further evidence for peroxidase-catalyzed production of phenoxyl radicals from colchicine metabolites, we performed model experiments using HRP/H_2O_2-catalytic system to detect radical formation by one-electron oxidation of ascorbate and ESR monitoring of the ascor-

bate radicals. We found that colchicine metabolites with hydroxy-groups were indeed oxidized to their phenoxyl radicals ESR-detectable by their subsequent reactions with ascorbate as ascorbate radicals.

We further established that phenoxyl radials generated by peroxidases (MPO in HL-60 cells or HRP in model system) from colchicine homologs were able to react with thiols and cause GSH oxidation. This high reactivity of phenoxyl radicals of colchicine metabolites is similar to that of phenoxyl radicals of etoposide or phenol. In contrast, the vitamin E homolog, PMC, protected GSH against peroxidase-catalyzed oxidation suggesting that its phenoxyl radicals are not reactive towards thiols (Kagan et al., 1999).

Taken together, the results demonstrate that even though all colchicine metabolites are capable of forming phenoxyl radicals by reaction with more reactive free radical species, they remain sufficiently reactive to cause not only consumption of glutathione and ascorbate but also oxidation of phospholipids. Thus, they are not chain-breaking antioxidants. This finding rules out the hypothesis that EIN, and probably other colchicine metabolites, can prevent lipid peroxidation via direct antioxidant action. It does not, however, rule out the possibility that they can protect against lipid peroxidation by other means such as modulation of neutrophil 'oxidative burst' associated with inflammation.

Acknowledgements

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