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Calcium antagonist and antiperoxidant properties of some hindered phenols

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1 The calcium antagonist and antioxidant activities of certain synthetic and natural phenols, related to BHA (2-*t*-butyl-4-methoxyphenol), were evaluated in rat ileal longitudinal muscle and in lipid peroxidation models respectively.

2 Compounds with a phenol or a phenol derivative moiety, with the exception of 2,2'-dihydroxy-3,-3'-di-*t*-butyl-5,5'-dimethoxydiphenyl (di-BHA), inhibited in a concentration-dependent manner the $BaCl_2$-induced contraction of muscle incubated in a Ca^{2+}-free medium. Calculated pIC_{50} (M) values ranged between 3.32 (probucol) and 4.96 [3,5-di-*t*-butyl-4-hydroxyanisole (di-*t*-BHA)], with intermediate activity shown by khellin < gossypol < quercetin < 3-*t*-butylanisole < BHA < nordihydroguaiaretic acid (NDGA) < 2,6-di-*t*-butyl-4-methylphenol (BHT) and papaverine.

3 The Ca^{2+} channel activator Bay K 8644 overcame the inhibition sustained by nifedipine, BHA and BHT, while only partially reversing that of papaverine.

4 BHA, BHT, nifedipine and papaverine also inhibited in a concentration-dependent fashion $CaCl_2$ contractions of muscle depolarized by a K^+-rich medium. This inhibition appeared to be inversely affected by the Ca^{2+}-concentration used.

5 The inhibitory effects of nifedipine, papaverine, BHA and BHT were no longer present when muscle contraction was elicited in skinned fibres by 5 μM Ca^{2+} or 500 μM Ba^{2+}, suggesting a plasmalemmal involvement of target sites in spasmolysis.

6 Comparative antioxidant capability was assessed in two peroxyl radical scavenging assay systems. These were based either on the oxidation of linoleic acid initiated by a heat labile azo compound or on lipid peroxidation of rat liver microsomes promoted by Fe^{2+} ions. Across both model systems, di-*t*-BHA, NDGA, BHT, di-BHA, BHA and quercetin ranked as the most potent inhibitors of lipid oxidation, with calculated pIC_{50} (M) values ranging between 7.4 and 5.7.

7 Of the 32 compounds studied only 15 phenolic derivatives exhibited both antispasmogenic and antioxidant activity. Within this subgroup a linear and significant correlation was found between antispasmogenic activity and antioxidation. These bifunctional compounds were characterized by the presence of at least one hydroxyl group on the aromatic ring and a highly lipophilic area in the molecule.

8 Di-*t*-BHA is proposed as a lead reference compound for future synthesis of new antioxidants combining two potentially useful properties in the prevention of tissue damage after ischaemia-reperfusion injury.

Keywords: Phenol derivatives; calcium antagonist; antioxidant; 2-*t*-butyl-4-methoxyphenol (BHA); 2,6-di-*t*-butyl-4-methylphenol (BHT)

Introduction

Superoxide formation ($O_2^{\cdot-}$) appears central to the pathological process attendant on ischaemia-reperfusion injury. In experimental animals, free radicals are generated in two main ways during ischaemia: when xanthine oxidase is formed subsequent to a calcium-triggered proteolytic attack on xanthine dehydrogenase (Battelli *et al.*, 1972; Della Corte & Stirpe, 1972) and during disorders of electron transport in anoxic-reoxygenated mitochondria (Boveris & Turrens, 1981; Naqui *et al.*, 1986). The direct effects of $O_2^{\cdot-}$ include membrane de-esterification of the apolar regions, lipid peroxidation after Fe^{2+} release (Deby & Goutier, 1990) and a redox cycle when hydrogen peroxide is converted to the reactive hydroxyl radical. This circular process perpetuates the formation of $O_2^{\cdot-}$ and an increased release of non-esterified fatty acids. This, in turn, may promote intracellular increase in calcium concentration due to anoxia. The formation of hypochlorous acid from reactive oxygen species may also contribute to tissue damage.

It is now recognized that disturbances in the homeostasis of intracellular Ca^{2+} may underlie a variety of toxicological and pathological processes. Several potential mechanisms have been identified whereby changes in intracellular free calcium trigger cytotoxic processes. Calcium ions activate phospholipase A_2 (PLA_2) and a number of proteinases and nucleases. The observation that sustained increase of intracellular Ca^{2+} causes membrane breakdown and subsequent cell damage is supported by the fact that PLA_2 inhibitors prevent ischaemic cell damage in liver and heart and by the finding that phospholipid hydrolysis is enhanced during tissue injury. Evidence that calcium ions mediate or propagate ischaemic cell damage (Nayler *et al.*, 1979; Katz & Reuter, 1979) is borne out by reports that Ca^{2+} increases the damage caused by oxygen free radicals to the mitochondrial electron transport chain (Malis & Bonventre, 1986). All these observations suggest that reperfusion or post-ischaemic tissue injury is a complex phenomenon to which several interrelated factors may contribute.

Different protective strategies might be envisaged to curtail tissue damage. There is some evidence for the clinical effectiveness of the enzyme superoxide dismutase, which catalyzes the dismutation of $O_2^{\cdot-}$ to form hydrogen peroxide and oxygen (Fridovich, 1986; Michelson, 1986; Marklund, 1986). This would not eliminate the noxious effects of hydrogen peroxide, itself a source of oxygen-derived radicals, in the presence of transition metals, though supplement with catalase might regress this problem. Tissue integrity is also

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challenged by the hypochlorous acid released by reactive oxygen species. Though xanthine oxidase inhibitors such as allopurinol and oxypurinol may be of value in certain cases, their applicability to man is still unclear (McCord, 1985).

From a strictly pharmacological point of view, tissue damage may be countered either upstream by compounds that scavenge the free radicals or downstream by limiting the increase of free intracellular calcium.

The effectiveness of hydroxyl or peroxyl radical scavengers has been established in animal models by the novel butylated hydroxytoluene (BHT) derivatives, such as the thiazolidinone derivative LY256548 (Ruterbories & Lindstrom, 1990), *p*-(pyrrolidinylmethylene)butylated hydroxytoluene (E-5110) (Shirota *et al.*, 1987) and 2-(allyl-1-piperazinyl)-4-n-amyloxyquinazoline fumarate (KB-5666) (Hara & Kogure, 1990).

Downstream protection may be afforded by calcium channel blockers such as verapamil and nifedipine which reduce calcium uptake from the extracellular space during or after ischaemia. Though some evidence has been marshalled for the clinical effectiveness of calcium antagonists (the Danish Study Group on Verapamil in Myocardial Infarction, 1990; the Multicenter Diltiazem Postinfarction Trial Research Group, 1988), the precise means by which they prevent ischaemia-reperfusion damage remains a matter for further elucidation (Cheung *et al.*, 1986; Nayler, 1992).

Results obtained in this laboratory (Sgaragli *et al.*, 1989) indicate that 2-*t*-butyl-4-methoxyphenol (BHA), besides its well-known antioxidant effect may also exhibit nifedipine-like spasmolytic activity. Molecules which combine both scavenging and calcium antagonist properties may be of particular value in protecting against ischaemia-reperfusion tissue damage. The focus of this paper is directed at identifying such dual-purpose molecules and comparing their performance against those that act simply as either calcium channel blockers or radical scavengers.

Methods

Animals

Male Sprague-Dawley rats (200–370 g body weight) were purchased from Società S. Morini S.p.a., S. Polo d'Enza, Italy. They were housed in a normal environmentally controlled animal room (20–22°C) with a 12 h alternating light/dark cycle, and had free access to food pellets and water. One kg of rat pellets contained: vitamin A 15,000 iu; vitamin D_3 1,500 iu; vitamin K_3 2 mg; vitamin E 30 mg; vitamin B_1 3 mg; vitamin B_2 5 mg; vitamin C 40 mg; vitamin B_6 3 mg; B_{12} 0.03 mg; D-pantothenic acid 15 mg; folic acid 1 mg; vitamin PP 30 mg; choline chloride 400 mg; manganese 60 mg; iron 150 mg; copper 5 mg; zinc 30 mg; iodine 1 mg; cobalt 0.2 mg; BHT 10 mg (Società S. Morini S.p.a., S. Polo d'Enza, Italy).

Assays on intact rat ileum longitudinal muscle preparations

These preparations were used in two distinct series of contractility experiments under isometric conditions (Basile, Comerio, Italy). In the first series, segments of longitudinal muscle (1.5–2.0 cm, in length; 60–80 mg, weight) were suspended under 500 mg tension in a 6 ml chamber filled with a modified Krebs-Henseleit solution containing (mM final concentration): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 5, $CaCl_2$ 2.5 and gassed with a 95% O_2:5% CO_2 mixture at 37°C. After 30 min equilibration, contractility was tested by stimulating the tissue electrically or chemically with acetylcholine (ACh). Five s train stimuli (60 V at 10 Hz for 2 ms) were delivered through two silver electrodes placed at the top and bottom of the bath chamber. After washing with the incubation solution, ACh was added at three different final concentrations (0.2, 1.6 and 10 μM). Muscle contraction was then elicited by the addition of $BaCl_2$ at different final concentrations. The subsequent assays were performed in the same medium deprived of $CaCl_2$. The omission of $CaCl_2$ from the bathing solution abolished any spontaneous activity as well as any electrically stimulated muscle contractions. Muscle contraction was elicited by the addition of $BaCl_2$ at different final concentrations every 15 min; between each addition extensive washing reduced tissue tension to baseline values. $BaCl_2$-induced responses were concentration-dependent and rapid in onset, an initial peak (phasic) being followed by a more sustained contraction (tonic).

The addition of Ba^{2+} to both incubation solutions, whether free or supplemented by Ca^{2+}, caused a concentration-dependent contraction of muscle tissue. ED_{50} (phasic phase) was 1.71 ± 0.14 mM ($n = 6$) in the presence of Ca^{2+} and 5.34 ± 0.06 mM ($n = 7$) in its absence. The synergistic action of Ca^{2+}, however, disappeared at the highest concentration of Ba^{2+} leaving the maximal tension unaltered. The presence of Ca^{2+} affected the constancy of response over time. Contractility with 2.5 mM Ca^{2+} decreased in a steady, and progressive manner in response to Ba^{2+}, dropping by approximately 40% over 4 h of incubation. In the absence of Ca^{2+}, this decrease took place in the first 90 min and stabilized thereafter.

In a separate series of experiments, the addition of 35 mM dimethylsulphoxide (DMSO) for up to 4 h had no effect on Ba^{2+}-elicited responses in a Ca^{2+}-deprived medium.

In the second experimental series, segments of longitudinal muscle were initially subjected to 500 mg tension and bathed by a MOPS-buffered physiological salt solution (MOPS-PSS) composed as follows (mM final concentration): NaCl 129.7, KCl 5.9, $CaCl_2$ 2.54, $MgCl_2$ 1.19, MOPS 10 and glucose 11.1. The pH of the solution was adjusted to 7.4 with NaOH. After equilibration the bath fluid was changed to a K^+-rich, Ca^{2+}-free MOPS-PSS which contained K^+ at 40 mM, sufficient to depolarize intestinal smooth muscle (Spedding, 1982). This produced a spasm which was dissipated by regular changes of the K^+-rich, Ca^{2+}-free bath fluid. The spasmogenic response to $CaCl_2$ was studied by constructing cumulative concentration-effect curves. The 15 min contact time for each concentration of $CaCl_2$ was sufficient to attain optimal isometric tension.

An initial concentration-effect curve was derived for $CaCl_2$ followed by two others. In test tissues, calcium antagonists were present for 30 min before and throughout the second and the third concentration-effect curves and increased 30 min before the third curve. Control tissues were treated similarly, without exposure to calcium antagonists. The inhibitory effect of all antagonists at the two concentrations was measured as the reduction (%) of the initial spasmogenic effect of $CaCl_2$ 0.1–10 mM.

Assays on skinned rat ileum longitudinal muscle preparations

Segments of rat ileum longitudinal muscle were skinned of their plasmalemma according to a procedure prescribed for vascular smooth muscle from rabbit renal arteries (Kreye *et al.*, 1983). Pieces of tissue (0.5 mm wide, 3–5 mm length) were attached with acrylate glue (Loctite) by both ends to the small part of two L-shaped stainless steel rods and kept in a vertical position under 200 mg tension. The skinning procedure consisted of an initial 30 min incubation in an aqueous solution (pH 7.4) containing 5 mM EGTA, 20 mM imidazole, 50 mM KCl, and 150 mM sucrose at 4°C, and a subsequent 1 h incubation in the same solution with the addition of 1% Triton X-100 and 0.5 mM dithioerythritol at 4°C, and followed by a final 30 min incubation in a solution containing 50% glycerol (v/v), 10 mM imidazole, 2 mM EGTA, 5 mM $MgCl_2$, 0.5 mM NaN_3, 3.75 mM ATP, 5 mM creatine phosphate and 0.5 mM dithioerythritol at − 20°C. The preparations were stored in the latter solution at − 20°C for up to 6 weeks. For the isometric contraction and relaxation studies,

the preparations, still attached to the rods, were transferred into 10 ml thermostatted organ bath chambers and connected to high sensitivity isometric transducers (Basile, Comerio, Varese, Italy). Contractile change was induced by alternating the 'relaxing' and 'contracting solution'. The relaxing solution (pCa > 8, pH 6.7) contained 20 mM imidazole, 4 mM EGTA, 10 mM $MgCl_2$, 1 mM NaN_3, 2 mM dithioerythritol, 7.5 mM ATP, 10 mM creatine phosphate and 10 units ml^{-1} of creatine kinase. The contracting solution, containing 5 μM $CaCl_2$, was a Krebs-Henseleit solution. For Ba^{2+}-induced contractions the contracting solution was nominally Ca^{2+}- and phosphate-free but contained 500 μM $BaCl_2$. The electrical transduction signals were amplified and displayed on a Basile strip recorder (Mod. Gemini). All contraction studies were performed at room temperature.

Comparative assessment of the antioxidant properties of phenol compounds: inhibition of lipid peroxidation

Phenol derivatives were assessed for their capacity to prevent lipid peroxidation by two experimental model systems. The first was based on the oxidation of linoleic acid initiated by 2,2'-azobis-2-amidinopropane hydrochloride (ABAP), a thermolabile azo compound which, on decomposition, forms radicals that abstract hydrogen atoms from linoleic acid. ABAP (11 mM) was added to a suspension of linoleic acid (33 mM) in 50 mM Na phosphate buffer pH 7.4, in the chamber of an O_2 electrode (Model 5300 Biological Oxygen monitor, Yellow Spring Instrument Co., Inc. Yellow Spring, Ohio, U.S.A.) thermostatted at 37°C (Wayner *et al.*, 1987). O_2 consumption was monitored for approximately 6 min before adding the antioxidant at different concentrations. O_2 consumption due to ABAP decomposition was determined separately and subtracted from the peroxidation rate of linoleic acid. In the second model, peroxidation of rat liver microsomes, by a peroxidizing mixture of 100 μM Fe^{2+}/Fe^{3+} and 100 μM ascorbic acid, was measured at 37°C as O_2 consumption. Reaction mixtures in a final volume of 3 ml contained 0.5 mg microsomal protein, with 57 mM ethanol or 47 mM DMSO vehicle and 20 mM KH_2PO_4-KOH buffer, pH 6.0.

Drugs: commercial sources and synthetic procedures

Phenol from Merk (Darmstadt, Germany) and phenol derivatives 3,5-di-*t*-butyl-4-hydroxyanisole (di-*t*-BHA) and 2,4,6-tri-*t*-butylphenol (TTP) from Aldrich-Chemie (Steinheim, Germany) were recrystallized once prior to use from ethanol. BHA and BHT from Fluka Chemie AG (Buchs, Switzerland) were recrystallized once prior to study from petroleum ether and ethanol respectively. 2,2'-Dihydroxy-3,3'-di-*t*-butyl-5,5'-dimethoxydiphenyl (di-BHA, dimer of BHA) was synthesized by direct oxidation of BHA as described elsewhere (Sgaragli *et al.*, 1980). 3-*t*-Butylanisole was synthesized from 3-*t*-butylphenol by methylation with diazomethane. After 24 h incubation at room temperature and normal pressure the mixture was washed with 2 N NaOH to remove the unreacted phenol. 3-*t*-Butylanisole was consequently purified by vacuum distillation. 1,4-Dimethoxy-2-*t*-butylbenzene was synthesized from BHA by methylation with CH_3I in the presence of Na methanolate. After 24 h incubation at room temperature and normal pressure the mixture was washed with 2 N NaOH to remove unreacted BHA. 1,4-Dimethoxy-2-*t*-butylbenzene was subsequently purified by vacuum distillation. Nifedipine, nordihydroguaiaretic acid (NDGA), gossypol and linoleic acid were purchased from Sigma Chemical Company (U.S.A.). Aldrich-Chemie (Steinheim, Germany) supplied 2,6-di-*t*-butyl-*p*-benzoquinone, 3,5-di-*t*-butyl-1,2-benzoquinone, 2-*t*-butylphenol, 3-*t*-butylphenol, 4-*t*-butylphenol, 4-*t*-butylphenyl-2,3-epoxypropylether, quercetin, khellin, eugenol, 2,4-di-*t*-butyl-6-(4-methoxybenzil) phenol, 3-hydroxyanisole, 1,8-dihydroxyanthraquinone, caffeic acid, trolox, anthraquinone, 4-*t*-butylbenzoic acid, 4-*t*-butylpyridine, *t*-butylbenzene and *t*-butylcyclohexane. Papaverine was purchased from Merk (Darmstadt, Germany). ABAP was purchased from Polysciences Inc. (U.S.A.). Silymarin, a mixture of three isomers (silybin, silidianin and silicristin) was a generous gift of Istituto Biochimico Italiano 'Giovanni Lorenzini' S.p.a. (Milan, Italy). Probucol was obtained from Lepetit S.p.a. (Milan, Italy). Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was a generous gift of Dr Franckoviack. All other compounds were of analytical grade and were used without further purification.

Compounds were diluted in DMSO or ethanol or water to give stock solutions stored at 4°C until use. All stock solutions were shielded from light with aluminium foil. Because of the known photosensitivity of the dihydropyridines, the experiments with nifedipine and Bay K 8644 were performed in the dark. The water used to prepare the calcium- and phosphate-free Krebs-Henseleit solution was first distilled and then passed through a NANOpure II deionization system (Barnstead-Sybron, Boston, U.S.A.), to obtain Type I Reagent Grade water (resistivity 18 MΩ).

Statistical analyses

Data are presented as means ± s.e.mean; n is the number of independent experiments. Statistical analysis was performed by Student's t test (paired to compare drug effects at different Ba^{2+} concentrations). P values < 0.05 were considered significant. The pharmacological response to each substance was described, where possible, by the pIC_{50} value (the negative $\log_{10}$ of the molar concentration at which the substance inhibits 50% of the maximum response). pIC_{50} values were estimated by linear regression analysis.

Results

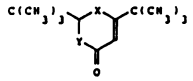
Inhibiting effects of hindered phenols in ileal smooth muscle: structure-activity

In a structure-activity relation study various model phenol compounds (see Table 1) were assessed to ascertain the requirements for inhibiting Ba^{2+}-induced muscle contraction in a nominally Ca^{2+}-free solution.

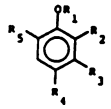
Table 2 shows pIC_{50} (M) values for some of the tested compounds (less than 10% inhibition was considered meaningless). Compounds which exhibited antispasmogenic activity were, at least, three orders of magnitude less potent than nifedipine ($pIC_{50} = 8.02 \pm 0.01$; $n = 5$). When either the hydroxyl- and *t*-butyl-moieties were present on the aromatic ring, compounds showed antispasmogenic properties, with pIC_{50} values ranging between 4.56 (2-*t*-butylphenol) and 4.08 (4-*t*-butylphenol). Compounds with only one of these groups on the ring were ineffective (phenol, *t*-butylcyclohexane, *t*-butylbenzene and 4-*t*-butylpyridine). This demonstrates that the presence of both groups is essential for activity. Additional tests included the study of the three different series of phenol derivatives obtained by substitution in the *o*-, *m*- and *p*-position. Among the *p*-substituted compounds, activity increased when the hydroxyl group was derivatized, even with a bulky moiety such as 2,3-epoxypropyl, but disappeared when the hydroxyl group was replaced by a carboxyl one as in the case of 4-*t*-butylbenzoic acid. Other carboxylic compounds, such as caffeic acid and trolox, were shown to be inactive. In the *m*-substituted series, activity was still maintained after derivatization of the hydroxyl group (3-*t*-butylanisole), while the substitution of the *t*-butyl moiety with a methoxy one made the 3-hydroxyanisole compound inactive. Finally, in the *o*-substituted derivatives (the closest series to our lead compounds BHA and BHT), the introduction of a *p*-methoxyl moiety into 2-*t*-butylphenol did not appreciably change the activity of the resulting BHA with

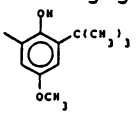
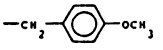
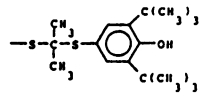
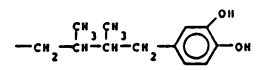
Table 1 General structures of phenol derivatives studied

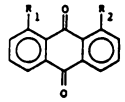
		X
1	*t*-Butylcyclohexane	C
2	*t*-Butylbenzene	C
3	4-*t*-Butylpyridine	N



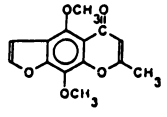
		X	Y
4	2,6-di-*t*-Butyl-*p*-benzoquinone	C=O	H
5	3,5-di-*t*-Butyl-1,2-benzoquinone	H	C=O



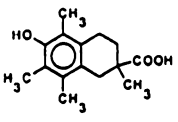
		R_1	R_2	R_3	R_4	R_5
6	Phenol	H	H	H	H	H
7	2-*t*-Butylphenol	H	$C(CH_3)_3$	H	H	H
8	BHA	H	$C(CH_3)_3$	H	OCH_3	H
9	1,4-Dimethoxy--2-*t*-butylbenzene	CH_3	$C(CH_3)_3$	H	OCH_3	H
10	3-*t*-Butylphenol	H	H	$C(CH_3)_3$	H	H
11	3-*t*-Butylanisole	CH_3	H	$C(CH_3)_3$	H	H
12	3-Hydroxyanisole	H	H	OCH_3	H	H
13	4-*t*-Butylphenol	H	H	H	$C(CH_3)_3$	H
14	4-*t*-Butylphenyl-2,3--epoxypropylether	CH_2-CH-CH_2 (O)	H	H	$C(CH_3)_3$	H
15	4-*t*-Butylbenzoic acid	COOH	H	H	$C(CH_3)_3$	H
16	TTP	H	$C(CH_3)_3$	H	$C(CH_3)_3$	$C(CH_3)_3$
17	di-*t*-BHA	H	$C(CH_3)_3$	H	OCH_3	$C(CH_3)_3$
18	BHT	H	$C(CH_3)_3$	H	CH_3	$C(CH_3)_3$
19	di-BHA	H	$C(CH_3)_3$	H	OCH_3	
20	2,4-di-*t*-Butyl-6-(4--methoxybenzil)phenol	H	$C(CH_3)_3$	H	$C(CH_3)_3$	
21	Probucol	H	$C(CH_3)_3$	H		$C(CH_3)_3$
22	Caffeic acid	H	OH	H	CH=CH-COOH	H
23	Eugenol	H	OCH_3	H	CH_2-CH=CH_2	H
24	NDGA	H	OH	H		H



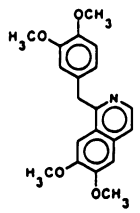
		R_1	R_2
25	Anthraquinone	H	H
26	1,8-Dihydroxyanthraquinone	OH	OH



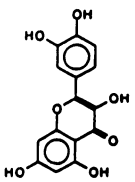
27 Khellin



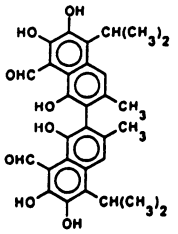
28 Trolox



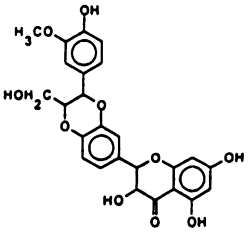
29 Papaverine



30 Quercetin



31 Gossypol



32 Silymarin

Table 2 Inhibition of Ba^{2+}-induced contractions in rat ileum longitudinal muscle preparations

Compounds	pIC_{50} (M)
Mono substituted hydrocarbons	
4-*t*-Butylpyridine	Inactive at 100 μM
t-Butylcyclohexane	Inactive at 200 μM
t-Butylbenzene	Inactive at 200 μM
Phenols	
Phenol	Inactive at 400 μM
o-*Substituted*	
2-*t*-Butylphenol	4.56 ± 0.07
BHA	4.34 ± 0.05
1,4-Dimethoxy-2-*t*-butylbenzene	4.30 ± 0.08
Khellin	4.03 ± 0.06
m-*Substituted*	
3-*t*-Butylanisole	4.29 ± 0.14
3-*t*-Butylphenol	4.24 ± 0.12
3-Hydroxyanisole	Inactive at 100 μM
p-*Substituted*	
4-*t*-Butylphenyl-2,3-epoxypropylether	4.39 ± 0.01
4-*t*-Butylphenol	4.08 ± 0.01
4-*t*-Butylbenzoic acid	Inactive at 200 μM
o-*di-Substituted*	
di-*t*-BHA	4.96 ± 0.06
BHT	4.68 ± 0.05
2,4-di-*t*-Butyl-6-(4-methoxybenzil)phenol	3.64 ± 0.31
TTP	3.34 ± 0.24
Probucol	3.32 ± 0.03
di-BHA	Inactive at 200 μM
poly-Substituted	
Trolox	Inactive at 1 mM
Natural polyphenol derivatives	
Papaverine	4.78 ± 0.08
NDGA	4.57 ± 0.03
Quercetin	4.26 ± 0.08
Gossypol	4.24 ± 0.08
Eugenol	3.87 ± 0.04
Silymarin	3.74 ± 0.13
Caffeic acid	Inactive at 5 mM
Quinones	
2,6-di-*t*-Butyl-p-benzoquinone	4.67 ± 0.01
3,5-di-*t*-Butyl-1,2-benzoquinone	4.66 ± 0.01
1,8-Dihydroxyanthraquinone	3.40 ± 0.68
Anthraquinone	Inactive at 200 μM

$BaCl_2$-induced contractions were developed at 37°C in a $CaCl_2$-free, modified Krebs-Henseleit solution gassed with a 95% O_2:5% CO_2 mixture. The various agents were added 5 min before $BaCl_2$ (6.25 mM). For further details see Methods section. Figures represent mean values ± s.e.mean ($n = 3-12$).

respect to the parent compound. Similarly little change occurred when the phenolic hydroxyl moiety was derivatized, giving rise to 1,4-dimethoxy-2-*t*-butylbenzene. Though lacking a *t*-butyl group, we considered khellin a lipophilic *p*-dimethoxy-benzene derivative of 1,4-dimethoxy-2-*t*-butylbenzene which showed a comparable pIC_{50} value to its parent compound. Compounds of a broader hindrance than BHA, namely BHT and di-*t*-BHA, characterized by the presence of 2,6-di-*t*-butyl groups, were among the most effective spasmolytics. Though diminished activity was still present when the *p*-methyl group was substituted by a *t*-butyl one (TTP) or by another BHT-analogue attached via an isopropylidenedithio bridge (probucol). This loss of activity might be a consequence of increased molecular size. In fact when di-*t*-BHA was substituted by a BHA radical in 2-position, the enlarged di-BHA molecule proved devoid of activity. However, molecular size alone could not account for suppression of activity as could be seen when the 2-*t*-butyl group of TTP was replaced by a 4-methoxybenzil radical, giving a 2,4-di-*t*-butyl-6-(4-methoxybenzil)phenol of greater efficacy than the parent compound.

Among the quinone derivatives, only those with two *t*-butyl groups were effective. The other test compounds were a series of natural polyphenolic derivatives. Of these the catechol-derivatizated papaverine and the catechol derivative NDGA were the most potent antispasmogenics and performed equivalently to BHT.

Mechanism of the antispasmogenic action of BHA, BHT and papaverine: reversal of the inhibition by Bay K 8644

As summarized in Figure 1, nifedipine, papaverine, BHA and BHT markedly inhibited in decreasing order of potency the Ba^{2+}-induced contraction of ileal preparations. Nifedipine was the strongest suppressor at concentrations three orders of magnitude lower than that of the other compounds.

When the same test was performed after addition of 1 μM Bay K 8644 there was an approximate 50% increase over control conditions in responsiveness to Ba^{2+}. The addition of Bay K 8644 to the preparations pre-incubated with BHA, BHT and nifedipine, abolished any inhibition except in the case of papaverine where inhibition was only partially reversed.

Studies with depolarized muscles

In K^+-depolarized tissues, $CaCl_2$ caused concentration-dependent contraction which became maximal at 10 mM. In control tissues the $CaCl_2$ concentration-effect curve showed no appreciable changes when repeated over time. The effects of BHA, BHT, papaverine and nifedipine are depicted in Figure 2. DMSO and ethanol, used to dissolve the drugs, had different effects on the preparations (data not shown). While DMSO, at 14.1 mM concentration had no effect, ethanol at 17.1 mM concentration increased the sensitivity of the preparation to $CaCl_2$ as could be seen in the leftward and upward shift of the $\log_{10}$ concentration-response curve. BHA (17.5 and 35 μM), BHT (10 and 20 μM), papaverine (8 and 25 μM) and nifedipine (5 and 10 nM) each antagonized $CaCl_2$ in a concentration-dependent manner. Figure 2 (a–d) shows that this antagonism comprised both a shift to the right of the log concentration-effect curve for $CaCl_2$ and a depression of the maximal response. The calculated mean pIC_{50} values were 4.47, 4.94, 4.84 and 9.01 for BHA, BHT, papaverine and nifedipine, respectively.

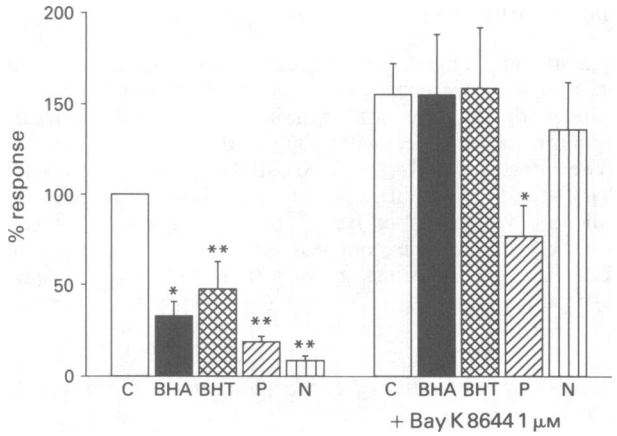


Figure 1 Inhibition of Ba^{2+}-induced contractions in rat ileum longitudinal muscle preparations and its reversal by Bay K 8644. Responses (%) were calculated with respect to paired controls (C). Columns represent mean values with s.e.mean ($n = 4-15$). BHA 50 μM, BHT 25 μM, papaverine 25 μM (P) (in 35 mM DMSO, final concentration) and nifedipine 50 nM (N; in 29 mM ethanol) were always present in the physiological solution, while Bay K 8644 was added 5 min before $BaCl_2$ (6.25 mM). For Methods see Table 2. The significance of differences was calculated by use of Student's *t* test. For abbreviations, see text. $^*P<0.05$; $^{**}P<0.01$. Tension developed in control + Bay K 8644 (1.01 ± 0.11 g) was significantly different ($P<0.01$; $n = 15$) from that developed in control alone (0.65 ± 0.09 g).

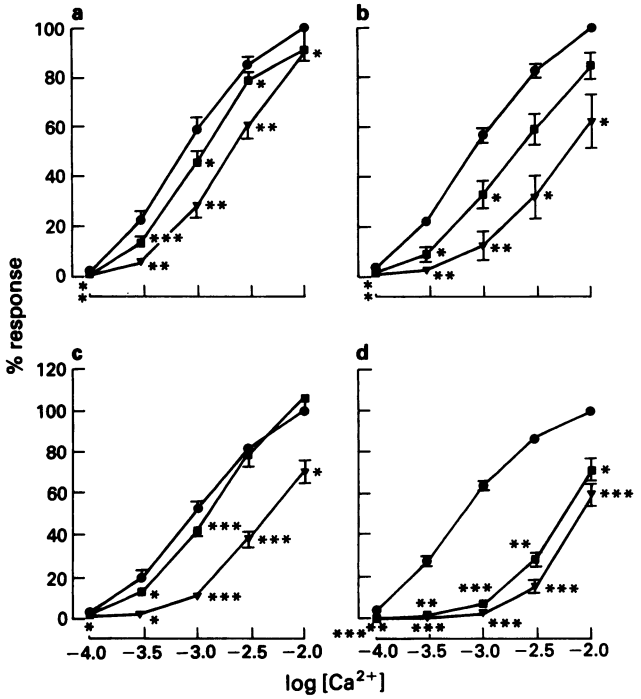


Figure 2 The effect of BHA, BHT, papaverine and nifedipine on the response to $CaCl_2$ of rat ileum longitudinal muscle in K^+-rich (40 mM), Ca^{2+}-free, MOPS-PSS. The abscissae indicate the concentration of $CaCl_2$ (M) on a $\log_{10}$ scale. The ordinates indicate spasm as a % of the maximal response to $CaCl_2$ in the absence of antagonists. Points represent mean values ± s.e.mean, ($n = 4-8$): (●) initial $\log_{10}$ concentration-effect curve for $CaCl_2$ in the presence of vehicles (DMSO for BHA, BHT and papaverine; ethanol for nifedipine), control; (■) second $\log_{10}$ concentration-effect curve for $CaCl_2$ obtained after tissue equilibration for 30 min in (a) BHA 17.5 μM, (b) BHT 10 μM, (c) papaverine 8 μM, (d) nifedipine 5 nM; (▼) third $\log_{10}$ concentration-effect curve for $CaCl_2$ obtained after an additional 30 min period of equilibration in (a) BHA 35 μM, (b) BHT 20 μM, (c) papaverine 25 μM, (d) nifedipine 10 nM. The significance of differences was calculated by use of Student's t test. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

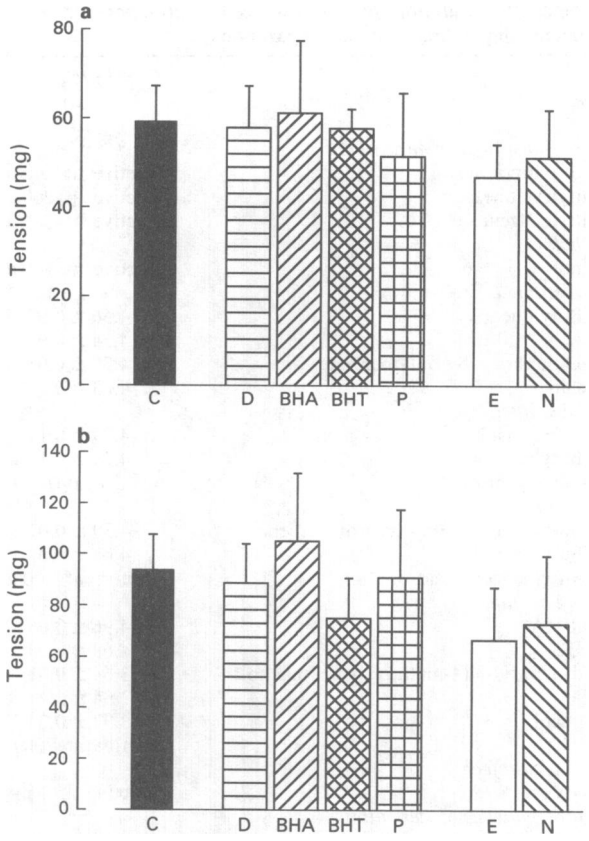


Figure 3 Effects of various agents on (a) Ba^{2+}- and (b) Ca^{2+}-induced contractions of rat ileum longitudinal skinned muscle. Contractions were developed at room temperature in a Krebs-Henseleit solution containing 0.5 mM Ba^{2+} or 5 μM Ca^{2+} gassed with 95% O_2:5% CO_2 mixture. Columns represent mean values with s.e.mean ($n = 4-14$). BHA 10 μM, BHT 50 μM, papaverine 50 μM (P) [in 140 mM DMSO (D) final concentration] and nifedipine 50 nM (N) [in 172 mM ethanol (E)] were added 5 min before $BaCl_2$ or $CaCl_2$ (C). The significance of differences was calculated by use of Student's t test.

Studies with skinned ileal preparations

Segments of skinned longitudinal ileum contracted in response to low concentrations of both Ba^{2+} and Ca^{2+}.

Under the experimental conditions, maximal contractile response was obtained with 500 μM Ba^{2+} or 5 μM Ca^{2+}.

The effects of BHA (100 μM), BHT (50 μM), papaverine (50 μM) and nifedipine (50 nM) on skinned preparations challenged with Ca^{2+} or Ba^{2+}, are summarized in Figure 3. As may be seen, the compounds were without inhibitory effects at concentrations greater than the IC_{50}s in intact preparations.

Antioxidant properties of hindered phenols: inhibition of lipid peroxidation

Among the group of antispasmogenic phenols, 27 compounds were assessed for their prevention of lipid peroxidation. Two model systems were employed as described previously (see Methods). Almost all compounds showed some degree of inhibition of lipid peroxidation as seen in Table 3. The degree of inhibition in linoleic acid was greater than that in the microsomal system. Ranking of inhibition according to potency was almost the same in both model systems, and with a wide range of potency (IC_{50}s 0.04–120 and 0.13–933 μM, respectively).

t-Butylbenzene, 1,8-dihydroxyanthraquinone, 4-*t*-butylphenyl-2,3-epoxypropylether, papaverine, khellin, 2,6-di-*t*-butyl-*p*-benzoquinone, 3-*t*-butylanisole and 1,4-dimethoxy-2-*t*-butylbenzene were inactive at concentrations ⩾ 100 μM. The vehicle, whether DMSO or ethanol, exerted no significant effect (data not shown) at the concentrations used.

Di-*t*-BHA, the most potent of the compounds tested, with antiperoxidant IC_{50} values of 0.04 and 0.13 μM in the linoleate and microsome system respectively, showed 100% inhibition at concentrations of 0.56 and 10 μM, respectively. BHA and BHT displayed IC_{50} values of 0.45 and 2.30 and 0.17 and 1.50 μM in the two model systems, respectively. Fifty percent inhibition of peroxidation by di-BHA required 0.22 and 1.0 μM concentration in both models. This indicates that the dimer maintains the antiperoxidant activity of the parent BHA. Quercetin and gossypol also exhibited remarkable antiperoxidant activity. The other compounds, in particular eugenol, silymarin, trolox and probucol showed IC_{50} values approximately two orders of magnitude higher than di-*t*-BHA.

The dihydropyridine derivative, nifedipine, protected the lipids against oxidation, but only at very high concentrations.

Discussion

This work demonstrates the dual action of some phenol derivatives – namely their antispasmogenic activity on longitudinal ileum musculature and their antioxidant property on microsomal and linoleate systems.

Table 3 Inhibition of lipid peroxidation (peroxyl radical scavenging property)

	Radical generating system/substrate	
	ABAP/linoleic acid	*Fe-ascorbic acid/microsomes*
Antioxidant	pIC_{50} (M)	pIC_{50} (M)
di-*t*-BHA	7.38	6.89
NDGA	6.80	6.01
BHT	6.77	5.82
di-BHA	6.66	6.00
3,5-di-*t*-Butyl-1,2-benzoquinone		5.98
BHA	6.35	5.64
Quercetin	6.30	5.66
TTP		5.63
2,4-di-*t*-Butyl-6-(4-methoxy-benzil)phenol		5.43
Gossypol	5.96	5.70
2-*t*-Butylphenol		4.91
3-*t*-Butylphenol		4.23
Eugenol	5.59	4.17
Trolox	5.31	4.17
4-*t*-Butylphenol		4.15
Probucol	5.09	3.51
Silymarin	5.03	4.43
Nifedipine	3.92	3.19
Caffeic acid		3.03
t-Butylbenzene	Inactive at 100 μM	
Papaverine	Inactive at 100 μM	
1,8-Dihydroxyanthraquinone	Inactive at 100 μM	
Khellin	Inactive at 150 μM	
2,6-di-*t*-Butyl-p-benzoquinone	Inactive at 313 μM	
4-*t*-Butylphenyl-2,3-epoxy-propylether	Inactive at 1 mM	
3-*t*-Butylanisole	Inactive at 1 mM	
1,4-Dimethoxy-2-*t*-butylbenzene	Inactive at 1 mM	

In the ABAP/linoleic acid assay samples containing a suspension of linoleic acid (33 mM) were incubated in 50 mM phosphate buffer pH 7.4 at 37°C. Peroxidation was initiated by 11 mM ABAP and the O_2 consumption was monitored by an O_2 electrode. After 6 min antioxidant was added at different concentrations.

In the Fe-ascorbic acid/microsomes assay samples containing rat liver microsomes (0.5 mg microsomal proteins) were incubated in 20 mM phosphate buffer pH 6.0 at 37°C. Peroxidation was started by adding 100 μM Fe^{2+}-Fe^{3+} and 100 μM ascorbic acid. After 6 min antioxidant was added at different concentrations.

Whereas previous research has focused on these two properties in isolation, our study points to a correlation between the two activites. It has been suggested that some phenol derivatives possess calcium antagonistic properties and other authors have shown how this restricts Ca^{2+} availability both in smooth muscle contraction (khellin, Ubeda *et al.*, 1991; quercetin, Abdalla *et al.*, 1989) and in immune response regulation (quercetin, kaempferol, myricetin and silymarin, Middleton & Kandaswami, 1992). Recent electrophysiological research provides evidence of some effects of NDGA on calcium channels. This compound, in fact, was shown to produce a reversible, concentration-dependent inhibition of Ca^{2+} channel currents with a pIC_{50} of 4.73 on two clonal anterior pituitary cell lines (Korn & Horn, 1990). The authors suggested that NDGA blocked Ca^{2+} currents by simply partitioning into the plasmalemma and interacting either directly with channel proteins, or with other membrane-bound Ca^{2+} channel modulators.

In the present study, muscle contraction was elicited by Ba^{2+} in a Ca^{2+}-free incubation medium and less, systematically, by Ca^{2+} in a K^+-rich medium.

Ba^{2+} induces phasic contraction via passage through voltage-operated Ca^{2+} channels of plasma membranes (Bülbring & Tomita, 1969; Inomata & Kao, 1985; Benham *et al.*, 1985) and/or by releasing Ca^{2+} from sarcoplasmic store sites (Northover, 1968; Somlyo *et al.*, 1974; Chi-Ming & Murphy, 1987). Phenol derivatives may exert antispasmogenic effects by inhibiting either or both of these mechanisms. Alternatively, phenols may inhibit the direct action of Ba^{2+}/Ca^{2+} on the contractile machinery. These possible *loci* of antispasmogenic action were examined by studying the effects of BHA, BHT and nifedipine on skinned fibres. The skinning procedure we adopted, based on Triton X-100, not only disrupts the plasma membrane (Cortijo *et al.*, 1987) but has also been found to destroy the functional integrity of both the sarcoplasmic reticulum and the mitochondria (Meisheri & Rüegg, 1983).

Failure to inhibit contraction in skinned fibres demonstates that an intact cell membrane or sarcoplasmic reticulum, or indeed both, are functional prerequisites for BHA and BHT action. Ca^{2+} and Ba^{2+} induce contractions by initially binding to calmodulin (Satoh *et al.*, 1987); the Ba^{2+}/Ca^{2+} thus complexed activates the contractile proteins (Rüegg *et al.*, 1984), so by-passing the binding sites seemingly required by BHA and BHT. Consequently, our results suggest that BHA and BHT act at the sarcoplasmic reticulum or certain plasmalemma components in close structural association with the voltage-operated Ca^{2+} channels.

Experiments with the Ca^{2+} channel activator, Bay K 8644 further narrowed the target site options for BHA and BHT to the plasmalemma alone. Bay K 8644 was found to reverse completely inhibition caused by the antioxidants and by nifedipine. It is now established that Bay K 8644 binds to the same pore site as nifedipine, but acts in an opposite fashion, that is, Bay K 8644 promotes the opening of the voltage-operated Ca^{2+} channels (Schramm *et al.*, 1983), thus pinpointing the plasmalemma as the main target site for the two antioxidants.

Though plasmalemma calcium channels seem therefore to be the main target sites of BHA and BHT antispasmogenic action, they seem less likely to be the unique means whereby papaverine exerts its effects. Bay K 8644, in fact, only partly reverses the antispasmogenic action, in support of the proposal that papaverine acts by a combination of phosphodiesterase inhibition (as with methylxanthines) and block of calcium channels.

Within the framework of spasmolytic function, it is well to remember that at concentrations one order of magnitude greater than those used here, BHA and BHT were shown to disrupt membrane structure (Sgaragli *et al.*, 1977). Among their pleiotypic effects on mitochondria we found that BHA increases the proton leak through the mitochondrial inner membrane and weakens the Δp generating system (proton motive force across the mitochondrial inner membrane), but has no effect on phosphorylation (Fusi *et al.*, 1992). The disruption of biomembranes or the inhibition of mitochondrial respiration (Fusi *et al.*, 1991) does not seem to occur at the antispasmogenic concentrations described here. In closing the discussion on BHA and BHT we suggest that spasmolytic action is a common feature of phenol derivatives and that this pharmacological property is exerted via the inhibition of Ca^{2+} influx into the cells through the voltage-operated Ca^{2+} channels. This mechanism, however, requires clarification.

Aside from their effects on contractility, the phenolic compounds also exhibited their more familiar distinctive antiperoxidant property. It is well established that phenols, in their capacity as hydrogen donors (Burton & Ingold, 1981), form stable phenoxy radicals and are therefore effective antioxidants (Valoti *et al.*, 1989). Experiments using the two model systems of lipid peroxidation, gave comparable results despite the higher complexity of the microsomal system as compared to the linoleate. In the microsomal system, the greater the degree of lipophilicity, the more effective the antioxidant agent was shown to be. Therefore it is conceivable that the compounds behaved in an equivalent pharmacokinetic manner in both model systems and that the differences in potency observed are a real reflection of their intrinsic antiperoxidant capability. While di-*t*-BHA, NDGA, BHT and di-BHA were the most potent, nifedipine and caffeic acid were the least effective scavengers of peroxyl radicals. Though the antiperoxidant action of nifedipine, described elsewhere (Janero *et al.*, 1988; Janero & Burghardt, 1989), is difficult to interpret because it was used only at stronger concentrations than those required for effective calcium antagonism, caffeic acid might be of more than theoretical interest as an antioxidizing agent. In mammals, in fact, caffeic acid might be found in sufficient concentrations as a metabolite of chlorogenic and neochlorogenic acid, abundant components of many foodstuffs, to be a reliable and readily forming antioxidant resource, though devoid of spasmolytic activity.

A highly significant ($P<0.01$) linear correlation was found when plotting the antispasmogenic versus antioxidant activity of the fifteen phenolic derivatives showing dual activity (Figure 4). The fifteen effective 'dual action' compounds all presented at least one hydroxyl group on the aromatic ring and a highly lipophilic area on the molecule. The compounds which exhibited only spasmolytic effects or only antioxidant effects did not and were therefore excluded from the correlation. Of the excluded compounds, those with no phenol moiety were bereft of antioxidant capability, whereas the highly polar phenol derivatives (e.g. caffeic acid and trolox) were devoid of antispasmogenic capacity. Di-BHA, though possessing both structural requirements for the dual activity,

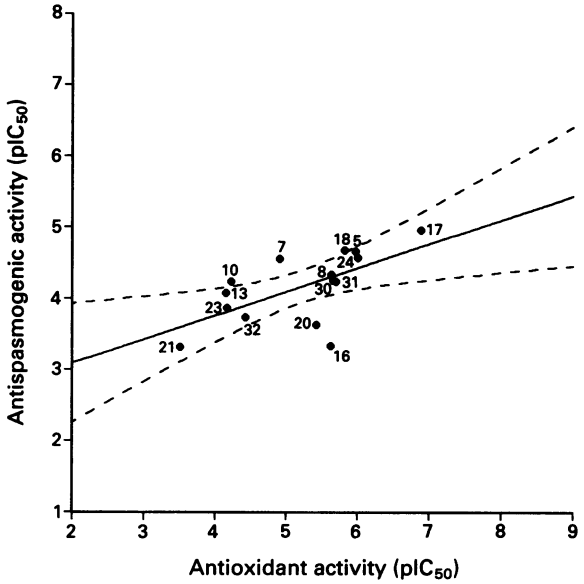


Figure 4 Comparison of pIC_{50} values for certain phenol derivatives. Antispasmogenic activity (intact musculature) versus peroxidation blockade (rat liver microsomal system). See Table 1 for key to compound numbers. Dashed lines represent 95% confidence intervals. Regression and correlation analysis showed $P<0.01$ and $r=0.62323$.

constitutes the exception i.e. though having lipophilic *t*-butyl and phenol moieties, this compound preserved the antioxidant capability of the parent without BHA's attendant antispasmogenic feature. This would seem to suggest that the phenol function, sterically hindered by a lipophilic moiety, is a necessary but insufficient condition for conferring dual action.

As to the linear correlation between the two activities in Figure 4, though significance was high the coefficient was low and the antispasmogenic property, though in an orderly relationship, consistently was less than the antioxidant behaviour of the compounds. Nevertheless, this suggests that some distinguishing structural feature determines dual behaviour and that di-*t*-BHA – structurally the simplest of all the bi-functional compounds – is of especial interest. This lead compound provides a fruitful starting point in the design of new drugs of high antioxidant and Ca^{2+}-blocking capabilities, thus potentially offering dual protection against tissue damage in ischaemia-reperfusion injury.

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