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THE INHIBITORY EFFECTS OF BOLDINE, GLAUCINE, AND PROBUCOL ON TPA-INDUCED DOWN REGULATION OF GAP JUNCTION FUNCTION

RELATIONSHIPS TO INTRACELLULAR PEROXIDES, PROTEIN KINASE C TRANSLOCATION, AND CONNEXIN 43 PHOSPHORYLATION

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Abstract—The naturally occurring antioxidant boldine and its di-methoxy analogue glucine, as well as the drug antioxidant probucol, all inhibit TPA-induced downregulation of gap junctional intercellular communication in WB-F344 rat liver epithelial cells in dose-dependent manners. The compounds were essentially 100% inhibitory to the effect of TPA (10 nM) at 50 μM each. Analysis of the mechanism of the antitumor promotive action of these agents *in vitro* revealed that boldine and probucol (both at 10 μM) totally inhibited the TPA-induced accumulation of intracellular oxidants. Additionally, boldine, glaucine, and probucol, each at 50 μM, inhibited TPA-induced translocation of protein kinase C (PKC) to the particulate fraction of the cells, with concomitant inhibition of TPA-induced hyperphosphorylation of gap junctional connexin 43 (cx43) and TPA-induced internalisation of cx43 protein from the plasma membrane of the cells. None of the compounds inhibited the binding of (^{3}H)-PDBu to TPA-specific binding sites in the cells. The results indicate that antioxidant molecules, irrespective of structure, possess common antitumor promotive potential in this model of gap junctional intercellular communication. The data also indicate that the compounds may interfere with the promotive function of TPA, at least in part, by the destruction of oxidants within the cells. Xanthine oxidase was excluded as a major source of such intracellular oxidants because allopurinol (50 μM) did not significantly affect either the accumulation of oxidants in the cells or the downregulation of gap junctional communication in response to TPA. Taken together, these data also suggest that TPA-induced oxidants play a role in the translocation of PKC to cellular membranes and it is at this level where the antioxidants may interfere in TPA-induced downregulation of gap junctional function.

Key words: antioxidants; gap junctional communication; TPA; oxidative stress; protein kinase C; connexin 43

GJIC plays a key role in growth control, development, and differentiation of cells. Conversely, disrupted GJIC is thought to be associated with the process of tumor promotion. Indeed, models of gap junctional function have been extensively used in screening for the activities of potential tumor promotive substances *in vitro* [1, 2]. One such agent is TPA, which has been proposed to function as a tumor promoter *in vivo* [3, 4] in association with the induction of oxidative stress. Previous work in this laboratory on TPA-induced inhibition of GJIC in control and glutathione (GSH)-depleted WB-F344 liver epithelial cells, indicated that the production of oxidants in cells in response to TPA functions in a cooperative manner with other cellular responses to the phorbol ester [5]. Using a series of well-defined synthetic redox-active selenium- and tellurium-containing antioxidants and GSH peroxidase mimetics, we have recently shown that TPA-induced intercellular oxidants may elicit their effects at the level of the translocation of PKC [6], the TPA receptor, which plays a key role in the phosphorylation of cx43, and the inactivation of gap junctions [7]. Despite this, it is uncertain whether these "anti-promotive" effects are specific to the redox properties of these xenobiotic agents. Similarly, the intercellular source of the oxidants produced in response to the treatment of the cells with TPA is uncertain.

Boldine (2,9-dihydroxy-1,10-dimethoxyaporphine) is a naturally occurring, aporphine alkaloid antioxidant extracted from the leaves of South American plant *Peumus boldus* Molina (Fig. 1). Boldine possesses potent antioxidative properties in biological systems undergoing free radical-mediated lipid peroxidation and has been used for many years in pharmacognacy [8, 9]. Similarly, the boldine analogue glaucine ((s)1,2,9,10-tetramethoxyaporphine) (Fig. 1) also possesses antioxidant activity. Additionally, the hypolipidemic drug probucol (4,4′-(isopropylidenedithio)-bis-(2,6 di-*tert*-butylphenol) (Fig. 1) has also been shown to possess antioxidative properties in biological systems undergoing lipid peroxidation [10–12].

In the present study we tested the efficacies of these

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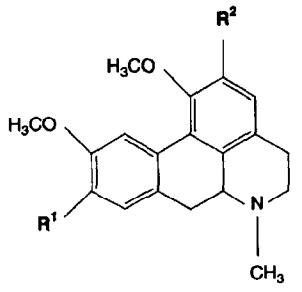
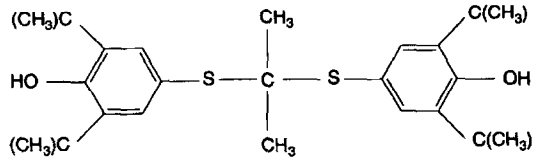
§ Abbreviations: GJIC, gap junctional intercellular communication; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; XO, xanthine oxidase; XD, xanthine dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PDBu, phorbol 12,13-dibutyrate; PS, Lα-phosphatidyl-L-serine; DAG, 1,2-dioleyl-sn-glycerol; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′7′-dichlorofluorescin; DMEM, Dulbecco's modified Eagle's medium; cx43, connexin 43.



R¹, R² = OH, boldine (2,9-dihydroxy-1,10-dimethoxyaporphine)

R¹, R² = OCH_3, glaucine (1,2,9,10 tetramethoxyaporphine)



Probucol, 4,4′-((1-methylethylidene)-bis-(thio))-bis-(2,6-bis(1,1dimethylethyl)phenol)

Fig. 1. The chemical structures of the antioxidants used in this study.

naturally occurring drug antioxidants in inhibiting TPA-induced downregulation of GJIC in WB-F344 cells, the accumulation of oxidants, and the translocation of PKC with concomitant phosphorylation of cx43. The results not only reinforce evidence for a potential role of intercellular oxidants and the translocation of PKC in this tumor-promotive effect of TPA *in vitro*, but also illustrate the generality of this "anti-promotive" effect of antioxidants. In addition, because TPA has been shown to stimulate the conversion of XD to its superoxide-generating form, (XO) [13], it was of interest to probe the effect of allopurinol, an inhibitor of this conversion, on the above TPA-induced effects.

MATERIALS AND METHODS

Chemicals and reagents

(γ-^{32}P)-ATP (5000 Ci/mmol) and (^{3}H)-PDBu (18.9 Ci/mmol) were obtained from New England Nuclear (Du Pont Medical Scandinavia, Sollentuna, Sweden). Boldine was extracted from the bark of *P. boldus* as described previously [14]. Briefly, the bark was extracted with methanol, the extract evaporated to dryness, and the residue partitioned between aqueous acid and chloroform to remove non-alkaloid contaminants. Total alkaloids were re-extracted with chloroform after adjusting the aqueous phase to pH 9. Boldine was finally isolated from the crude extract by several recrystalisations from chloroform and the alkaloid obtained (a 1:1 boldine-chloroform complex) was demonstrated chromatographically (TLC) pure and identified by IR and NMR spectroscopy. Glaucine, probucol, allopurinol, TPA, and MTT, catalase, sodium azide, PDBu, histone type III-S, PS, DAG, DTT, PMSF, leupeptin, and 3,3′-diaminobenzidine tetrahydrochloride were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The working solutions of boldine and glaucine were prepared freshly in phosphate-buffered saline (PBS). Stock solutions of probucol and allopurinol were prepared in distilled dimethyl sulfoxide (DMSO) (dried over anhydrous sodium sulphate) and stored at −20°C until diluted in medium for use. DMSO was always present at less than 0.1% (v/v). Lucifer Yellow CH (di-lithium salt) was from Aldrich Chemical Co. (Dreieich, Germany) and dissolved in 0.33 M $LiCl_2$ to 10% (w/v) for microinjection. DCFH-DA was obtained from Kodak (New York, NY, U.S.A.), dissolved in 100% ethanol and kept at −20°C until use (maximum 2 weeks).

Polyclonal rabbit antibodies raised against a synthetic peptide containing 20 amino acids of the C-terminal end of cx43 were kindly provided by Dr. Edgar Rivedal (Norwegian Radium Hospital, Montebello, Oslo, Norway). Peroxidase-conjugated anti-rabbit IgG was from Daco A/S (Glostrup, Denmark). Low-molecular-weight standards for gel electrophoresis were from Pharmacia LKB (Piscataway, NJ, U.S.A.). All media, serum, HEPES, and materials for the culture of the cells were obtained from GIBCO BRL Life Technologies (Lab, Design, Stockholm, Sweden)

Cell culture

WB-F344 rat liver epithelial cells were the kind gift of Dr. Lars Wärngård, Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden. The cells were cultured and incubated in DMEM supplemented with foetal calf serum (FCS, 10% v/v) and glutamine (4 mM) (growth medium). Cells were grown by conventional monolayer techniques on culture plastic (Falcon) in a humidified incubator under an atmosphere of 5% CO_2 in air.

Evaluation of intercellular oxidant tonus

The intercellular flux of oxidants (the sum of peroxides and free radical species within the cell) in the cells was monitored by their abilities to accumulate oxidized, fluorescent products from DCFH-DA [15]. Briefly, confluent WB cells on 6-well plates were incubated in DMEM without FCS but supplemented with 2 mM HEPES, sodium azide (25 mM), and DCFH-DA (10 μM) for 20 min, followed by incubation with one of the agents under study for 5 min. The cells were then treated with TPA (10 nM) for 1 hr at 37°C. Reactions were halted by removing the medium and the cells were washed (PBS 2x) and then lysed in 40 mM Tris-HCl, pH 7.4, containing SDS (0.2%) and catalase (100 units) for 10 min at 37°C. The lysate was then collected, centrifuged (2000 × g for 10 min at 4°C), and the fluorescence of the supernatant determined on a Shimadzu Spectrofluorimeter (Model RF 510-LC), λ_{ex} = 504, λ_{em} = 525 nm.

Preparation of subcellular fractions and assay of protein kinase C

Preparation and measurement of PKC was generally according to Kass *et al.* [16]. Briefly, confluent WB cells in 100 mm dishes were incubated for 1 hr with different compounds as indicated, then washed twice with PBS and twice with buffer A (20 mM Tris-HCl, pH 7,5, 250

mM sucrose, 10 mM EGTA, 2 mM EDTA, 0.1 mM PMSF, 100 μg/mL leupeptin). The cells were scraped into 1 mL of ice-cold buffer A, then sonicated and centrifuged at 100,000 × g for 1 hr at 4°C to separate cytosolic fraction and particulate fractions. Both fractions were then partially purified by using Whatman DE-52 ion-exchange chromatography. The Ca^{2+}/PS/DAG-dependent PKC activities of the fractions were assayed by the stimulated incorporation of ^{32}P from (γ-^{32}P)-ATP into histone H1. The standard reaction mixture consisted of 30 mM Tris-HCl buffer, pH 7.5, supplemented with 7 mM $MgCl_2$, 300 μg/mL histone H1, 10 μM (γ-^{32}P)-ATP, 20 μg/mL PS, 1 μg/mL DAG, 5 mM $CaCl_2$, and fraction protein (0.05 mg/assay) in a final volume of 150 μL. The mixtures were incubated at 30°C for 10 min and the reactions halted by adding ice-cold 25% trichloroacetic acid. The samples were kept on ice for 30 min, the acid-insoluble fraction collected on a membrane filter (Sartorius SM 11306, 0.45 μm), and its radioactivity determined by liquid scintillation spectrometry. Kinase activity was expressed as pmol of P incorporated/min/mg fraction protein. Background blanks were obtained by replacing the fraction sample with BSA.

[^{3}H]-PDBu binding to intact cells

Confluent WB cells on 6-well plates were preincubated with compounds under study for 5 min in DMEM without FCS, but supplemented with 20 mM HEPES and then exposed to (^{3}H)-PDBu (50 nM, 0.2 μ Ci/well) for a further 5 min. The assays were terminated by aspirating the media, the plates rinsed 3 times with ice-cold PBS, the cells solubilized with 0.5 mL of 1N NaOH and the samples neutralized with 0.5 mL of 1N HCl and quantified by liquid scintillation counting. Specific binding of (^{3}H)-PDBu to PKC in the cells were determined as the difference between total cellular binding in the absence and presence of TPA (10 μM).

Analysis of the phosphorylation status and immunocytochemical localization of cx43

After the different treatments, the confluent cells on 100 mm dishes were washed twice with PBS and lysed in 200 μL of SDS-sample buffer (2% SDS, 60 mM Tris-HCl, 0.1 M DTT, pH 6.8). The cells wre further homogenized by sonication for 2 × 5 s (40 watts) and the cell debris then precipitated by centrifugation (6000 × g, 5 min, 4°C) and the supernatant kept at −20°C until analysis. SDS-PAGE (Bio-Rad, Mini Protean) was performed on 10% acrylamide gels, with 10 μg/lane of sample loaded (heated at 100°C for 3 min before loading). The proteins were separated and then electroblotted onto nitrocellulose membranes. The membranes were initially incubated with anti-cx43-antibodies, diluted 1/1000, for 2 hr and then with peroxidase-conjugated antirabbit IgG (1/1000). They were then washed and stained with the peroxidase substrate, 3,3′-diaminobenzidine tetrahydrochloride, according to the manufacturer's recommended procedures.

For immunocytochemical analysis, the cells were grown on glass slides at the density used above and the treated cells washed with PBS and fixed in acetone at −20°C. The slides were then washed with PBS and incubated overnight with anti-cx43 antibodies diluted 1:1500 in PBS supplemented with 3% BSA and 5% nonfat milk. Subsequently the plates were incubated with biotinylated anti-rabbit IgG (1:200 in PBS) for 1 hr at 37°C and then with fluorescein-avidin D (25 μ g/mL, Vector Labs.) for 30 min at 37°C and the glasses mounted in Vectasheild Mounting Medium (Vector Labs.) and analyzed by confocal microscopy (Bio Rad MRC 600, equipped with a krypton-argon laser. λ_{em} 488 nm).

Gap junctional communication assay

Gap junctional intercellular communication was assessed by the efficacy of intercellular diffusion of Lucifer Yellow dye from a single microinjected cell to its neighboring cells. The cellular incubations and microinjection were performed according to our previous report [5]. Briefly, confluent cells on 35 mm plates were incubated in growth medium minus FCS, but supplemented with 20 mM HEPES, for 60 min with compounds and/or TPA (10 nM). After incubation, microinjection was performed. Dye-coupled cells were counted 5 min after each injection. The numbers of injections per dish averaged 14 ± 2. The effects of the test compounds are expressed in terms of % of communicating cells/injection in the absence of compound and TPA. The data were obtained from 2 separate dishes in each case.

Evaluation of cytotoxicity

The acute cytotoxicity of the treatment regimens was assayed in each case using the MTT reduction assay described by Carmichael *et al.* [17], with slight modifications [5]. The results were expressed as the fractional absorbance of the test sample compared to the appropriate control in %. The data were obtained from 3 separate incubations for each treatment and dose used.

Statistical appraisals

Groups of test data (means ± SD) were compared using Students *t*-Test for unpaired observations. Statistically different values were denoted at $P < 0.05$.

RESULTS

In agreement with previous results [5], TPA treatment (10 nM, 1 hr) of WB-F344 cells resulted in a >95% inhibition of GJIC (Fig. 2). For each of the compounds tested, a dose-dependent inhibition of the TPA-induced downregulation of GJIC was noted (Fig. 2). The naturally occurring aporphrine antioxidants boldine and glaucine were equipotent, resulting in nearly 100% reinstatement of the control communication at 50 μM of the respective compound. On the other hand, the drug antioxidant probucol and the XO inhibitor allopurinol were less effective in this respect, facilitating the reinstatement of 88% and 35% of the control communication at 50 μM of the compounds, respectively. It will be noted that, under the conditions employed, none of the compounds induced acute, sublethal cytotoxicity at the concentrations used, as assessed by the MTT reduction assay.

The ability of the test compounds to interact with cellular oxidants produced in the cells in response to TPA treatment was also probed. It is clear from Fig. 3 that TPA (10 nM, 1h) treatment significantly ($P < 0.001$) induced the accumulation of fluorescent metabolites of DCFH-DA in the cells, indicating an elevation in intercellular oxidants during this time. Similarly, it is clear from Fig. 3 that co-incubation of these TPA-treated cells with either boldine or probucol caused a dose-dependent decrease in the accumulation of oxidants in the cell.

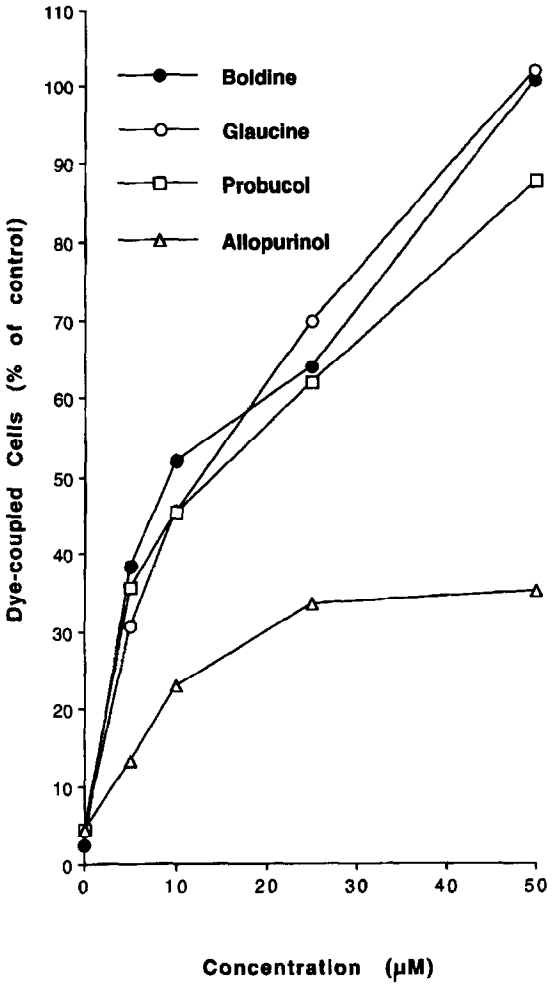


Fig. 2. The inhibitory actions of boldine, glaucine, probucol, and allopurinol on TPA-induced downregulation of GJIC in WB-F344 cells. Confluent WB cells were coincubated with TPA (10 nM) and the compounds at various concentrations for 60 min. After treatment, GJIC was determined by microinjection/dye transfer as described in Materials and Methods. The data are expressed as a % of the number of communicating cells per injection in control cells. Control injections routinely coupled 50–60 cells. The data were obtained from 12–16 injections per dish and pooled from 3 separate dishes for each point. The data are the means of the above observations, with the SD (<10% on all points) omitted in each case for clarity.

Indeed, both compounds were 100% effective in depressing the TPA-induced accumulation of oxidants (*i.e.* insignificantly different from controls) when present at only 10 μM each. On the other hand, allopurinol was relatively ineffective in this respect, with 25 and 50 μM depressing accumulation significantly ($P < 0.05$) by 15%–20% (Fig. 3) It will be noted that glaucine could not be studied for its potential antioxidative effects because it was shown to interact with the DCFH-DA probe, in the absence of TPA, to increase fluorescent intensity.

Table 1 shows that TPA (10 nM, 1 hr) treatment of the cells significantly decreased ($P < 0.05$) the Ca^{2+}/DAG/PS-dependent PKC activity associated with cytosol fractions of WB-F344 cells from 18.9 ± 4.0 to 10.2 ± 1.8 pmol P/min/mg protein. Again, with the exception of allopurinol, all of the antioxidant compounds inhibited TPA-induced translocation significantly ($P < 0.05$) at 50 μM. It will be noted that TPA-induced PKC activity in particulate fraction was not strictly significant from the controls, but it is clear from the data in the Table that the treatment of the cells with the antioxidants, particularly with boldine, prevented the TPA-induced increase in particulate PKC activity.

To test if the above inhibition of the activation of PKC by the antioxidants is due to interference in phorbol ester binding to the protein, we tested the effect of each compound at 50 μM on the sequestration of (^{3}H)-PDBu by the cells. To determine to what extent this effect was due to specific binding to PKC in the cells, the control binding of PDBu was carried out in the presence of TPA (10 μM). It will be noted that nonspecific binding of PDBu amounted to 21 ± 1%, $n = 4$. It will also be noted that PDBu binding was optimal by 5 min of incubation, hence the choice of this period for the study. The data in Table 2 clearly show that none of the compounds tested inhibited the binding of PDBu to PKC in the cells at the concentration tested.

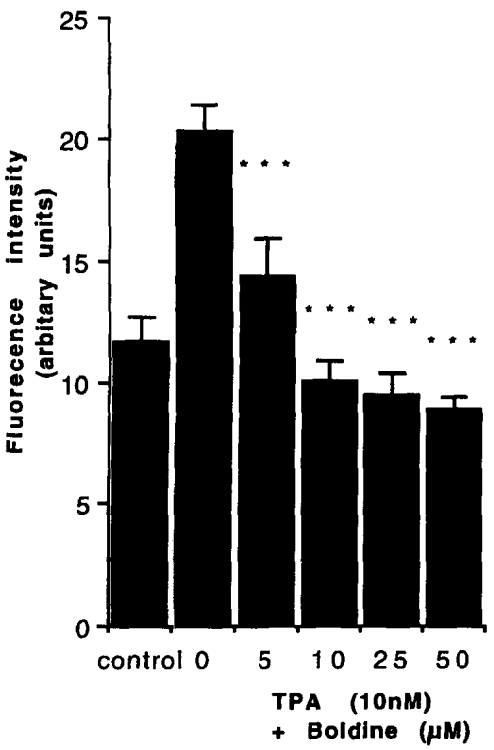
As PKC has been shown to be involved in the phosphorylation of connexin proteins in the gap junctions of WB-F344 cells [6], it was interesting to explore if the compounds tested were able to interfere with TPA-induced phosphorylation of cx43 in the cells. It can be seen from Fig. 4 that 3 protein bands cross-react with the polyclonal antibody to cx43 in control cells (Lane 1) and that TPA (10 nM, 1 hr) treatment of the cells caused a marked shift in the pattern of cross-reaction, with the major species cross-reacting at 46 KDa (Lane 2). Both boldine and glaucine, when co-incubated with TPA at 50 μM, totally suppressed TPA-induced phorphorylation of cx43 (Lanes 3 and 4, respectively). Similarly, probucol (50 μM) prevented TPA-induced phosphorylation of cx43 (Lane 5), while allopurinol was shown to be ineffective (Lane 6).

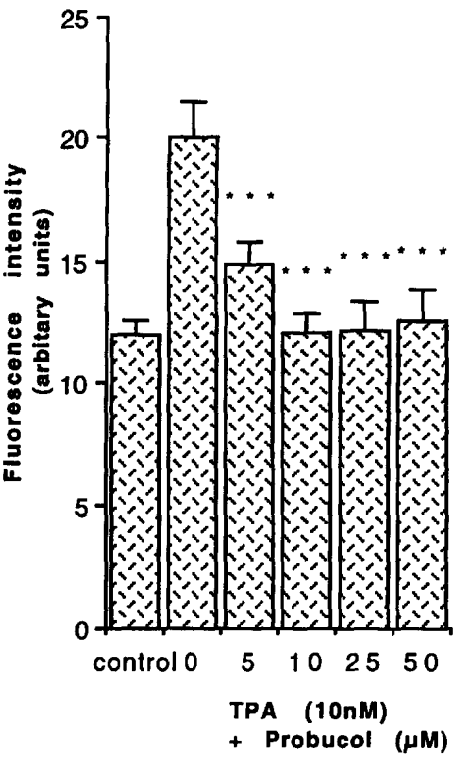
Finally, the treatment of the cells with TPA (10 nM) for 60 min resulted in internalization of connexin 43 molecules (Fig. 5a *versus* 5b). However, in the presence of either boldine, glaucine, or probucol (Fig. 5c, d and e, respectively), all at 50 μM, the internalization of cx43 was hindered. In this respect, allopurinol was totally without effect (Fig. 5f).

DISCUSSION

Cellular oxidative stress has been implicated in the molecular mechanism of tumor promotion of agents such as TPA [18, 19]. Consequently, it has been proposed that antioxidants may exert antitumor promotive effects through the destruction of free radicals and other oxidants implicated in the tumor-promotion process [20].

Alterations to gap-junctional function have been proposed as a central component in the promotion of initiated cells to rapid clonal expansion. Indeed, *in vitro* studies of GJIC have been used to screen for tumor-promotive substances and to study molecular aspects of their action [21]. Several studies have indicated that TPA induces a downregulation of GJIC in association with the increased production of oxidants in cells. We previously demonstrated, in a rat liver WB-F344 epithelial cell model of GJIC, that the efficacy of TPA in the downregulation of GJIC is potentiated if the cellular antioxidant network is compromised by the depletion of





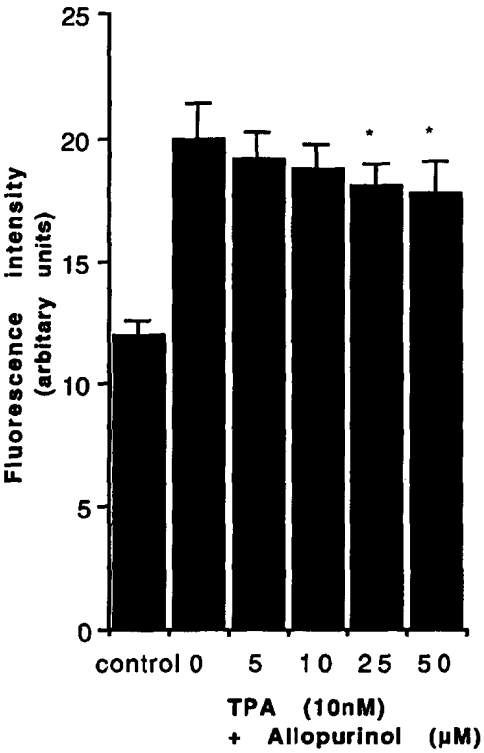


Fig. 3. The effects of boldine, probucol, and allopurinol on TPA-induced accumulation of oxidants in WB-F344 cells. Confluent cells were preloaded with DCFH-DA in control medium as described in Materials and Methods. The cells were then washed (3 × PBS), resuspended in medium, and treated with different concentrations of the test agents and TPA (10 nM) for 60 min. The cells were then lysed in 40 mM Tris-HCl, pH 7.4, containing SDS (0.2%) and catalase (100 units) for 10 min at 37°C. The lysate was then collected, centrifuged (2000 × g for 10 min at 4°C), and the fluorescence of the supernatant determined at γ_{ex} 504 nm, λ_{em} 525 nm. Results are expressed as mean values ± SD and are calculated from two experiments with triplicate samples. * denotes $P < 0.05$ and *** denotes $P < 0.001$. It should be noted that glaucine was not studied because the compound increased the fluorescence of DCFH-DA in the absence of TPA in the cells.

Table 1. Boldine, glaucine and, probucol, but not allopurinol, inhibit the translocation of protein kinase C in TPA-treated WB-F344 cells

Treatment	PKC activity (pmol P/min/mg protein)	
	Cytosolic fraction	Particulate fraction
Control cells	18.9 ± 4.0	3.6 ± 1.2
TPA (10 nM)	10.2 ± 1.8*	7.5 ± 2.6
+ Boldine (50 μM)	19.3 ± 4.1†	1.9 ± 0.3‡
+ Glaucine (50 μM)	14.3 ± 1.3†	3.7 ± 2.2
+ Probucol (50 μM)	13.6 ± 0.9†	6.6 ± 3.0
+ Allopurinol (50 μM)	10.7 ± 3.9	7.3 ± 3.0

Confluent cells were pre-incubated with test compounds (50 μM) for 5 min followed by exposure to TPA (10 nM) for 5 min. The cells were then harvested, subcellular fractions were prepared, and the Ca^{2+}/DAG/PS-dependent PKC activity partially purified and assayed as described in Materials and Methods. Values are means ± SD of triplicate assays. * denotes a significant ($P < 0.05$) difference from PKC activity in the soluble fraction of control cells, † and ‡ denote significant ($P < 0.05$) differences from PKC activity of the respective fractions of TPA-treated cells.

cellular GSH [5]. Similarly, we showed that a variety of redox-active selenium- and tellurium-containing xenobiotics, possessing either GSH-peroxidase-mimetic or antioxidant activities, possess powerful inhibitory effects on TPA-induced downregulation of GJIC in the cells [6]. This, further indicates that an oxidative stress component exists in the molecular mechanism of this *in vitro* tumor-promotive effect of TPA. Despite these observations, it is uncertain whether or not these "antipromotive" effects were specific to the redox properties of these xenobiotic agents. Thus, the potential of other antioxidants, particularly those of natural origin, have not been tested in this respect. Similarly, the intercellular source of the oxidants produced in response to the treatment of the cells with TPA is uncertain.

The naturally occurring aporphrine alkaloid boldine

Table 2. The failure of the test compounds to inhibit the binding of [^{3}H]-PDBu to PKC in intact WB-F344 cells

Treatment	Specific binding of (^{3}H)-PDBu (dpm/10^6 cells)
Control cells	2107 ± 201
+ Boldine (50 μM)	2011 ± 187
+ Glaucine (50 μM)	2057 ± 95
+ Probucol (50 μM)	1906 ± 187
+ Allopurinol (50 μM)	1931 ± 162

Confluent cells were preincubated with compounds at 50 μM for 5 min and then exposed to [^{3}H]-PDBu (50 nM, 0.1 μci/well) at 37°C for 5 min. The assays were terminated by aspirating the media, the plates rinsed 3 times with ice-cold PBS, the cells solubilized with 0.5 mL of 1N NaOH, and the samples neutralised with 0.5 mL of 1N HCl and quantified by liquid scintillation counting. Specific binding of (^{3}H)-PDBu to PKC in the cells was determined as the difference between total cellular binding in the absence and presence of nonlabelled TPA (10 μM). These values are the means ± SD of 2 experiments with triplicate samples in each case.

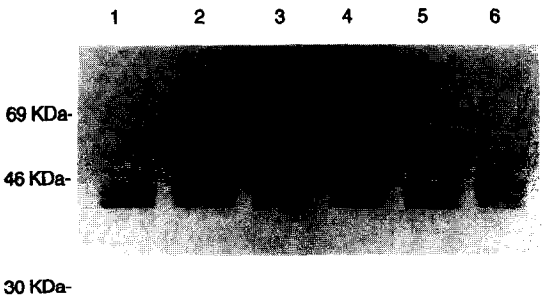


Fig. 4. Boldine, glaucine, and probucol interfere with TPA-induced hyperphosphorylation of connexin 43 in WB-F344 cells. Cell were treated with the compounds (50 μM) and TPA (10 nM) for 1 hr and the cellular protein extracted, separated by SDS PAGE, electroblotted, and immunostained with a primary anti-cx43 antibody and a secondary peroxidase-conjugated antibody, as described in Materials and Methods. Lane 1 = control cell, Lane 2 = control cells treated with TPA (10 nM) for 1 hr. Lanes 3–7 = control cells coincubated with boldine, glaucine, probucol, or allopurinol, respectively, and TPA (10 nM) for 1 hr.

and its O-methylated analogue glaucine (Fig. 1) possess potent antioxidative properties both *in vitro* [8, 9, 22] and *in vivo* [23]. It is clear from Fig. 2 that both boldine and glaucine, when co-incubated with TPA, prevented the downregulation of GJIC in dose-dependent manners. Similarly, the hypolipidemic drug probucol, which also possesses antioxidative capacity, also effectively interfered with TPA's effect on GJIC in WB-F344 cells. These observations add potential anti tumor-promotive properties to the pharmacodynamic spectrum of these antioxidants. Additionally, because the aporphine alkaloids and probucol bear no structural resemblance to each other or to the chalcogenine-containing antioxidants previously tested [6], it is likely the redox activity of the compounds that elicits their remidial effects on TPA-induced downregulation of GJIC.

The inhibition of GJIC by TPA in WB-F344 cells has been previously shown to be related to the PKC-dependent phosphorylation of gap junctional cx43 proteins. Similarly, the activity of PKC itself has been shown to be sensitive to redox events on the protein [14]. With this in mind, it was interesting to investigate the mode of action of the antioxidants under study. The intercellular oxidant probe DCFH-DA has previously been used to indicate the production of oxidants within the intercellular milieu. Thus, the reagent has been shown to undergo peroxidase-dependent co-oxidation with H_2O_2, yielding fluorescent metabolites that accumulate in the cells [24]. Similarly, it has been proposed that DCFH-DA undergoes free radical-mediated oxidation to fluorescent products [25]. From the data in Fig. 3, it is clear that both boldine and probucol inhibited the accumulation of oxidants in TPA-treated cells in dose-dependent manners. This ability has been noted previously for several redox-active chalcogenine-containing antioxidants and GSH-peroxidase mimetics [6]. It should be noted that glaucine interacted with the DCFH-DA in the absence of TPA, precluding its further study. The reasons underlying this effect are uncertain, but this interaction has been noted with other compounds, particularly those able to undergo redox-cycling in cells [6]. Finally, it will be noted that the compounds totally inhibited the accumulation of oxidants at 10 μM, and 50 μM of each was

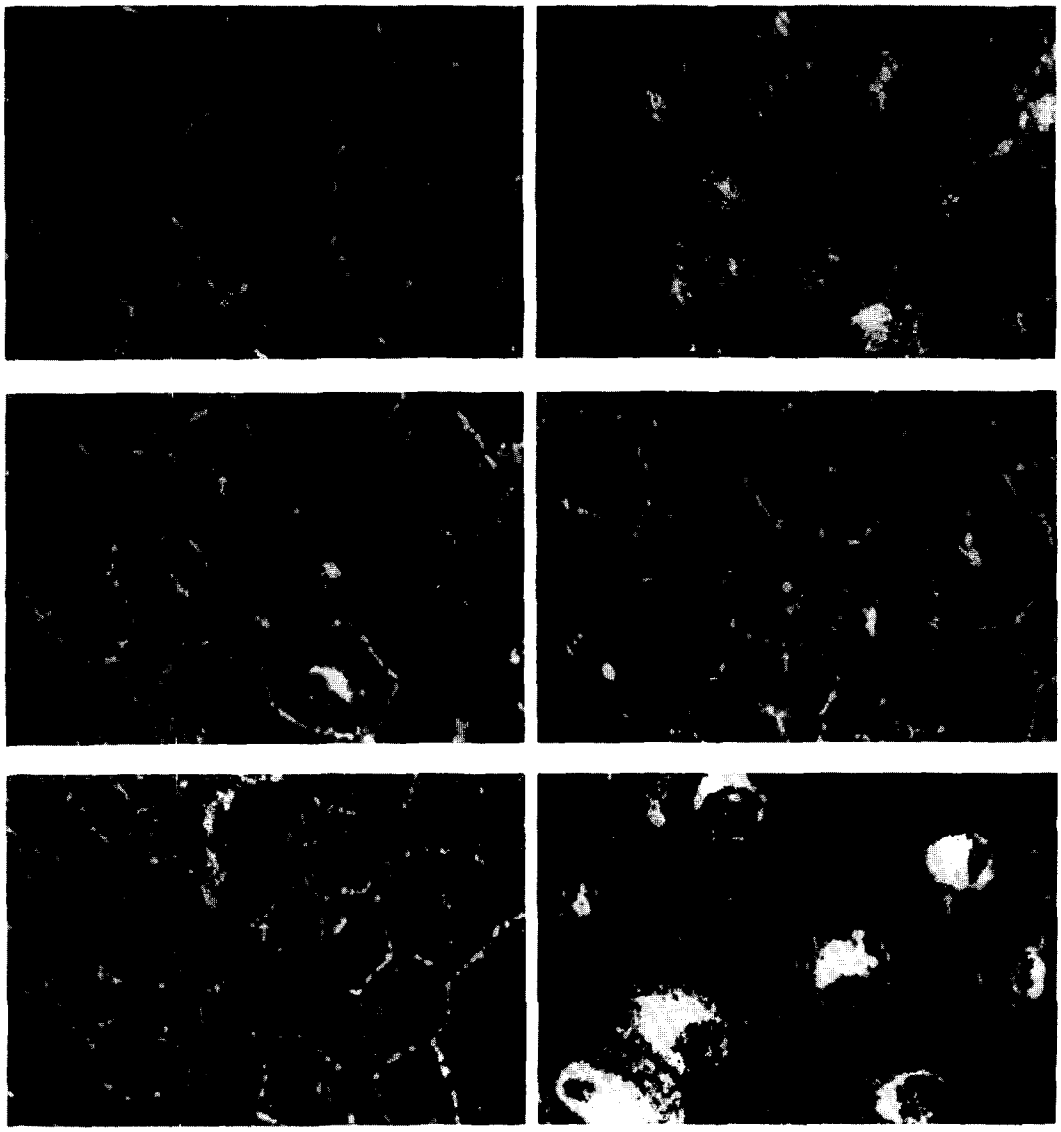


Fig. 5. The effects of boldine, glaucine, probucol, and allopurinol on TPA-induced internalization of connexin 43 in WB F344 cells. The cells were grown to confluency on glass slides and treated with TPA (10 nM) for 1 hr in the presence and absence of the test compounds. The slides were then washed, fixed, stained first with rabbit anti-cx43 antibody and then with anti-rabbit IgG-biotin conjugate and fluorescein avidin D, and finally analyzed by fluorescence confocal microscopy, all as described in Materials and Methods. The images are, from top left to bottom right, control cells (a), cells treated with TPA for 60 min (b) and cells treated with TPA for 60 min in the presence of boldine (c), glucine (d), probucol (e), or allopurinol (f), each at 50 μM.

required to approach complete protection of GJIC in the cells (Fig. 3 *versus* Fig. 2). This suggests that the intercellular production of oxidants in the cells may only play a partial role in the effect of the phorbol ester on GJIC. Indeed, previous experiments in these cells have suggested that TPA-derived oxidants interact synergistically with other effects of TPA, (*e.g.* the activation of PKC) [5, 6]. Thus, the induction of oxidative stress in the cells alone is not sufficient to cause an inhibition of GJIC [5].

As mentioned above, one critical link between oxidative stress and gap-junctional function may lie at the level of PKC itself. The data in Table 1 indicate that boldine totally inhibited the TPA-induced decline in cytosolic PKC activity at 50 μM. This was reflected in inhibition of TPA-induced elevation in PKC activity associated with the particulate fraction, suggesting that boldine effectively prevents the translocation of PKC. A similar, but less potent, effect was noted for glaucine, and probucol's effect was limited. These observations may be interpreted in several ways. First, the compounds may interfere with TPA's binding to the kinase itself. This seems, however, unlikely, as none of the compounds were able to significantly affect the binding of (^{3}H)-PDBu to PKC at 50 μM (Table 2). Here, it will be noted that the cellular PDBu-binding was standardized to PKC content by determining the nonspecific binding in the presence of unlabelled TPA. A second explanation lies in the ability of the compounds to inhibit the accumulation of oxidants in the cells. Previous experiments have shown that oxidants induce structural changes on

the PKC protein that alter the catalytic efficiency of the enzyme [14, 26]. The present data suggest that oxidants may also be involved in the control of translocation of PKC in its active form to membrane compartments of the cell. It will be noted that the present data are in agreement with data obtained previously with chalcogenine-containing antioxidants and GSH-peroxidase-mimetics [6].

One of the early events involved in TPA-induced downregulation of GJIC in WB-F344 cells is the PKC-dependent phosphorylation of cx43 in the gap junctions, themselves. Thus, any effect of the antioxidant compounds on the translocation of PKC should be reflected in the phosphorylation status of the cx43 molecule, because association with the plasma membrane is a prerequisite for phosphorylation of gap-junctional proteins. The data shown in Fig. 4 clearly confirm that compounds that prevent the translocation of PKC in response to TPA, such as boldine and glaucine at 50 μM, prevent the hyperphosphorylation of cx43. On the other hand, probucol only partially prevented this hyperphosphorylation and was only partially effective in preventing translocation of PKC at 50 μM. It is noted that the multiple bands of cross-reaction with the anti-cx43 antibody represent different phosphorylation states of the protein, with the band of highest molecular weight representing the most heavily phosphorylated form [27].

Previous studies by Matesic and co-workers [28] and work from this laboratory [6] have shown that the kinetics of TPA-induced phosphorylation of cx43 and the inhibition of GJIC in WB F344 closely parallel each other. These authors propose that phosphorylation plays a role in the rapid inactivation of the protein. In the present experiments a 1-hr incubation time was chosen to detect any remedial effects of the compounds on GJIC. During this time, the authors demonstrated complete internalization of the cx43 molecules in response to TPA. In the present study, we confirmed a considerable internalization of cx43 in the cells 1 hr after treatment with TPA (Fig. 5b *versus* 5a) and, interestingly, all of the antioxidants tested effectively prevented this internalization in the presence of TPA (Fig. 5c–3 *versus* b). The close correlation between the abilities of these redox-active compounds to inhibit phosphorylation of cx43 and prevent its internalization might suggest that the hyperphosphorylation of the protein serves as a signal to internalization. However, using different promotive stimuli, Matesic *et al.* have suggested that the internalization of cx43 is independent of the phosphorylation status of the protein [28].

The source of the intercellular oxidants arising from TPA treatment is uncertain. It is well established that one of the pleiotrophic actions of TPA is the stimulated conversion of XD to XO. Xanthine oxidase effectively releases $\cdot O_2^-$, and is known to be one of the major sources of intercellular oxidants in many cell types [25]. With this in mind, it is interesting to note that allopurinol, an effective inhibitor of XO but not an antioxidant, at the doses studied in the present work [29] was only weakly inhibitory fo TPA-induced downregulation of GJIC (Fig. 2) and TPA-induced accumulation of oxidants in the cells (Fig. 3). Additionally, allopurinol was without effect on TPA-induced translocation of PKC (Table 1), TPA-induced hyperphosphorylation of cx43 (Fig. 4) and TPA-induced internalization of cx43 (Figure 5f *versus* b). Together, these data suggest that the contribution of XO in these cells to the production of oxidants in response to TPA is marginal, perhaps amounting to 10%–20%. Thus, the source of the oxidants derived from TPA treatment that act synergistically with other effects of the tumor promoter is, at present, unknown.

In summary, boldine, glaucine, and probucol have been shown to interfere with TPA-induced downregulation of GJIC in WB-F344 cells. Investigation of the mechanism of the antipromotive effects of these compounds *in vitro* has revealed that a close relationship exists between the induction of oxidative stress in the cells, *via* the activation of components other than XO, the translocation of PKC to membrane compartments, and the phosphorylation of cx43. Thus, the present data reinforce the premise that the induction of oxidative stress may play an important support role in the mechanism of the promotive activity of TPA. Additionally, these data, taken together with those obtained with other structurally nonrelated redox-active compounds in these cells [6], reveal the generality of the antipromotive activity of antioxidants in this *in vitro* model of tumor promotion.

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