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Structure-Activity Relationships of Phenothiazines and Related Drugs for Inhibition of Protein Kinase C

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SUMMARY

Phenothiazines are known to inhibit the activity of protein kinase C. To identify structural features that determine inhibitory activity against the enzyme, we utilized a semiautomated assay [*Anal. Biochem.* **187**:84–88 (1990)] to compare the potency of >50 phenothiazines and related compounds. Potency was decreased by trifluoro substitution at position 2 on the phenothiazine nucleus and increased by quinoid structures on the nucleus. An alkyl bridge of at least three carbons connecting the terminal amine to the nucleus was required for activity. Primary amines and unsubstituted piperazines were the most potent amino side chains. We selected 7,8-dihydroxychlorpromazine (DHCP) (IC_{50} = 8.3 μM) and 2-chloro-9-(3-[1-piperazinyl]propylidene)thioxanthene (N751) (IC_{50} = 14 μM) for further study because of their potency and distinct structural features. Under standard (vesicle) assay conditions, DHCP was noncompetitive with respect to phosphatidylserine and a mixed-type inhibitor with respect to ATP. N751 was competitive with respect to phosphatidylserine and noncompetitive with respect to ATP. Using the mixed micelle assay, DHCP was a competitive inhibitor with respect to both phosphatidylserine and ATP. DHCP was selective for protein kinase C compared with cAMP-dependent protein kinase, calmodulin-dependent protein kinase type II, and casein kinase. N751 was more potent against protein kinase C compared with cAMP-dependent protein kinase and casein kinase but less potent against protein kinase C compared with calmodulin-dependent protein kinase type II. DHCP was analyzed for its ability to inhibit different isoenzymes of protein kinase C, and no significant isozyme selectivity was detected. These data provide important information for the rational design of more potent and selective inhibitors of protein kinase C.

Protein kinase C, a Ca^{2+}- and phospholipid-dependent protein kinase, is widely recognized as a major element in the signal transduction pathway for molecules that act by inducing the turnover of phosphatidylinositol (1). Diacylglycerol, one of the second messengers produced by phosphatidylinositol turnover, and the tumor-promoting phorbol esters directly activate protein kinase C by increasing its affinity for calcium and phospholipid (2). The enzyme is widely distributed in mammalian tissues, and at least seven isoenzymes are known to exist (3). Protein kinase C mediates the effects of a large number of hormones and is involved in many aspects of cellular regulation and carcinogenesis (2). The enzyme is also thought to play a role in certain types of resistance to cancer chemotherapeutic agents (4–7) and may constitute a target for future chemotherapeutic strategies.

Inhibitors of protein kinase C are potentially valuable tools for elucidating the role of the enzyme in cellular processes. However, most of the currently available drugs are nonselective toward this kinase, compared with other kinases such as cyclic nucleotide-dependent protein kinases and calmodulin-dependent protein kinases. Therefore, we examined the phenothiazine and thioxanthene classes of protein kinase C inhibitors to evaluate the structural features that determine inhibitory activity and potential selectivity. The phenothiazines are particularly well suited for this type of study for several reasons. First, the few derivatives that have been studied are relatively potent, with K_i or IC_{50} values approaching 10 μM (8–10). Second, a large number of phenothiazine congeners have been synthesized for their potential activity as antipsychotic agents and are readily available for analysis. Structure-activity studies offer a way to understand which molecular structures affect activity, and this information may be used in the rational design of more effective drugs.

Experimental Procedures

Materials. The materials used in these experiments were obtained from the following sources: 2-trifluoromethyl-10-(3-[1-piperazinyl]propyl)phenothiazine was generously donated by Dr. A. Shepartz and Mr. B. Cuenoud, Yale University (New Haven, CT); 2-chloro-10-(2-dimeth-

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ABBREVIATIONS: DHCP, 7,8-dihydroxychlorpromazine; PDBU, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ylaminoethyl)phenothiazine,2-chloro-10-(3-dimethylamino-2-hydroxypropyl)phenothiazine, 7,8-diacetoxychlorpromazine, 6,9-dihydroxychlorpromazine, DHCP, 7,8-dimethoxychlorpromazine, 7,8-dioxochlorpromazine, 7,8-dioxochlorpromazine 7-semicarbazone, and 3,7,8-trihydroxychlorpromazine were generously donated by Drs. Stephen Kennedy and Steven Zalcman, National Institute of Mental Health (Rockville, MD); 2-chloro-10-(4-dimethylaminobutyl)phenothiazine, 1-chloropromazine, 3-chloropromazine, 4-chloropromazine, chlorproethazine, chlorpromazine, chlorpromazine sulfoxide, desmethylchlorpromazine, didesmethylchlorpromazine, 3,8-dihydroxychlorpromazine, 7-hydroxychlorpromazine, penfluridol, pimozide, promazine, R-6033, 2-thiomethylpromazine, trifluopromazine, and desipramine were generously donated by Dr. Walter Prozialeck, Philadelphia College of Osteopathic Medicine (Philadelphia, PA); trifluoperazine was from Dr. Alfred Brown, Smith Kline and French Laboratories (Philadelphia, PA); fluphenazine was from Dr. S. J. Lucania, E. R. Squibb and Sons (Princeton, NJ); 2-chloroimipramine was from Geigy Pharmaceuticals (Summit, NJ); thioxanthenes were from Dr. John Hyttel, H. Lundbeck (Copenhagen, Denmark); haloperidol, imipramine, prochlorperazine, promethazine, perphenazine, quinacrine, diolein, histone H1 (type IIIs), casein, cAMP-dependent protein kinase, and phosphatidylserine were from Sigma (St. Louis, MO); [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham (Arlington Heights, IL); and [^{3}H]PDBU was from New England Nuclear (Boston, MA). Calmodulin-dependent protein kinase type II (rat brain) and synapsin I (bovine brain) were generously donated by Dr. Fred Gorelick, Yale University School of Medicine; hydroxylapatite-purified rat brain α and β(I/II) and partially purified human recombinant γ and δ protein kinase C isozymes were from Sphinx Pharmaceuticals (Durham, NC).

Purification of protein kinase C. Protein kinase C was partially purified from rat brain as previously described (11).

Assay of protein kinase activity. Protein kinase C was assayed using a semiautomated 96-well plate assay, as previously described (11). Drugs were dissolved in water or dimethyl sulfoxide (final concentration, ≤2.5%) before addition to the wells. Drugs were screened by generating triplicate dose-response curves with nine drug concentrations from 0 to 1 mM. Vesicle assay reactions (100 μl) contained 12.5 units (nmol P_i/min) of protein kinase C, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 200 μg/ml histone, 1 μg/ml diolein, 8 μg/ml phosphatidylserine, 100 μM $CaCl_2$, and 25 μM [γ-^{32}P]ATP. Mixed micelle reactions (used for some kinetic determinations) were performed as previously described (29). Reactions (250 μl) contained 0.05 units of protein kinase C, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 300 μg/ml histone, 0.3% Triton X-100, 10 mol % phosphatidylserine, 2 mol % diacylglycerol, 100 μM $CaCl_2$, and 30 μM [γ-^{32}P]ATP. Protein kinase C isozymes were assayed in 250-μl reactions containing 20 μl of partially purified isozyme, 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 200 μg/ml histone, 1.76 μg/ml diolein, 40 μg/ml phosphatidylserine, 100 μM $CaCl_2$, 95 μM EGTA, and 20 μM [γ-^{32}P]ATP.

cAMP-dependent protein kinase was assayed according to a slight modification of a previously described procedure (12). Enzyme activity was assayed with the 96-well plate method (11), where reactions were stopped by the addition of 100 μl of 25% trichloroacetic acid. Enzyme activity was corrected for cAMP-independent kinase activity by assaying in the absence of cAMP.

Calmodulin-dependent protein kinase was assayed by a modification of a previously described procedure (13). Reactions (100 μl) contained 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1.5 mM $CaCl_2$, 25 μM ATP, 25 μg/ml calmodulin, 200 μg/ml bovine serum albumin, 250 μg/ml synapsin I, and 250 ng of calmodulin-dependent protein kinase type II. Reactions were initiated by the addition of ATP, containing 10^6 cpm of [γ-^{32}P]ATP, and were terminated by the addition of 100 μl of 25% trichloroacetic acid. The precipitated proteins were filtered over 0.45-μm Millipore filters and washed with 10 ml of ice-cold 5% trichloroacetic acid. Incorporation of radiolabeled phosphate into precipitated proteins was determined by Cerenkov counting. Enzyme activity was corrected for calmodulin-independent kinase activity by assaying in the absence of calcium and calmodulin.

Casein kinase was assayed in 250-μl reactions containing 10 μl of partially purified casein kinase, 20 mM Tris, pH 7.5, 5 mM NaF, 5 mM casein, and 20 μM of [γ-^{32}P]ATP.

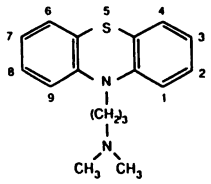
Assay of phorbol ester binding. [^{3}H]PDBU binding was determined as previously described (14).

Cell proliferation assay. The effect of drugs on the proliferation of A2780 ovarian carcinoma cells was determined with a microplate assay, as previously described (27). Cells were plated on day 1, at a density of 5000 cells/well, in RPMI 1640 medium containing 10% fetal bovine serum. Drug was added on day 2, and cell density was determined on day 4.

Results

Effect of modifying the phenothiazine nucleus. Fig. 1 shows the structures and IC_{50} values for inhibition of protein kinase C for a series of promazine derivatives having different substitutions on the phenothiazine nucleus. The parent compound, promazine, inhibited protein kinase C with an IC_{50} of 46 μM. Chloro substitution at position 2 had no effect, whereas this substitution at position 1, 3, or 4 decreased potency. Substitutions at position 2 had the following order of potency: H = Cl = SCH > CF_3. Oxidation of the sulfur at position 5 abolished activity.

The most potent compounds were the dihydroxylated (quinoid) analogs of chlorpromazine, DHCP and 6,9-dihydroxychlorpromazine, which had IC_{50} values of 8.3 and 9.7 μM, respectively. The oxidized form of DHCP, 7,8-dioxochlorpromazine, was also potent (IC_{50} = 15 μM). Addition of a third hydroxyl at position 3 of DHCP decreased the potency of that compound by 6-fold. Substitution of methoxy or acetoxy groups at positions 7 and 8 significantly decreased potency, as did substitution of a semicarbazone at position 7 on 7,8-dioxochlorpromazine.



Substituent	Position	Name	IC_{50} for PKC Inhibition (μM)
		Promazine	46 ± 8
—Cl	1	1-Chloropromazine	69 ± 10 [a]
—Cl	2	Chlorpromazine	50 ± 5 [b]
—Cl	3	3-Chloropromazine	82 ± 4 [c]
—Cl	4	4-Chloropromazine	290 ± 30 [c]
—Cl;—OH	2;7	7-Hydroxychlorpromazine	58 ± 10
—Cl;—OH;—OH	2;3;8	3,8-Dihydroxychlorpromazine	100 ± 20
—Cl;—OH;—OH	2;7;8	7,8-Dihydroxychlorpromazine	8.3 ± 0.1
—Cl;—OH;—OH	2;6;9	6,9-Dihydroxychlorpromazine	9.7 ± 0.8
—Cl;—OH;—OH;—OH	2;3;7;8	3,7,8-Trihydroxychlorpromazine	49 ± 7
—Cl;—OCH_3;—OCH_3	2;7;8	7,8-Dimethoxychlorpromazine	220 ± 10
—Cl;—$OCOCH_3$;—$OCOCH_3$	2;7;8	7,8-Diacetoxychlorpromazine	150 ± 30
—Cl;=O;=O	2;7;8	7,8-Dioxochlorpromazine	15 ± 1
—Cl;=$NNHCONH_3$;=O	2;7;8	7,8-Dioxochlorpromazine 7-Semicarbazone	66 ± 4
—S—CH_3	2	Thiomethylpromazine	50 ± 4
—CF_3	2	Trifluopromazine	170 ± 30
=O	5	Chlorpromazine Sulfoxide	>1000

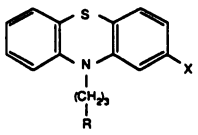
[a] $p < .05$
[b] p-not significant
[c] $p \leq .01$

Fig. 1. Effect of modifying the phenothiazine nucleus on activity against protein kinase C. IC_{50} values for inhibition of protein kinase C (mean ± standard deviation) were determined from triplicate dose-response curves generated with nine drug concentrations in the vesicle assay, as described in Experimental Procedures. The IC_{50} equals the concentration of drug required to inhibit 50% of the enzyme activity, compared with vehicle controls. *p* values are for comparison with promazine.

Effect of modifying the side chain amino group. Fig. 2 shows the structures and IC_{50} values for inhibition of protein kinase C for a series of chlorpromazine and trifluopromazine derivatives that have substitutions on the side chain amino group. The primary amine didesmethylchlorpromazine was more potent than the tertiary amine chlorpromazine, and the secondary amine desmethylchlorpromazine was less potent than chlorpromazine. Incorporation of the amino group into a piperazine ring increased potency dramatically [compare trifluopromazine and 2-trifluoromethyl-10-(3-[1-piperazinyl]propyl)phenothiazine]. However, if the piperazine ring contained substitutions on the nitrogen at position 4, potency was not significantly increased, as can be seen when comparing chlorpromazine and prochlorperazine or trifluopromazine and trifluoperazine. Also, substitution of a hydroxyethyl group for the methyl group at position 4 on the piperazine ring did not alter potency. However, replacing the methyl groups of chlorpromazine with ethyl groups (chlorproethazine) significantly decreased potency.

Effect of modifying the length of the alkyl bridge. Fig. 3 shows the structures and IC_{50} values for inhibition of protein kinase C for a series of chlorpromazine and promazine derivatives with different alkyl bridge lengths. A three-carbon bridge was found to be optimal. Decreasing the chain length from three carbons to two carbons, as in 2-chloro-10-(2-dimethylaminoethyl)phenothiazine and promethazine, significantly decreased potency. Also, the addition of a hydroxyl group to the side chain, as in 2-chloro-10-(3-dimethylamino-2-hydroxypropyl)phenothiazine, decreased potency. Increasing the chain length to four carbons, as in 2-chloro-10-(3-dimethylaminobutyl)phenothiazine, did not alter potency.

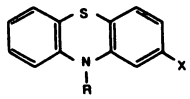
Inhibition of protein kinase C by compounds structurally related to phenothiazines. Fig. 4 shows the structures and IC_{50} values for inhibition of protein kinase C for a series of compounds that are structurally similar to the phenothiazines. The potency of R-6033 was increased by >10-fold by the addition of a fluorophenyl group, as in pimozide. Penfluridol and pimozide, which are potent anticalmodulin agents (15), inhibited protein kinase C with IC_{50} values of 49 and 76 μM, respectively. The remaining compounds were relatively poor inhibitors of protein kinase C (IC_{50} values of 280 to >1000 μM).



X	R	Name	IC_{50} for PKC Inhibition (μM)
—Cl	—NH_2	Didesmethylchlorpromazine	37 ± 6 [a]
—Cl	—NH—CH_3	Desmethylchlorpromazine	95 ± 6 [b]
—Cl	—N(—CH_3)—CH_3	Chlorpromazine	50 ± 5
—Cl	—N(—CH_2—CH_3)—CH_2—CH_3	Chlorproethazine	120 ± 2
—Cl	—N(piperazine)—N—CH_2—CH_2—OH	Perphenazine	63 ± 13 [c]
—Cl	—N(piperazine)—N—CH_3	Prochlorperazine	44 ± 5
—CF_3	—N(—CH_3)—CH_3	Trifluopromazine	170 ± 30 [d]
—CF_3	—N(piperazine)—N—CH_2—CH_2—OH	Fluphenazine	93 ± 18
—CF_3	—N(piperazine)—N—CH_3	Trifluoperazine	100 ± 30
—CF_3	—N(piperazine)—NH	2-Trifluoromethyl-10-(3-[1-piperazinyl] propyl)phenothiazine	4.7 ± 0.3

[a] $p < .05$ compared to chlorpromazine
[b] $p = .01$ " " "
[c] p=not significant compared to prochlorperazine
[d] p= " " " trifluoperazine

Fig. 2. Effect of modifying the type of side chain amino group on activity against protein kinase C. IC_{50} values (mean ± standard deviation) were determined as described for Fig. 1.



X	R	Name	IC_{50} for PKC Inhibition (μM)
—Cl	—CH_2—CH_2—N(—CH_3)—CH_3	2-Chloro-10-(2-dimethylaminoethyl) phenothiazine	310 ± 10
—Cl	—CH_2—CH_2—CH_2—N(—CH_3)—CH_3	Chlorpromazine	50 ± 5
—Cl	—CH_2—CH(OH)—CH_2—N(—CH_3)—CH_3	2-Chloro-10-(3-dimethylamino-2-hydroxypropyl)-phenothiazine	230 ± 10
—Cl	—CH_2—CH_2—CH_2—CH_2—N(—CH_3)—CH_3	2-Chloro-10-(4-dimethylaminobutyl) phenothiazine	56 ± 17
—H	—CH_2—CH(—CH_3)—N(—CH_3)—CH_3	Promethazine	290 ± 10
—H	—CH_2—CH_2—CH_2—N(—CH_3)—CH_3	Promazine	46 ± 8

Fig. 3. Effect of modifying the length of the side chain amino group on activity against protein kinase C. IC_{50} values (mean ± standard deviation) were determined as described for Fig. 1.

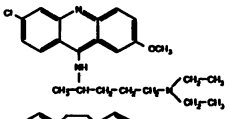
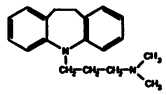
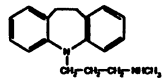
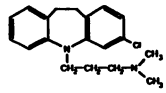
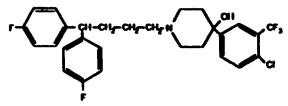
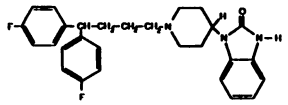
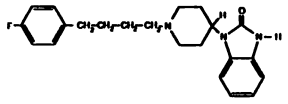
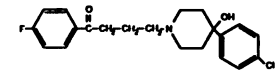
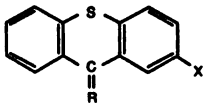
Compound	Structure	IC_{50} for PKC Inhibition (μM)
Quinacrine		850 ± 110
Imipramine		520 ± 10
Desipramine		540 ± 100
2-Chloroimipramine		280 ± 60
Penfluridol		49 ± 11
Pimozide		76 ± 3
R-6033		930 ± 100
Haloperidol		>1000

Fig. 4. Effect of compounds structurally related to phenothiazines on protein kinase C activity. IC_{50} values (mean ± standard deviation) were determined as described for Fig. 1.

Effect of thioxanthene stereoisomers on protein kinase C activity. The effect of thioxanthenes on the activity of protein kinase C is shown in Fig. 5. These compounds inhibited protein kinase C with IC_{50} values in the range of 14–100 μM (Fig. 5). Substitution of a trifluoro group for a chloride on the nucleus did not produce consistent changes in activity (compare chlorprothixene with compound 796 and clopenthixol with flupenthixol). As seen with the phenothiazines, an unsubstituted piperazine ring on the side chain increased activity (as in compound 751). No consistent stereospecificity was observed and, overall, there was little difference (usually <2-fold) between stereoisomers.

Characterization of DHCP and N751. Two compounds identified in this study, DHCP and the thioxanthene N751, were selected for further analysis because of their potency and distinct structural features. Fig. 6 shows the effects of three concentrations of each drug on enzyme activity with respect to phosphatidylserine. Fig. 6A shows a Lineweaver-Burke plot for inhibition of protein kinase C by DHCP with respect to phosphatidylserine. The data indicate noncompetitive inhibition with respect to phosphatidylserine (K_i = 26 μM). However, the kinetics of the thioxanthene N751 were competitive with respect to phosphatidylserine (K_i = 13 μM) (Fig. 6B). At the highest concentration tested, we observed mixed-type inhibition with N751.

We next investigated the ability of DHCP and N751 to compete with ATP for activation of the enzyme. Fig. 7A demonstrates mixed-type inhibition by DHCP with respect to ATP



X	R	Name	IC_{50} for PKC Inhibition (μM)
—Cl	=CH—CH$_2$—CH$_2$—N(CH$_3$)$_2$	*cis*-762 (B)	59 ± 4
		trans-762 (A)	91 ± 6
—Cl	=CH—CH$_2$—CH$_2$—N(CH$_3$)$_2$	*cis*-chlorprothixene	80 ± 22
		trans-chlorprothixene	49 ± 1
—Cl	=CH—CH$_2$—CH$_2$—N(CH$_2$CH$_3$)$_2$	*cis*-768 (B)	46 ± 1
		trans-768 (A)	45 ± 3
—CF$_3$	=CH—CH$_2$—CH$_2$—N(CH$_3$)$_2$	*cis*-796 (A)	45 ± 5
		trans-796 (B)	70 ± 2
—Cl	=CH—CH$_2$—CH$_2$—N(CH$_2$)	*cis*-753 (B)	86 ± 3
		trans-753 (A)	90 ± 5
—Cl	=CH—CH$_2$—CH$_2$—N(NH)	751	14 ± 2
—Cl	=CH—CH$_2$—CH$_2$—N(N—CH$_2$CH$_2$OH)	*trans*-7006	91 ± 15
—Cl	=CH—CH$_2$—CH$_2$—N(N—CH$_2$CH$_2$OH)	*cis*-clopenthixol	65 ± 10
		trans-clopenthixol	37 ± 2
—CF$_3$	=CH—CH$_2$—CH$_2$—N(N—CH$_2$CH$_2$OH)	*cis*-flupenthixol	71 ± 4
		trans-flupenthixol	29 ± 3
—Cl	—CH$_2$—CH$_2$—CH$_2$—N(N—CH$_2$CH$_2$OH)	789	100 ± 12

Fig. 5. Effect of thioxanthene stereoisomers on protein kinase C activity. IC_{50} values (mean ± standard deviation) were determined as described for Fig. 1.

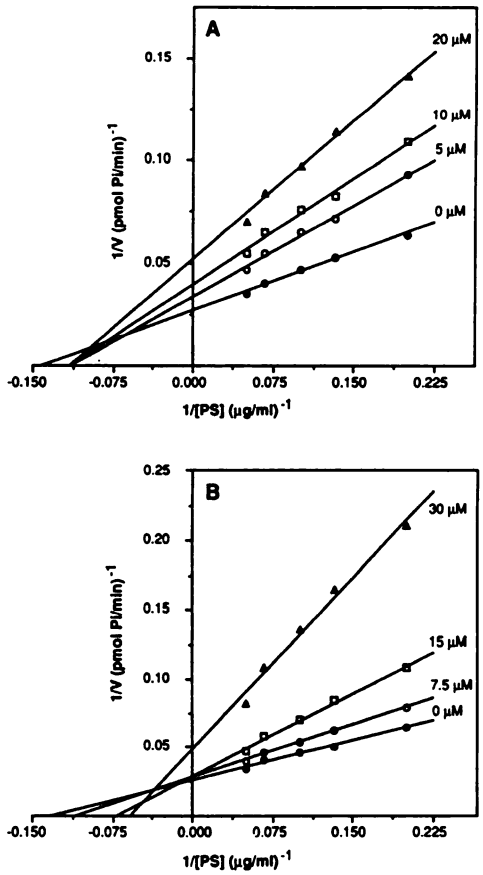


Fig. 6. Inhibition of protein kinase C by DHCP and N751 with respect to phosphatidylserine. Double-reciprocal plots were generated for the interaction between drug and phosphatidylserine (*PS*). A, Effect of DHCP at 0 μM (●), 5 μM (○), 10 μM (□), and 20 μM (△). B, Effect of N751 at 0 μM (●), 7.5 μM (○), 15 μM (□), and 30 μM (△).

(K_i = 10 μM). Fig. 7B demonstrates noncompetitive inhibition by N751 with respect to ATP (K_i = 9 μM). The mechanism of inhibition shifted to mixed-type at 30 μM.

To determine whether DHCP or N751 interacted with the phorbol ester binding site on protein kinase C, we examined the abilities of the drugs to inhibit [^{3}H]PDBU binding to the enzyme. Fig. 8 demonstrates that N751 (IC_{50} = 44 μM) was more potent than DHCP (IC_{50} = 310 μM) as an inhibitor of phorbol ester binding.

Activity of DHCP and N751 against other protein kinases. To determine the selectivity of DHCP and N751 for protein kinase C, we tested the ability of these drugs to inhibit cAMP-dependent protein kinase, calmodulin-dependent protein kinase type II, and casein kinase, enzymes that play important roles in transmembrane signaling and cellular regulation. DHCP had an IC_{50} of 200 μM for inhibition of cAMP-dependent protein kinase, 65 μM for inhibition of calmodulin-dependent protein kinase type II, and 218 μM for inhibition of casein kinase (Table 1). Therefore, DHCP was 24-fold more potent against protein kinase C compared with cAMP-dependent protein kinase, 8-fold more potent compared with calmodulin-dependent protein kinase type II, and 26-fold more potent compared with casein kinase.

N751 had an IC_{50} of 100 μM for inhibition of cAMP-dependent protein kinase, 6.6 μM for inhibition of calmodulin-dependent protein kinase type II, and >218 μM for inhibition of casein

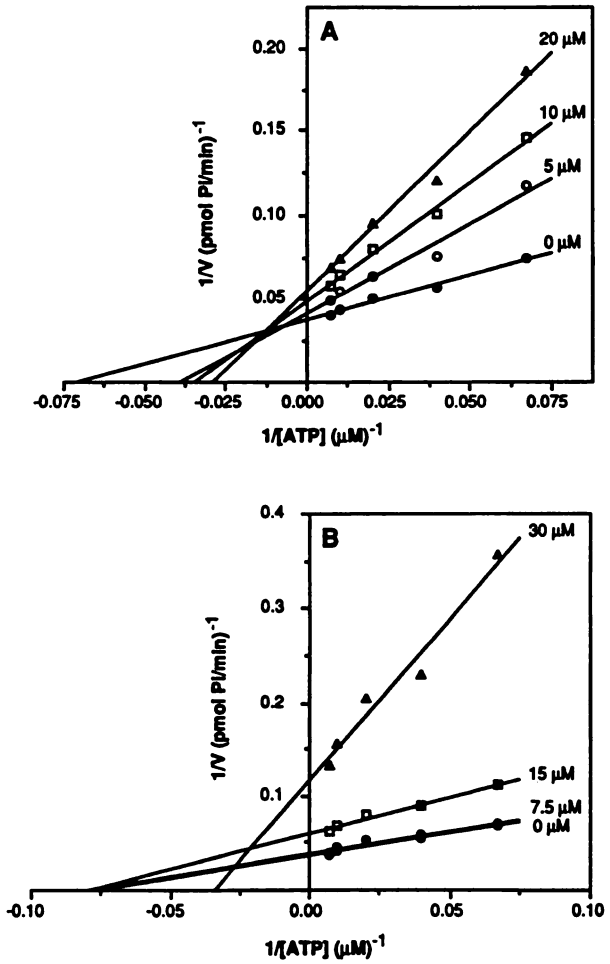


Fig. 7. Inhibition of protein kinase C by DHCP and N751 with respect to ATP. Double-reciprocal plots were generated for the interaction between drug and ATP. Reactions were set up with various concentrations of [γ-^{32}P]ATP and initiated by the addition of enzyme. A, Effect of DHCP at 0 μM (●), 5 μM (○), 10 μM (□), and 20 μM (△). B, Effect of N751 at 0 μM (●), 7.5 μM (○), 15 μM (□), and 30 μM (△).

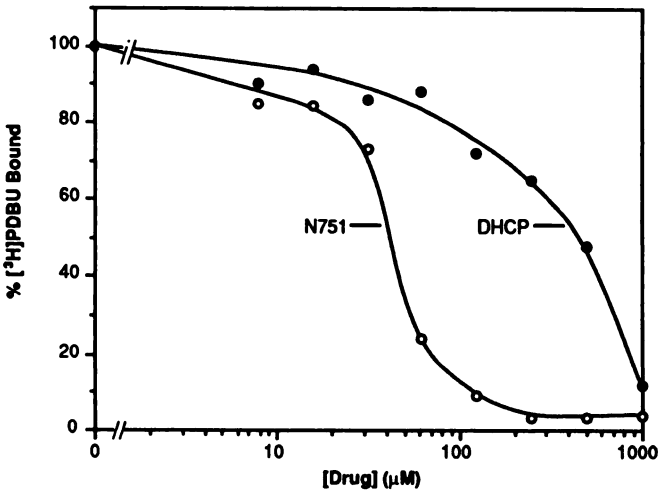


Fig. 8. Inhibition of phorbol ester binding to protein kinase C by DHCP and N751. The effect of DHCP (●) and N751 (○) on [^{3}H]PDBU binding to partially purified protein kinase C was determined in the absence and presence of 30 μM PMA. Specific binding was determined by subtracting the counts obtained in the presence of unlabeled PMA from counts obtained in the absence of unlabeled PMA. The concentration of [^{3}H]PDBU was 30 nM. Each *point* represents the average of two determinations.

TABLE 1

Effect of DHCP and N751 on protein kinase C, cAMP-dependent protein kinase, calmodulin-dependent protein kinase, and casein kinase

IC_{50} values for inhibition of protein kinase C (PKC) and cAMP-dependent protein kinase (PK) were determined as described for Fig. 1. IC_{50} values for inhibition of calmodulin-dependent protein kinase were determined from dose-response curves generated with five concentrations of drug. IC_{50} values for inhibition of casein kinase were determined from dose-response curves generated with three concentrations of drug.

Drug	Enzyme			
	PKC	cAMP-dependent PK	Calmodulin-dependent PK II	Casein kinase
	IC_{50} (μM)			
DHCP	8.3	200	65	218
N751	14	100	6.6	>218

kinase (Table 1). Therefore, N751 was 7-fold more potent against protein kinase C compared with cAMP-dependent protein kinase, and at least 15-fold more potent compared with casein kinase, but was 2-fold more potent against calmodulin-dependent protein kinase type II compared with protein kinase C.

Kinetics for inhibition of protein kinase C by DHCP, utilizing the mixed micelle assay. DHCP was subjected to further analysis because it was both active against and selective for protein kinase C. We used the mixed micelle assay to determine the inhibition kinetics in a well defined lipid environment (29). Fig. 9 shows the Lineweaver-Burke plots for inhibition of protein kinase C by DHCP with respect to phosphatidylserine and ATP. The data indicate competitive inhibition with respect to phosphatidylserine (Fig. 9A) and competitive inhibition with respect to ATP (Fig. 9B).

Inhibition of protein kinase C isozymes by DHCP. We next determined whether DHCP could selectively inhibit several of the known isozymes of protein kinase C. Table 2 shows the IC_{50} values for inhibition of the α, β(I/II), γ, and δ isozymes of protein kinase C. Although some differences were seen, the data indicate no significant selectivity for any of the isozymes.

Effect of DHCP on cellular proliferation. To determine whether DHCP had effects on intact cells, we studied its ability to inhibit the proliferation of A2780 ovarian carcinoma cells. Fig. 10 shows the dose-response curve for cell growth inhibition by DHCP. In this cell line, DHCP inhibited growth with an IC_{50} of 8 μM.

Discussion

The establishment of structure-activity relationships is important for the rational design of potent and selective enzyme inhibitors. Phenothiazines have been shown to inhibit protein kinase C with IC_{50} values in the 10^{-5} M range (10), and numerous structurally modified congeners are available for testing. Therefore, in the present study we analyzed phenothiazines and related molecules to determine which structural features influence activity and to discover more selective drugs.

Phenothiazines containing quinoid structures on the phenothiazine nucleus were among the most potent and selective inhibitors of protein kinase C from this class. This structure-activity relationship is further supported by the presence of quinoid structures in other inhibitors of protein kinase C, such as quercetin (16), doxorubicin (4, 10, 17, 18), calphostin C (19),

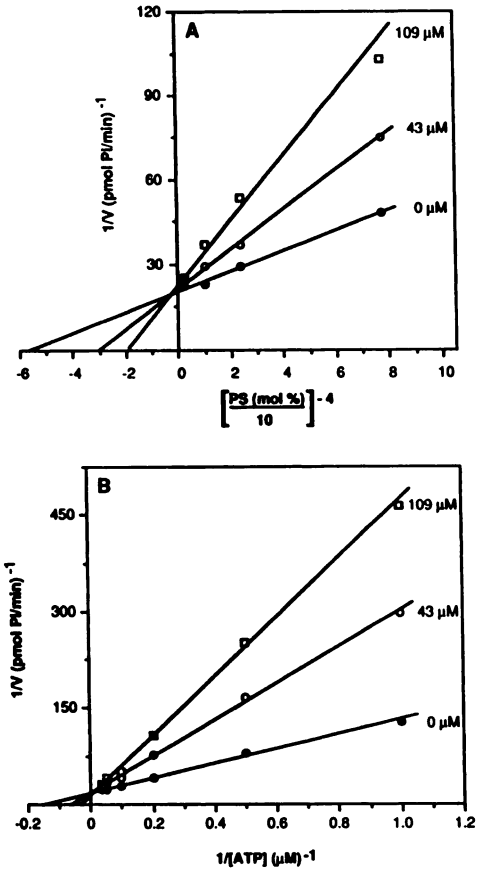


Fig. 9. Mixed micellar kinetics for inhibition of protein kinase C by DHCP. Reactions were carried out using three concentrations of drug, 0 μM (●), 43 μM (○), and 109 μM (□), which represent 0, 1, and 2 mol %, respectively. A, Kinetics with respect to phosphatidylserine. The *abscissa* was plotted as 1/[phosphatidylserine]^n, where *n* represents the apparent Hill number. B, Kinetics with respect to ATP.

TABLE 2

Effect of DHCP on isolated isozymes of protein kinase C

IC_{50} values for inhibition of protein kinase C isozymes were determined in the vesicle assay from dose-response curves generated with three concentrations of drug.

Isozyme	IC_{50}
	μM
α	24
β(I/II)	15
γ	18
δ	31

apomorphine (20), and LY 170198 (20). In an aerobic environment and in the presence of reducing equivalents, quinoid compounds may act as redox catalysts to produce hydrogen peroxide and superoxide anions directly, and hydroxyl radicals indirectly, all of which have the potential to inhibit enzyme activity (21). In fact, Gopalakrishna and Anderson (22, 23) have shown that protein kinase C can be inhibited by high concentrations of hydrogen peroxide. Therefore, the quinoid phenothiazines may inhibit protein kinase C through an oxidative or reductive mechanism.

A piperazine ring with a secondary nitrogen at position 4 was another important structure for activity. This relationship is supported by two observations. First, two of the most active compounds in our study, 2-trifluoromethyl-10-(3-[1-piperazinyl]propyl)phenothiazine (Fig. 2) and the thioxanthene N751 (Fig. 5), had piperazine rings with secondary nitrogens at position 4. Second, the widely used isoquinolinesulfonamide protein kinase inhibitor H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine] also has a piperazine ring with a secondary nitrogen at position 4 (24). Therefore, a piperazine ring with a secondary nitrogen at position 4 appears to be an important structural feature.

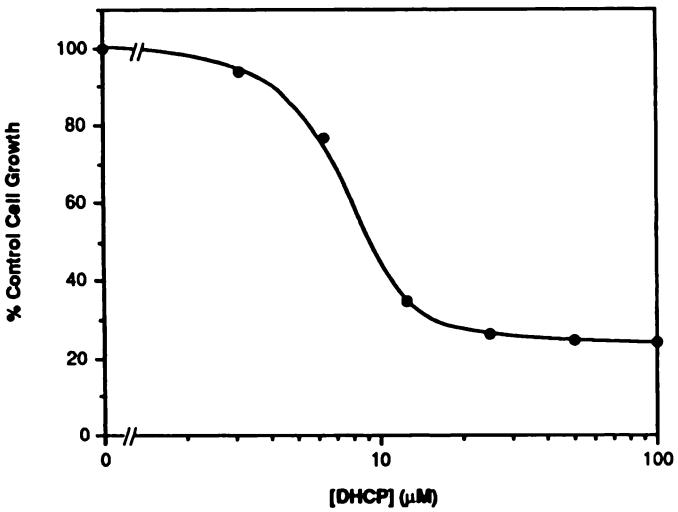


Fig. 10. Effect of DHCP on cellular proliferation. DHCP was evaluated for its ability to inhibit the growth of A2780 ovarian carcinoma cells in a microplate assay. Cells were plated on day 1, drug was added on day 2, and cell density was determined on day 4.

The order of potency for methyl substitution on the side chain amino group of chlorpromazine was primary > tertiary > secondary, whereas the order of potency for methyl substitution on the amino group of sphingosine has been shown to be primary > secondary > tertiary (25). Therefore, primary amino groups appear to be important determinants for activity. However, the most potent compound we studied, 2-trifluoromethyl-10-(3-[1-piperazinyl]propyl)phenothiazine, contains only secondary and tertiary amines. Furthermore, Hannun and Bell (26) determined that acridine orange, an aminoacridine protein kinase C inhibitor with only tertiary and quaternary amino groups, was more potent than other inhibitors in that class that contained only primary and secondary amines. The same group also showed that acetylation of the amino group on doxorubicin significantly decreased its potency against protein kinase C (18). Therefore, simple correlations between the degree of substitution of amino groups and potency cannot be made. It is likely that both the degree of substitution and the placement of amino groups within the three-dimensional structure of the molecule are important determinants for activity.

Because protein kinase C interacts with hydrophobic molecules, we investigated whether hydrophobicity plays a role in the ability of the phenothiazines to inhibit the enzyme. In fact, it has been previously demonstrated that, with phenothiazines having substitutions made only on the nucleus, inhibitory activity against calmodulin (15), cellular proliferation (27), and multidrug resistance (27) correlate with hydrophobicity (octanol/buffer partition coefficient). However, with a correlation coefficient of 0.47, this phenomenon did not hold for inhibition of protein kinase C.[2]

[2]D. T. Aftab and W. N. Hait, unpublished observations.

The thioxanthenes are structurally related to the phenothiazines, differing by a carbon substitution for the nitrogen at position 10 on the phenothiazine nucleus and by a double bond connecting that carbon to the side chain. These classes of compounds share several pharmacological properties, including antipsychotic activity (30). The thioxanthenes were generally equal in potency to their phenothiazine homologues (see Figs. 2 and 5). However, some of the structure-activity relationships that existed for the phenothiazines did not hold for the thioxanthenes. For example, trifluoro substitution at position 2 significantly decreased potency, compared with chloro substitution, in the phenothiazines (Fig. 1) but not the thioxanthenes (compare chlorprothixene with compound 796 and clopenthixol with flupenthixol in Fig. 5).

We chose two compounds, DHCP and N751, for further analysis because they represent two important structural features for activity against protein kinase C; DHCP contains a quinoid structure on the phenothiazine nucleus, whereas N751 contains an unsubstituted piperazine ring on the side chain. To determine whether they inhibited by either of two common mechanisms for protein kinase C inhibitors, namely competition with ATP or phosphatidylserine, kinetic analyses were carried out with both drugs. We identified another compound containing an unsubstituted piperazine ring, 2-trifluoromethyl-10-(3-[1-piperazinyl]propyl)phenothiazine (Fig. 2), but, because of its structural similarity to N751 and its limited availability, we did not perform additional kinetic analyses with this drug.

In the vesicle assay described by Nishizuka and co-workers (8), the kinetics of inhibition by DHCP were noncompetitive with respect to phosphatidylserine and mixed with respect to ATP. These results are in contrast to studies of other phenothiazines analyzed with the same vesicle assay (8–10). However, the quinoid nucleus of DHCP may be conducive to oxidative or reductive mechanisms that could contribute to its activity against protein kinase C (see above in Discussion). We have determined that DHCP rapidly catalyzes the production of hydrogen peroxide in the presence of oxygen and reducing equivalents. However, neither catalase nor superoxide dismutase could protect protein kinase C from inhibition by DHCP.[2] Therefore, production of reactive forms of oxygen is probably not involved in the mechanism of inhibition. However, DHCP also lost most of its potency when the reactions were carried out under nitrogen.[2] Therefore, the mechanism of action of DHCP may be related to the potential ability of the drug to form intermediate structures as a result of redox cycling (21).

The inhibition kinetics of the thioxanthene N751 were competitive with respect to phosphatidylserine and noncompetitive with respect to ATP. This is consistent with other studies on the mechanism of action of structurally related phenothiazines such as chlorpromazine and trifluoperazine (8–10).

We directly compared the potency of two lead compounds, DHCP and N751, against protein kinase C, cAMP-dependent protein kinase, calmodulin-dependent protein kinase type II, and casein kinase. N751 was selective for protein kinase C compared with cAMP-dependent kinase and casein kinase; however, it was more active against the calmodulin-dependent kinase. DHCP was selective for protein kinase C compared with all of the other kinases tested. In contrast, the widely used inhibitor H-7 was more active against at least two protein kinases (including cAMP-dependent protein kinase) other than protein kinase C (24).

Based on its activity and selectivity for protein kinase C, DHCP was subjected to further analysis, utilizing the mixed micelle assay, to determine the inhibition kinetics in a well defined lipid environment. We observed competitive inhibition with respect to both ATP and phosphatidylserine. The ATP kinetics were similar in both the vesicle and mixed micelle assay systems. Thus, DHCP revealed mixed-type inhibition in the vesicle assay and competitive inhibition in the mixed micelle assay. These data indicate that DHCP interacts at the ATP site on protein kinase C.

DHCP was noncompetitive with respect to phosphatidylserine in the vesicle assay but competitive when analyzed by the mixed micelle assay. When transforming the phosphatidylserine data from the mixed micelle assay for double-reciprocal analysis, it is necessary to plot 1/[phosphatidylserine]^n, where n is the apparent Hill number. This treatment of the data is required because of the highly cooperative nature of phosphatidylserine stimulation of protein kinase C activity in the mixed micelle system (29). Therefore, the fact that cooperativity is not evident in the vesicle system makes meaningful comparisons between phosphatidylserine kinetics for the two assay systems difficult.

Because protein kinase C is made up of a family of at least seven isozymes, it is possible that different isozymes play different roles in cellular regulation (3). Consequently, isozyme-specific inhibitors would be valuable tools for elucidating these putative roles. Therefore, we tested the ability of DHCP to inhibit the α, β(I/II), γ, and δ isozymes of protein kinase C. Although some differences in potency were observed, DHCP was not distinctly isozyme selective.

Protein kinase C is considered to play a key role in numerous cellular processes, including cell growth and differentiation and extracellular signal transduction (1, 3). Therefore, the activity of DHCP in several cellular systems has been studied. DHCP inhibited A2780 ovarian carcinoma cell proliferation (IC_{50} = 8 μM) (Fig. 10) and epidermal growth factor-induced DNA synthesis in BALB/MK murine epidermal keratinocytes (IC_{50} = 12.5 μM),[3] and it enhanced the scatter factor-induced scattering of Madin-Darby canine kidney cells (5–10 μM) (28). These effects, as well as the effect on partially purified protein kinase C, occur with similar potency (~10 μM). Also, other protein kinase inhibitors, such as H-7 and staurosporine, have similar effects on these systems at concentrations close to those required to inhibit protein kinase C *in vitro* (28).[2, 3] Therefore, the effects of DHCP on whole cells are consistent with its ability to inhibit protein kinase C.

Phenothiazines have numerous pharmacological actions, some of which might be explained by inhibition of protein kinase C and others by their known effects such as antagonism of dopaminergic, muscarinic, and α-adrenergic receptors (30). The clinical utility of inhibitors of protein kinase C might be limited by the expression of the enzyme in almost every tissue. The ultimate clinical use of these inhibitors will depend on factors such as the pharmacodynamics and pharmacokinetics of the particular drug and the relative concentrations of targets/receptors in the tissues where the drug is distributed.

The structure-activity relationships for substitutions on the

[3] M. Reiss, personal communication.

phenothiazine nucleus for inhibition of protein kinase C are in contrast to those for anticalmodulin activity. For example, quinoid structures on the nucleus increase potency against protein kinase C but decrease potency against calmodulin (15). Also, the order of potency of substitutions at position 2 for inhibition of protein kinase C is not the same as that for inhibition of calmodulin-dependent phosphodiesterase (15). These phenomena indicate that selectivity between protein kinase C and other kinases can be achieved through the rational design of inhibitors from the phenothiazine class.

Acknowledgments

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