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Pharmacological Properties of Fluphenazine-Mustard, an Irreversible Calmodulin Antagonist

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SUMMARY

We describe an improved synthesis and properties of fluphenazine-mustard, a potent phenothiazine having an alkylating chloroethylamine chain in its structure. The drug possesses anticalmodulin activity equivalent to the parent compound, but unlike fluphenazine dihydrochloride, the mustard derivative irreversibly antagonizes the ability of calmodulin to activate cyclic nucleotide phosphodiesterase. This property is partially calcium-dependent and can be overcome by coincubation with excess fluphenazine dihydrochloride. The compound irreversibly inactivated calmodulin when incubated with intact cells and caused single-stranded breakage of DNA. Fluphenazine-mustard possesses potent antiproliferative and cytotoxic properties against malignant cell lines that are likely to be mediated through both of these actions.

Phenothiazines are an important class of antipsychotic drugs that bind to and inhibit various enzymes, receptors, and regulatory proteins, such as protein kinase C (1), dopamine receptors (2), and calmodulin (3). The binding of the phenothiazines to calmodulin has been carefully studied and shown to involve both hydrophobic and electrophilic interactions (4). The association of the penothiazines with calmodulin is usually reversible but can be made irreversible by exposure of the calmodulin drug complex to ultraviolet light (5, 6) or by treatment with peroxidase-hydrogen peroxide (5). Recently, drugs with carbamoylating properties, such as norchloropromazine isothiocyanate (7), or alkylating properties, such as fluphenazine-mustard (Smith, Kline and French 7171-A) (8) and phenoxybenzamine (9), have been shown to irreversibly inactivate calmodulin.

Since several targets of the phenothiazines are involved in the regulation of cellular proliferation, we have previously studied the effect of these and related compounds on growth and clonogenicity of malignant cells (10–12). In particular, we have concentrated on the interaction of drugs with calmodulin, a calcium-binding protein believed to regulate the effects of calcium on normal and abnormal cellular proliferation (13–15). For example, Chafouleas *et al.* (14, 16) and Sasaki and Hidaka (17) have shown that progression of cells into and through the cell cycle is associated with unique changes in calmodulin content and that the progression is blocked by antagonists of calmodulin. Also, certain transformed cells have a greater concentration of calmodulin than their normal counterparts (18, 19) and may require less calcium to sustain cellular division (20).

We now report an improved synthesis of fluphenazine-mustard, a potent phenothiazine that incorporates an alkylating chloroethylamine chain into its structure, the characterization of fluphenazine-mustard's interaction with calmodulin, and its effect on DNA and growth of malignant cells.

Materials and Methods

Fluphenazine dihydrochloride was prepared by a modification of the method of Yale and Sowinski (21). Accordingly, 4.8 g (0.1 mol) of a 50% dispersion of sodium hydride in mineral oil (rather than sodium amide) was added cautiously, in small portions, to a stirred solution of 26.9 g (0.1 mol) of 2-trifluoromethylphenothiazine in 250 ml sieve-dried DMSO (rather than toluene) at 20–25°C. The resulting red solution was added dropwise to a stirred solution of 1-bromo-3-chloropropane (63.0 g, 0.4 mol) in 75 ml sieve-dried DMSO. After the addition was completed, the solution was stirred at 50°C for 1 hr; it was then poured into 1 l ice-water, and the mixture was extracted with ether. The ether extracts were washed with water, dried ($MgSO_4$), decolorized (decolorizing carbon), and concentrated. The residue was dissolved in 150 ml acetonitrile, and the solution was extracted three times with 50-ml portions of *n*-hexane to remove mineral oil. After the acetonitrile extracts were concentrated the remaining solid was recrystallized from methanol-water to give 15.2 g (49.5%) of nearly colorless crystalline (10-(3-chloropropyl)-2-trifluoromethylphenothiazine, m.p.

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; EGTA, ethylene glycol bis(β-aminoethyl esther)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IR, infrared spectrometry; MS, mass spectrometry; NBP, 4-(*p*-nitrobenzyl)pyridine.

72–73°C (m.p. 70–71°C in Ref. 21). This material gave a single spot on TLC (silica, 1:1 *n*-hexane:ethyl acetate); R_F = 0.82 IR, ^{1}H-NMR, and MS were consistent for the assigned structure.

A mixture of 12.3 g (0.04 mol) of the above chloropropylated phenothiazine, 1-(2-hydroxyethyl)piperazine (10.4 g, 0.08 mol), sodium iodide (6.0 g, 0.04 mol), and 250 ml of 2-butanone was stirred and refluxed for 16 hr. After the mixture was concentrated to 100 ml, it was poured into 500 ml of 2 N hydrochloric acid at 10°C. The mixture was extracted twice with ether, and the aqueous solution was made alkaline with concentrated ammonium hydroxide. After the mixture was extracted with ethyl acetate, the organic solution was washed with water, dried ($MgSO_4$), and concentrated. The resulting liquid was dissolved in ethanol, and the solution was made acidic with hydrogen chloride to give 19.6 g (96%) of fluphenazine dihydrochloride, m.p. 235–237°C (m.p. 231–233°C in Ref. 21) after recrystallization from ethanol.

Fluphenazine dihydrochloride, thus obtained, was converted to fluphenazine-mustard by modification of the method of Anderson *et al.* (22). Accordingly, fluphenazine dihydrochloride (17.9 g, 0.035 mol) in 400 ml chloroform was saturated with gaseous hydrogen chloride, and 8.3 g (0.07 mol) thionyl chloride were added. After the mixture was stirred at 25°C for 16 hr (rather than at reflux temperature), it was filtered. Recrystallization of the solid from ethanol gave 15.6 g (84%) of chemically stable pale yellow needles of (2-chloroethyl)-4-[3-(2-trifluoromethyl-10-phenothiazinyl)propyl]piperazine dihydrochloride (fluphenazine-mustard), m.p. 227–229°C (m.p. 209–211°C in Ref. 22). TLC (silica, 1:3:60 concentrated ammonium hydroxide:ethanol:chloroform) R_F = 0.85; MS (CH − CI) m/e = 456 (M + 1); ^{1}H-NMR, and IR consistent for structure.

$C_{22}H_{25}ClF_3N_3S$ 2HCl

Calculated: C 49.96 H 5.15 N 7.95
Found: C 49.93 H 5.12 N 7.79

The structure of the parent compound and its mustard derivative are shown in Fig. 1.

The biological activity of calmodulin was measured by its ability to activate a sensitive cyclic nucleotide phosphodiesterase prepared from murine cerebrum as previously described (10). Phosphodiesterase activity was measured by the luciferin-luciferase method (23). Times of incubation and concentrations of tissue were adjusted to ensure linearity.

The effect of drugs on calmodulin activity was measured by their ability to inhibit by 50% (IC_{50}) the activation of phosphodiesterase by 2 U (6 ng) of calmodulin (1 U equals the concentration of calmodulin required to produce half-maximal activation of the enzyme). In other studies, 6 ng calmodulin were incubated for 5–30 min with various concentrations of drugs or vehicle in a total volume of 500 μl of 60 mM glycyl-glycine buffer, containing 30 mM ammonium acetate, 4 mM magnesium chloride, 0.3 mM phosphoenol pyruvate, 7 mM dithiothreitol, 0.1 mM calcium chloride, pH 8.0. The reaction was terminated by adding 20 μl of 25 mM sodium thiosulfate to neutralize alkylating activity. Tubes were placed on ice, and the samples were immediately transferred into dialysis bags having a molecular weight cut-off of 3,500

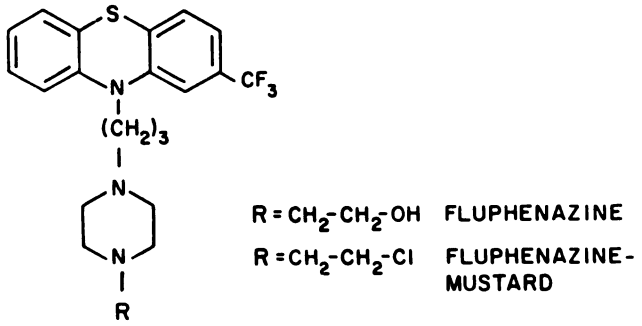

Fig. 1. Structure of fluphenazine and fluphenazine-mustard.

D (Spectrum Industries, Los Angeles, CA) then dialyzed for 24–48 hr against 3 l of 50 mM Tris buffer containing 0.1 mM calcium chloride, pH 7.4. After dialysis the remaining biological activity of calmodulin in this preparation was assayed as described above.

To determine the effect of drugs on intracellular calmodulin, 20 × 10^6 L1210 cells were incubated for 60 min with 50 μM fluphenazine dihydrochloride or fluphenazine-mustard. Cells were washed twice by centrifugation at 200 × *g* × 10 min at 4°C then resuspended in 20 ml drug-free Fisher's medium. Greater than 95% of cells were viable as estimated by trypan-blue exclusion. A portion of the medium from the second wash was removed and found to be free of anticalmodulin activity. Cells were resuspended in phosphate-glycine buffer containing 0.088 M NaH_2PO_4, 0.023 M Na_2HPO_4, 0.64 M glycine, and 0.001 M EGTA, (pH 6.0) and disrupted by sonication (10.05-sec pulses at 100 W-sec) using a Tekmar Sonic Disrupter (Tekmar/Hereaus, Cincinnati, OH). The sonicate was heated in a boiling water bath for 5 min, precipitated proteins removed by centrifugation at 3,600 × *g* × 20 min and the supernatant dialyzed against 0.005 M Tris buffer containing 1 mM $CaCl_2$ (pH 7.6) for 48 hr. The biological activity of calmodulin in this preparation was assayed as described above.

Leukemic cell lines were grown in Fisher's medium supplemented with 5% horse serum as previously described (11). C_6 and 9L astrocytoma cells were grown in monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (10). All cell lines were maintained in logarithmic growth at 37°C in an atmosphere of 5% CO_2-95% air and were checked monthly and found to be free of contamination by mycoplasma, bacteria, or fungi.

Cells were exposed to a range of concentrations of drug for 1–48 hr then counted with an electronic counter. In addition, the cytotoxic effects of drugs were determined by a soft-agar clonogenic assay (24). In these studies, cells were exposed to drugs for 24 hr, washed by centrifugation at 100 × *g* × 10 min, then resuspended in drug free medium; 100–1,000 cells were added to culture tubes containing Fisher's medium supplemented with 15% horse serum and 0.3% agar. After 14 days incubation at 37°C in an atmosphere of 95% air-5% CO_2, microscopic colonies were stained with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and counted 24 hr later using a dissecting microscope.

The effect of drugs on DNA breakage was determined by the alkaline elution method described by Kohn (25). The alkylating activity of drugs was determined using 4-(*p*-nitrobenzyl) pyridine as previously described (26). Concentration of protein was determined by the method of Lowry (27).

Calmodulin was obtained from Calbiochem-Behring (La Jolla, CA) or purified from L1210 leukemic cells as previously described (10). Nitrogen-mustard and luciferin-luciferase were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemotherapeutic agents were obtained through commercial sources. All chemicals were analytical grade and were purchased from commercial sources.

Results

To determine if fluphenazine-mustard maintained pharmacological activity, its effect on calmodulin-activated phosphodiesterase was compared with that of fluphenazine. Figure 2 demonstrates the IC_{50} for fluphenazine-mustard (4 μM) was similar to that of fluphenazine (10 μM). In the absence of calmodulin there was less than 5% inhibition of phosphodiesterase activity by either drug.

To study whether the phenothiazine derivative possessed alkylating activity, it was assayed by the NBP technique (23). Figure 3 demonstrates the dose related alkylation of NBP by fluphenazine-mustard. The parent compound, fluphenazine dihydrochloride, had no alkylating activity.

To characterize whether fluphenazine-mustard could "irreversibly" antagonize calmodulin, 6 ng calmodulin were prein-

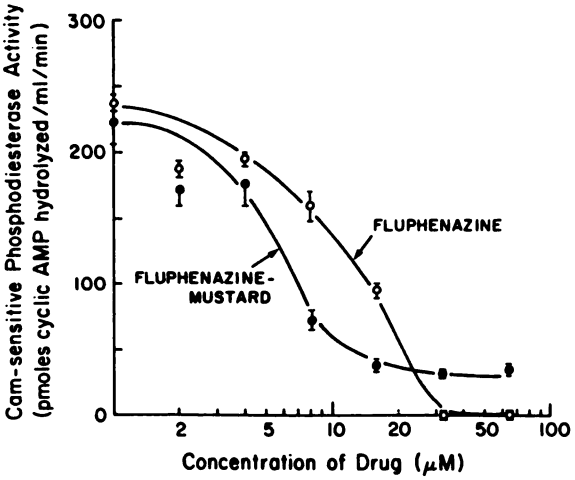

Fig. 2. The effect of fluphenazine and fluphenazine-mustard on the biological activity of calmodulin. Calmodulin was purified from L1210 leukemic lymphocytes as previously described (11) and assayed by the activation of cyclic nucleotide phosphodiesterase. Drugs were dissolved in water and their effect on calmodulin-activated phosphodiesterase was determined in the presence and absence (data not shown) of 2 units calmodulin. In the absence of calmodulin there was less than 5% inhibition of enzymic activity at the highest concentration of drug. Each point represents the mean ± standard deviation of triplicate determinations.

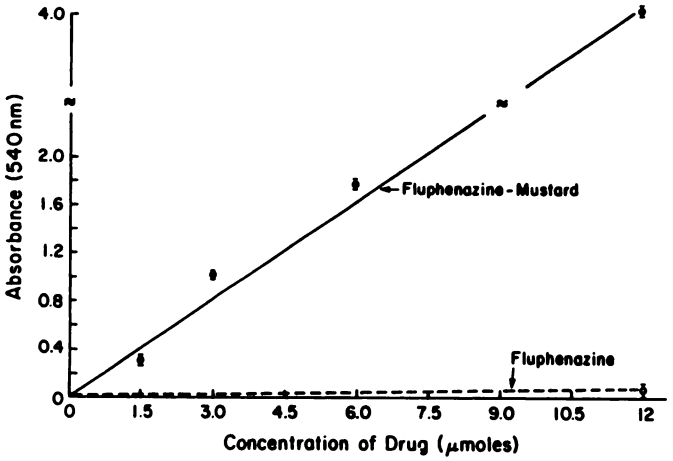

Fig. 3. Measurement of the alkylating activity of fluphenazine-mustard. Alkylating activity of fluphenazine-mustard and fluphenazine dihydrochloride was determined by the 4-(*p*-nitrobenzyl) pyridine technique as described in Materials and Methods. Each point represents the mean of three determinations.

cubated with 10 μM of either fluphenazine-mustard, fluphenazine dihydrochloride, nitrogen mustard, or vehicle then dialyzed. Irreversible inhibition was defined as the inability to reverse the inactivation of calmodulin after 48 hr dialysis. Fig. 4 shows the irreversible inhibition of calmodulin by fluphenazine-mustard, whereas the inhibition of calmodulin by fluphenazine dihydrochloride was completely reversible. Nitrogen mustard had no demonstrable effect on calmodulin activity. In addition, we studied other alkylating agents and DNA binding drugs and found they had little effect on calmodulin activity (Table 1).

To test the calcium requirement for the irreversible inhibition of calmodulin by fluphenazine-mustard, calmodulin (50 ng) was incubated for 30 min with 400 μM EGTA or 400 μM EGTA plus 1 mM $CaCl_2$ in 50 mM Tris buffer, adjusted to pH 7.4 in the presence or absence of 20 μM fluphenazine-mustard.

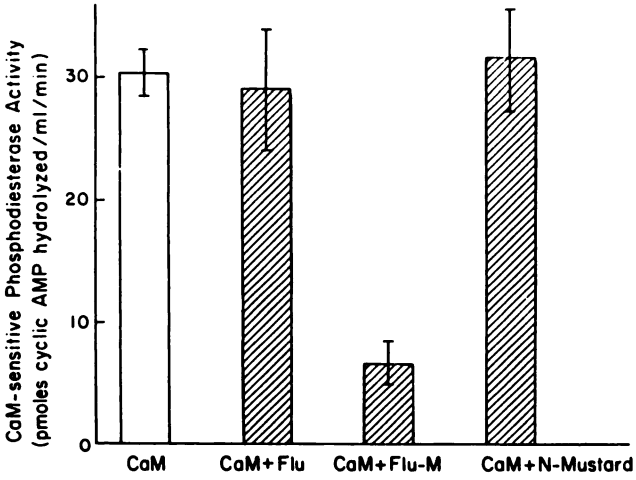

Fig. 4. Irreversible inhibition of the biological activity of calmodulin (CaM) by fluphenazine-mustard (Flu-M). Calmodulin (6 ng) was incubated for 30 min with 20 μM of either fluphenazine-mustard, fluphenazine dihydrochloride, or nitrogen mustard. The samples were then dialyzed for 24 hr and assayed for biological activity as described in Materials and Methods. Each point represents the mean ± standard deviation of quadruplicate samples from a representative of three similar experiments.

TABLE 1

Effect of alkylating agents and DNA binding drugs on activity of calmodulin

Drugs were dissolved in water or ethanol, and for each the effect of the drugs and their vehicle and preservatives were tested. CaM was preincubated with the drug for 15 min then assayed for its ability to activate a sensitive phosphodiesterase as described in Materials and Methods. Each drug was tested in quadruplicate at a concentration of 100 μM. Drugs were freshly prepared and used immediately.

Drug	Inhibition
	%
Phenothiazines	
Fluphenazine-mustard	90
Fluphenazine dihydrochloride	5
Alkylating Agents	
Mechlorethamine (nitrogen mustard)	5
Triethylenethiophosphoramide (thiotepa)	13
Nitrosoureas	
N,N-Bis(2-chloroethyl-*N*-nitrosourea) (carmustine)	0
2-Deoxy-2-[[(methylnitrosoamino)-carbonyl]amino]-D-glucopyranose	0
5-(3,3-dimethyl-l-triazeno)-imidazole-4-carboxamide	0
cis-diaminedichloroplatinum	0
Epidophyllotoxins	
VP-16	33
Antibiotics	
Doxorubicin	0
Mitomycin C	0
Mithramycin	0

Samples were dialyzed for 48 hr then assayed for biological activity. Figure 5 demonstrates that in the presence of EGTA, calmodulin lost 27% of its biological activity. When calmodulin was incubated with fluphenazine-mustard in the presence of EGTA, the activity of calmodulin was inhibited by 66%. However, in the presence of excess calcium, fluphenazine-mustard completely inhibited the biological activity of calmodulin.

To determine whether fluphenazine-mustard interacted at or near the same binding site on calmodulin as the parent compound, we studied the effect of fluphenazine dihydrochloride on the activity of the mustard derivative. When 6 ng calmodulin were incubated with 1.25 μM fluphenazine-mustard for 5 min,

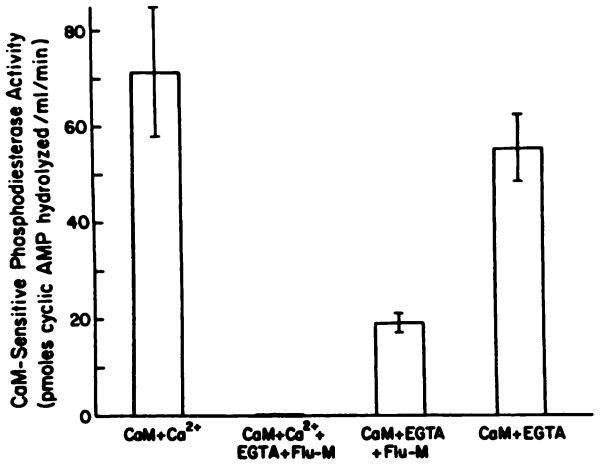

Fig. 5. Enhancement by calcium of the inhibition of calmodulin (CaM) by fluphenazine-mustard (Flu-M). Calmodulin (6 ng) was incubated with 20 μM fluphenazine-mustard for 30 min in the presence of 400 μM EGTA or 400 μM EGTA plus 1 mm $CaCl_2$. Samples were dialyzed for 24 hr then assayed for biological activity as described in Materials and Methods. In the presence of calcium, calmodulin activated phosphodiesterase three-fold. *Bars*, mean ± standard deviation of three dilutions of the calmodulin preparation, each assayed in triplicate.

TABLE 2

Effect of phenothiazines on intracellular calmodulin

Cells (20×10^6 L1210) were incubated in the presence or absence of 50 μM fluphenazine dihydrochloride or fluphenazine-mustard. Cells were washed twice and checked for viability. Cells were disrupted and calmodulin activity assayed as described in Materials and Methods. Biological activity is expressed as ng calmodulin/10^6 cells. Values represent means ± standard deviation from three samples from a representative experiment.

Treatment	Biological activity of calmodulin
Vehicle	19 ± 2
Fluphenazine dihydrochloride	17 ± 6[a]
Fluphenazine-mustard	10 ± 2[b]

[a] Not significant.
[b] $p < 0.05$ vs. vehicle.

neutralized with thiosulfate, then dialyzed for 24 hrs, 35% of the biological activity was lost. However, in the presence of excess (50 or 100 μM) fluphenazine dihydrochloride, the inhibition of calmodulin by fluphenazine-mustard was completely prevented.

The effect of the drugs on intracellular calmodulin was studied by incubating L1210 with 50 μM fluphenazine or fluphenazine dihydrochloride for 60 min. After several washes, the cells were disrupted and assayed for biologically active calmodulin. Table 2 demonstrates that cells exposed to the mustard derivative but not the parent compound had decreased biologically active calmodulin compared with cells exposed to vehicle.

To determine the effect of fluphenazine-mustard on DNA integrity, L1210 cells were exposed for short periods to the phenothiazines, and DNA breakage was determined by alkaline elution. Figure 6 demonstrates that fluphenazine-mustard caused time and concentration-dependent breaks in DNA and that fluphenazine-mustard had the greatest effect.

The effect of fluphenazine-mustard compared with that of fluphenazine dihydrochloride on the proliferation of several malignant cell lines in serum-containing medium is shown in Table 3. The potency of the drugs was similar against the two leukemic lines, L1210 and HL-60, and against the two brain tumor lines, C_6 astrocytoma and 9L glioma.

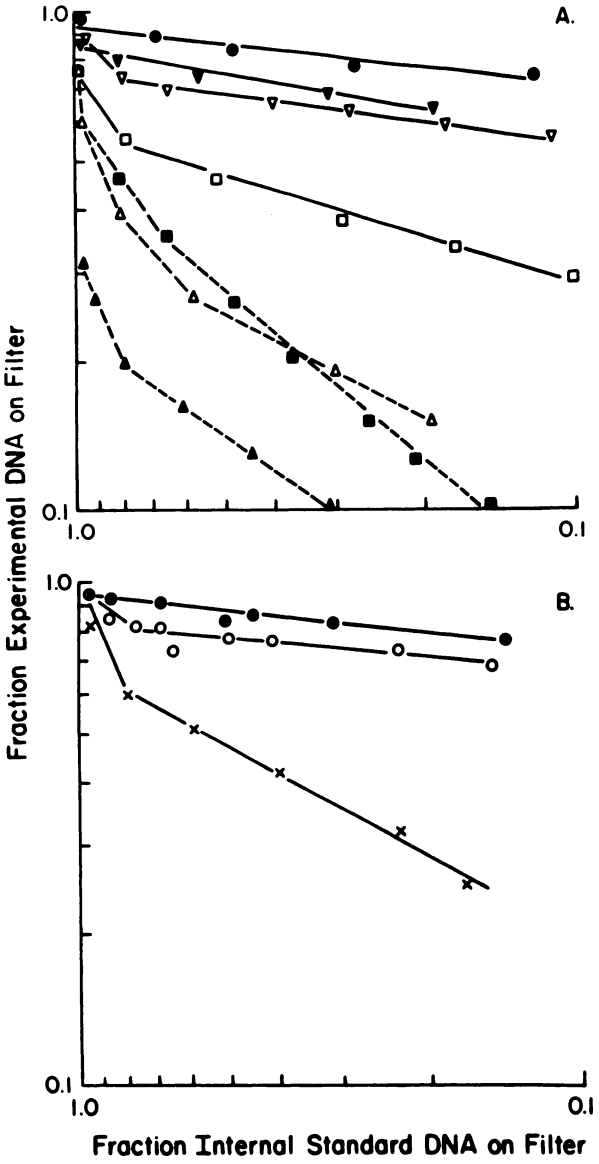

Fig. 6. Effect of fluphenazine and fluphenazine-mustard on DNA breakage. Exponentially growing L1210 cells were incubated with drug or with vehicle then assayed for DNA breaks by alkaline elution techniques (25). *A*, effect of time of exposure. Cells were exposed to 50 μM drug or vehicle. ●—● vehicle; ▼—▼, ■—■, ▲—▲ fluphenazine-mustard 1, 2 and 4 hr, respectively; ▽—▽, □—□, △—△ fluphenazine, 1, 2 and 4 hr respectively. *B*, effect of concentration of drug. Cells were exposed to vehicle (●—●), 20 μM (○—○) or 50 μM (×—×) fluphenazine-mustard for 4 hr.

The effect of the agents on clonogenicity of L1210 cells is shown in Figure 7. Fluphenazine dihydrochloride appeared more potent than the mustard derivative (IC_{50}, 18 and 60 μM, respectively).

Since fluphenazine-mustard might also irreversibly bind to serum proteins, thereby making the molecule less accessible for interaction with cells, we tested the effect of exposing leukemic cells in serum-free medium to 0–50 μM fluphenazine dihydrochloride or fluphenazine-mustard for 60 min. Cells were then washed three times by centrifugation at $100 \times g \times 10$ min, resuspended in drug-free medium containing 10% fetal bovine serum and incubated for 48 hr. Figure 8 demonstrates that under these conditions fluphenazine-mustard was a far more

TABLE 3

Effect of fluphenazine and fluphenazine-mustard on growth of malignant cell lines in serum-containing medium

Cells were grown in culture as described in Materials and Methods. After 24 h growth, a sterile solution of phenothiazine (0–100 μM) was added to each flask and allowed to incubate at 37°C for 24 h. Media was then discarded and cells washed and resuspended in drug free media. Surviving cells were counted 48 h later. Each value represents the concentration of drug required to produce 50% inhibition of cell growth compared with control cultures that were exposed to the vehicle alone (distilled water).

Cell line	Fluphenazine	Fluphenazine-mustard
	IC_{50} (μM)	
L1210 (murine leukemia)	10	20
HL-60 (human leukemia)	20	20
C_6 (rat astrocytoma)	13	21
9L (rat glioma)	18	19

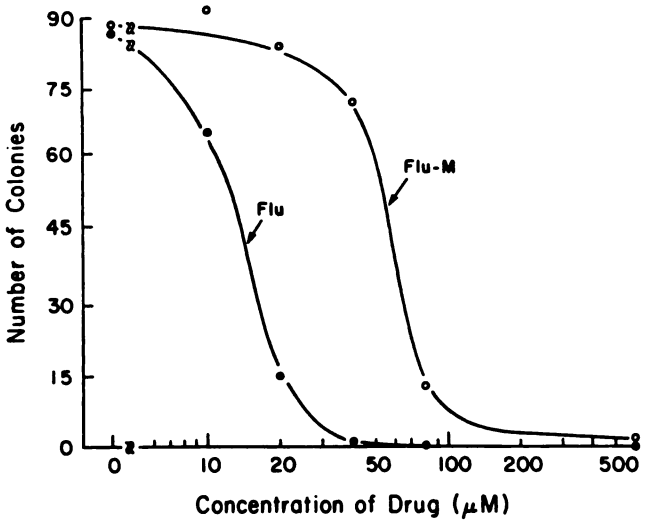

Fig. 7. Effect of fluphenazine dihydrochloride (Flu) and fluphenazine-mustard (Flu-M) in serum-containing medium on the clonogenicity of L1210 cells. Cells in the exponential phase of growth were exposed to drug for 24 hr. Cells were washed by gentle centrifugation and cloned in soft agar as described in Materials and Methods. Cloning efficiency was greater than 90%. Each point represents the mean of three culture tubes from one of three similar experiments.

potent inhibitor of cell growth (IC_{50} 1.5 μM) than the parent drug (IC_{50} 30 μM).

Discussion

These studies describe an improved synthesis and pharmacological characteristics of fluphenazine-mustard. As originally shown by Weiss *et al.* (8), the drug was an irreversible antagonist of calmodulin and, in addition, had a broad spectrum of antiproliferative and cytotoxic activity.

The binding of phenothiazines and related drugs to calmodulin is enhanced by calcium and involves hydrophobic, electrophilic, and geometric interactions. For example, Prozialeck and Weiss (4) have shown that the major structural features required for this interaction were a large hydrophobic region consisting of at least two aromatic rings and a positively charged side chain amino group separated by at least three atoms from the hydrophobic region. Drugs that irreversibly inhibited calmodulin also appeared to conform to this model. Thus fluphenazine-mustard irreversibly bound to calmodulin while nitrogen mustard and several other alkylating agents did not (Table 1). However, phenoxybenzamine, a drug that shares

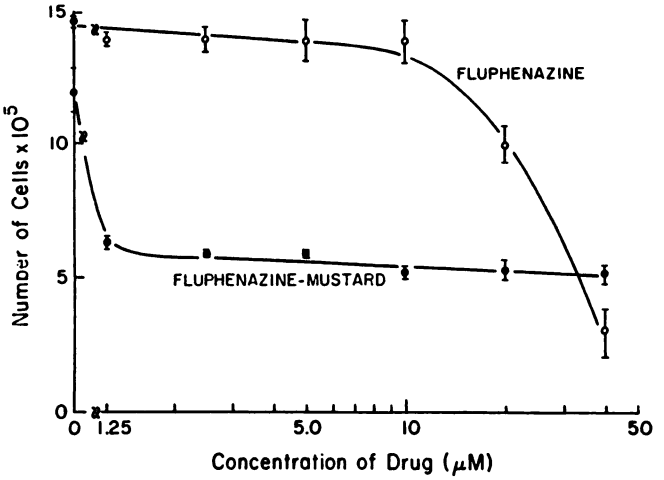

Fig. 8. Effect of fluphenazine dihydrochloride and fluphenazine-mustard on cellular growth in serum-free medium. Exponentially growing HL-60 cells were exposed for 1 hr to 0–50 μM fluphenazine dihydrochloride or fluphenazine-mustard in serum-free medium, then washed extensively and resuspended in drug-free medium containing 10% serum. Cells were counted 48 hr later. Each point represents mean ± standard error of triplicate samples.

certain structural features with the phenothiazines, also irreversibly inhibited the molecule (9). Similarly, Harrison *et al.* (28) found that only the cyclohexyl derivatives of the nitrosoureas, such as 1-(2-chloroethyl)-3-cyclohexyl and 3-(4-methylcyclohexyl)-1-nitrosourea irreversibly angtagonized calmodulin. It is likely that the carbamoylating cyclohexyl-isocyanate moiety liberated during the degradation of the parent compound interacted with and was responsible for the anti-calmodulin effect. This may be similar to the interaction of norchlorpromazine isocyanate (7) with calmodulin.

The binding of calcium to calmodulin induces structural changes in calmodulin that expose the phenothiazine binding domain (29). Further support for the interaction of fluphenazine-mustard with calmodulin at or near this region was obtained by studies of the effects of fluphenazine and calcium. For example, a molar excess of fluphenazine completely blocked the inhibition of calmodulin by fluphenazine-mustard. This effect could not be demonstrated without adding sodium thiosulfate after the incubation to quench the alkylating species that presumably continued to bind to the protein during the period of dialysis. Similarly, the binding of norchlorpromazine isocyanate to calmodulin in the presence of calcium could be blocked by high concentrations of trifluoperazine (7). Furthermore, the irreversible inhibition of calmodulin by fluphenazine-mustard was enhanced by calcium and was attenuated but not completely abolished by the calcium chelator, EGTA (Fig. 5). This inhibition by the phenothiazine in the absence of added calcium could not be explained by the presence of calcium associated with calmodulin since dialysis of calmodulin for 24 hr against excess EGTA still could not completely abolish the effect.

The availability of fluphenazine-mustard should allow identification of important intracellular targets for the phenothiazines. For example, Table 2 demonstrates that cells exposed to the alkylating-derivative had less biologically active calmodulin suggesting *in situ* binding. Although these experiments were carefully controlled to ensure equal numbers of viable cells and equal concentrations of protein for each experimental condition

and although we could find no extracellular drug present in the final wash, it was still possible that some of the drug bound to the molecule after cellular disruption.

The mechanism of the DNA damaging effects of phenothiazines and other calmodulin antagonists is unresolved. Although Ohnishi and McConnell (30) proposed a direct damaging effect of chlorpromazine through production of free radicals, Waring (31) could not demonstrate this effect on supercoiled phage DNA. Similarly Lazo *et al.* (32) found that pimozide, a diphenylbutylpiperidine calmodulin antagonist, did not directly damage isolated DNA but increased DNA damage in whole cells exposed to bleomycin. These studies and those of Chafouleas *et al.* (33) suggested that calmodulin antagonists indirectly damage DNA through inhibition of a calmodulin-mediated process of DNA repair. Our studies demonstrated that both fluphenazine and fluphenazine-mustard damaged DNA in intact cells and that the mustard-derivative was more effective (Fig. 6). The availability of this irreversible ligand should allow identification of the nuclear sites of action of this class of drugs.

One of the many pharmacological effects of phenothiazines and related compounds is the inhibition of malignant cell growth (10–12, 34, 35). Fluphenazine-mustard retained this effect but in serum-containing medium was no more potent than fluphenazine dihydrochloride (Table 2, Fig. 5). One possible explanation for this observation was that the mustard derivative also irreversibly bound to serum proteins present in the medium, making it less accessible to cellular sites of action. In fact, when cells were exposed to fluphenazine-mustard or fluphenazine in serum-free medium, the inhibitory effect of the mustard derivative on cell growth was far greater than that of the parent drug (Fig. 8).

The increased damage to DNA and the irreversible inhibition of calmodulin by fluphenazine-mustard could account for the increased effect of this drug against malignant cell growth. Further studies are in progress to identify other cellular sites of action. The potential role for this compound as an unique antitumor agent will require further investigation.

Acknowledgments

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