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STRUCTURE-ACTIVITY RELATIONSHIPS OF PHENOTHIAZINES IN INHIBITING LYMPHOCYTE MOTILITY AS DETERMINED BY A NOVEL FLOW CYTOMETRIC ASSAY

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Abstract—Lymphocyte motility is highly dependent on rapid changes in cell shape. The human T-lymphoma cell line, MOLT-4, is constitutively shape-changing and motile, and both of these properties can be inhibited by the phenothiazine, chlorpromazine, as assessed by video analysis and migration across polycarbonate filters. In this paper, the light-scattering facility of a flow cytometer has been used to establish a simpler and more quantitative means of measuring changes in shape. By this method, the structure activity relationship (SAR) of phenothiazines and related compounds has been determined. The most active compounds had the tricyclic phenothiazine nucleus with a constrained dialkylaminoalkyl substituent at the nitrogen. The SAR for inhibition of lymphocyte motility differs from those reported for neuroleptic effects and for inhibition of PKC or calmodulin. Phenothiazine concentrations that inhibited lymphocyte shape-changing resulted in reduced F-actin concentrations. This indicates that the probable mode of action is disruption of mechanisms regulating actin polymerisation.

Key words: lymphocyte; motility; phenothiazine; flow cytometry; shape; actin

Initiation and perpetuation of chronic inflammation requires extravasation of lymphocytes into inflammatory sites. This is a multistep process involving initial adhesion of the lymphocytes to activated endothelium and subsequent induction of motility, so that the lymphocyte can migrate between endothelial cells and through connective tissue. Inhibition of either the initial adhesion or the induction of motility would be expected to dampen chronic inflammation. Although there has been a great deal of work on lymphocyte-endothelial adhesion, regulation of lymphocyte motility has been relatively unexplored.

In a previous study, we found that lymphocyte motility could be inhibited by chelerythrine, calphostin C, sphingosine, H7, and H8; these agents inhibit a variety of serine-threonine kinases, but have in common the ability to inhibit PKC‡ [1]. We originally tested phenothiazines for inhibition of lymphocyte motility because they too have been reported to inhibit PKC [2]. There is also evidence that phenothiazines have immunosuppressive and anti-inflammatory activity in addition to their neuroleptic effects [3, 4, 5].

In preliminary experiments, the prototype phenothiazine, chlorpromazine, was found to potently inhibit motility of human peripheral blood lymphocytes. Inhibition of motility was seen at concentrations below that reported for effects on PKC [2] and calmodulin [6]. As this suggested a potentially novel mode of action, it was of interest to study the SAR for phenothiazine inhibition of lymphocyte motility.

For studies on SAR, we needed a simpler assay than those currently in use. Existing *in vitro* assays of lymphocyte motility usually involve migration across filters and are too cumbersome for large numbers of samples. Therefore, we have exploited the fact that ability to shape change rapidly is essential for lymphocyte motility [7, 8, 9]. Non-motile lymphocytes are spherical, whereas their motile counterparts are irregular, constantly shape-changing cells. The proportion of irregular cells in a lymphocyte population correlates with motility, and can be used as a measure of motility [8, 10]. Although this can be determined by simple microscopic observation, this method is slow and tedious, and we have found that the light-scattering facility of a flow cytometer offers a simple and quantitative means of measuring changes in lymphocyte shape. As a source of motile lymphocytes, we have selected a stable, spontaneously motile variant of a human T lymphocyte line (MOLT-4) [10].

MATERIALS AND METHODS

Chemical compounds

Compounds are numbered as in Fig. 4. Mepazine (**6**) was a gift from Byk Gulden, Konstanz, Germany. Most of the other compounds were purchased from commercial sources (Aldrich, Sigma, RBI). The structures for **26** and **38** are corrected versions of those shown in the Aldrich Rare Chemicals Collection. Other compounds were prepared by analogy to published methods. Amides (e.g. **34, 50, 51**) were prepared from the corresponding acid [11], and amines (e.g. **37, 43, 52**) either from the corresponding amide by reduction or by reductive alkylation of a less substituted amine. Where these routes were not appropriate (e.g. **42, 44**), the 10-unsubstituted phenothiazine was derivatised directly with the relevant electrophile [12]. New compounds showed spectroscopic and analytical properties consistent with the proposed structures. Stock solutions were made at 50 mM in DMSO, although 12.5 mM was used for a minority of compounds with lower solubility. In all cases, stocks

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‡ Abbreviations: PKC, protein kinase C; SAR, structure activity relationship.

were stored in the dark at –20°C. Other reagents were purchased from Sigma (Poole, U.K.).

Conventional shape-change assay

The T lymphocytes were a spontaneously motile variant of human MOLT-4 cells grown in 10% FCS in RPMI 1640 medium [10]. In some experiments, a non-motile variant of the cells [10] was used as a negative control. Growth medium was used as the diluent in this and other assays. The test compound (50 µl) was mixed with 50 µl cell suspension (5×10^6/ml) in a well of a 96-well cluster plate. After 1 hr at 37°C, a 100-µl volume of 3.7% formaldehyde in PBS was added to fix the cells. The well was then examined at ×400 magnification, and between 100 and 200 cells were scored as either "shape-changed" or "non-shape-changed." The criterion for non-shape-changed was that at least three-quarters of the cell approximated to a circle. Each dilution of test reagent was tested in triplicate, and results were expressed as the mean % shape-changed cells ± *SD* [1]. Since the test reagents were made up as stock solutions in DMSO, solvent controls were included in all assays. With the data shown in the Results section, the corresponding concentrations of solvent were without effect.

Time lapse video

Cells were suspended in growth medium with 1 mM HEPES, and placed in the wells of 96-well cluster plates, which were then sealed with tape to prevent evaporation. The cluster plate was placed on the stage of a Zeiss Axiovert 35 microscope, and maintained at 37°C by means of a thermostatically controlled fan heater. For video analysis, a Panasonic WV-BL600 camera was used with a Panasonic time lapse video cassette recorder. Recordings were made over 1 hr and replayed at ×160.

Transmigration across polycarbonate filters

Cells (5×10^6/ml) were incubated with the test compound for 30 min before addition of 100 µl suspension to the upper chamber of a Costar Transwell insert (8 µm pores). The insert was immediately placed in the well of a 24-well cluster plate containing 600 µl growth medium without inhibitor. Control wells contained cells that had been preincubated without inhibitor before addition to the insert. After 4 hr at 37°C, the inserts were removed, and the number of cells in the lower chamber was determined with the use of a Neubauer counting chamber. For each inhibitor concentration, triplicate cultures were set up, and results were expressed as the % cells migrated compared with controls with no inhibitor. Typically 10–20% of the lymphocytes transmigrated in the control wells over this time period.

Flow cytometric assay

Cells (100 µl, 10^6/ml) were incubated with 100 µl phenothiazine dilution for 30 min at 37°C before addition of 500 µl 5% formaldehyde in PBS to fix the cells. Initially, compounds were tested at a range of doubling dilutions (100–3.125 µM, final culture concentration) and examined visually to establish an approximate IC_{50}. For the flow cytometer, a range of 3 or 4 dilutions encompassing the approximate IC_{50} was chosen. Each sample was analysed using the forward/side scatter facility of a FACScan (Becton Dickinson), and 10,000 cells were screened. Round and irregular cells have different forward scatter, and an estimate of the proportion of round and irregular cells in any sample was determined (see Results section).

F-actin measurement

Cells (100 µl, 10^6/ml) were incubated with 100 µl phenothiazine dilution for 30 min at 37°C before addition of 200 µl prewarmed solution containing 7.4% formaldehyde, 0.2 mg/ml lysophosphatidyl choline, 0.22 µM fluoresceinated phalloidin in PBS. After a further 20 min at 37°C, the cells were washed ×2 with PBS and resuspended in 400 µl PBS containing 1% formaldehyde, 1% FCS. The mean fluorescence intensity of 10,000 cells was measured using the FACScan flow cytometer.

RESULTS

Validation of the assay

When viewed by time lapse video, motile MOLT-4 cells have a constantly changing, irregular shape. Treatment of the cells with the prototype phenothiazine, chlorpromazine, caused rounding up of the cells, with complete inhibition of pseudopod extension. This is dose-dependent, as shown in Fig. 1a, where rounding up of the cells was assessed by conventional microscopy after 30 min treatment ("shape change assay"). With the higher concentrations, effects were seen within 5 min. As expected, motility of the cells was also inhibited. This was measured by ability to cross a polycarbonate filter (Fig. 1b).

As shown in Fig. 2, the irregular motile MOLT-4 cells and round, non-motile variants have distinct light-scattering characteristics. A round cell will have a uniform forward scatter irrespective of its orientation on passage through the detector orifice. In contrast, a more elongated, motile cell can have a number of different light-scattering profiles, depending on its orientation through the orifice, including one particular orientation that appears round. Therefore, it is not surprising that the forward scatter profiles of round and irregular cells overlaps. Nevertheless, there is one particular region of the scattergram that is unique to irregular motile cells (R2 in Fig. 2). For motile cells, the following ratio is approximately 0.3.

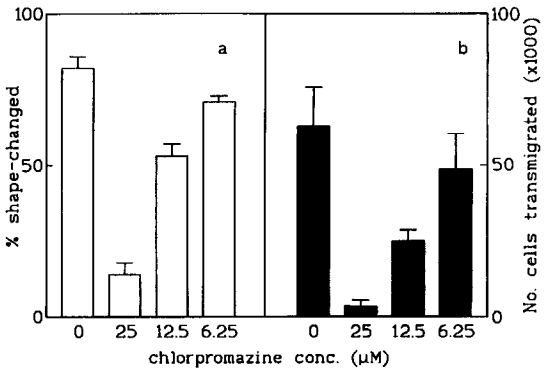


Fig. 1. Concentration dependence of chlorpromazine in inhibiting (a) shape change and (b) transmigration across polycarbonate filters of motile MOLT-4 cells. The data are mean ± standard deviation of a single representative experiment.

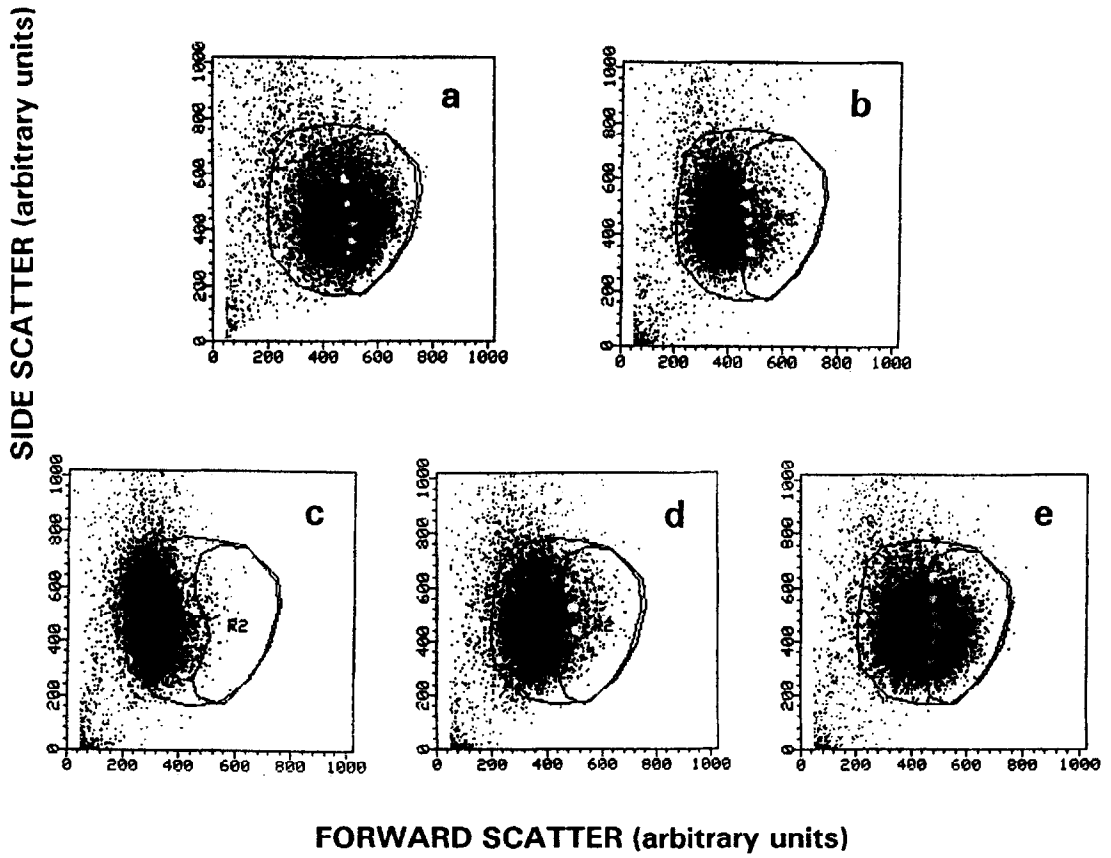


Fig. 2. Plots of forward scatter vs side scatter for (a) motile MOLT-4 and (b) non-motile MOLT-4. (c, d, and e) are motile MOLT-4 cells treated, respectively, with 25, 12.5, or 6.25 μM chlorpromazine. Region 1 (R1) includes all cells. Region 2 (R2) is the area selected to be unique to irregular cells. In practice, R2 was defined as that part of R1 that contains less than 5% non-motile MOLT-4 cells.

$$\frac{\textit{No. cells in the region unique to motile cells } (R2)}{\textit{No. cells in region encompassing motile + non-motile cells } (R1).}$$

For non-motile cells it approaches zero. Therefore, determination of this ratio gives a simple method of determining the proportion of irregular, shape-changed cells. The ratio was determined after treatment with different concentrations of phenothiazine, and the concentration necessary to cause a reduction to half the level of the untreated motile cells was determined by least squares analysis (=IC_{50}). This method correlated well ($r = 0.96$) with the conventional shape change assay (Fig. 3).

Structure activity relationships of phenothiazines and related compounds

The results summarised in Fig. 4 are based on 20 separate experiments in which typically 6–8 compounds were tested per experiment. Because this is a bioassay, some inter-experimental variation was expected. For standardisation, trifluoperazine (**3**) was included in every experiment. Over the 20 experiments, the IC_{50} for trifluoperazine was 4.5 ± 1.9 μM (*mean ± standard deviation*), with a range of 1.3–7.7 μM. The IC_{50}s given in Fig. 4 are standardised against this mean value for trifluoperazine. Each compound was tested in two separate experiments, and the IC_{50}s in Fig. 4 are means.

Phenothiazine nucleus. Changes to the tricyclic phenothiazine nucleus generally result in less active compounds. For example, replacing the sulphur atom in **12** (IC_{50} = 12 μM) with an oxygen atom (**36**, IC_{50} = 58 μM) or an ethylene unit (**41**, IC_{50} = 90 μM) causes a significant decrease in potency. Removing the bridging group altogether (**39**, IC_{50} = 82 μM) also reduces potency. One exception to this generalisation is the observation that

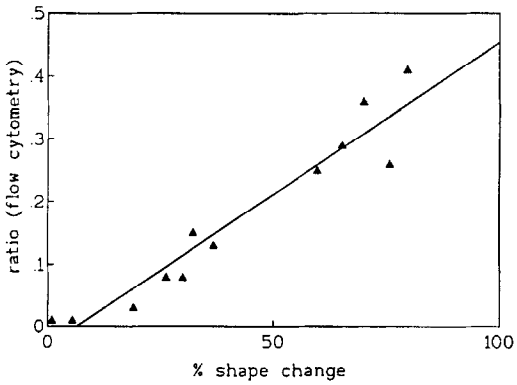
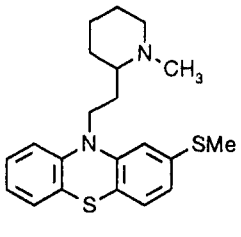
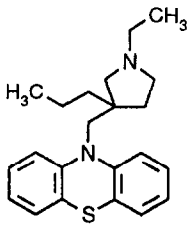


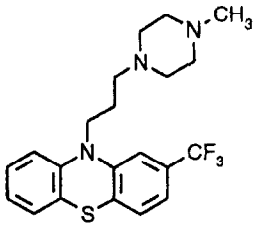
Fig. 3. Correlation between conventional shape-change assay (%shape-change) and flow cytometry. The data are pooled from two separate experiments in which motile MOLT-4 cells were treated with 0, 3.1, 6.3, 12.5, or 25 μM chlorpromazine.



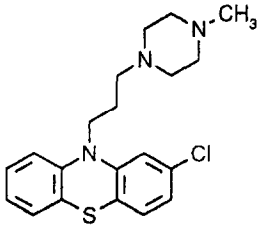
1 (IC_{50} = 4 μM)



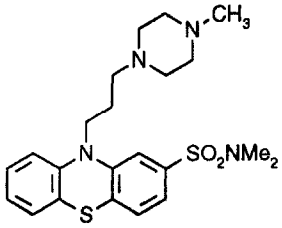
2 (4 μM)



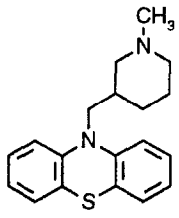
3 (4.5 μM)
Trifluoperazine



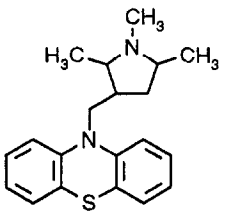
4 (6 μM)



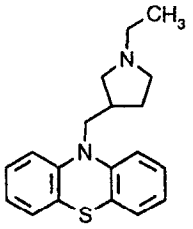
5 (7 μM)



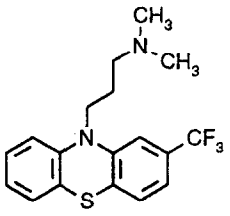
6 (7 μM)



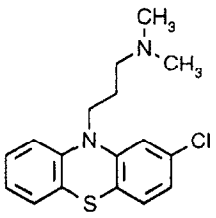
7 (7 μM)



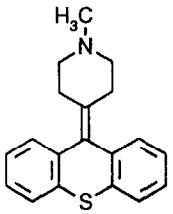
8 (7 μM)



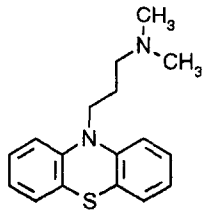
9 (7 μM)



10 (7 μM)
Chlorpromazine



11 (9 μM)

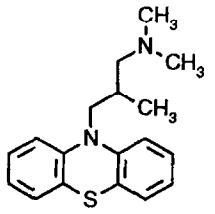


12 (12 μM)

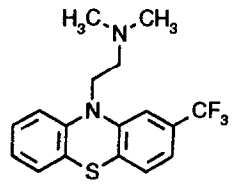
Fig. 4. Structures of phenothiazines and related compounds tested in the flow cytometric assay. The figures to the right of each structure are the IC_{50}s in μM. As outlined in the results section, this is corrected vs the trifluoperazine standard, which was included in all assays.

the phenothiazine nitrogen atom can be replaced with a trigonal carbon. The resulting thiaxanthenes are approximately equipotent with the corresponding phenothiazines. For example, phenothiazine **10** (IC_{50} = 7 μM) and thiaxanthene **21** (IC_{50} = 22 μM) differ in potency by a factor of 3, and phenothiazine **15** (IC_{50} = 13 μM) and the corresponding thiaxanthenes **16** and **17** (IC_{50} = 14 μM) are equipotent. The conservative nature of this replacement is further illustrated in other ring systems. The nitrogen atom in **41** (IC_{50} = 90 μM) can be replaced by trigonal carbon (**32**, IC_{50} = 49 μM), and once again the potency changes by a factor of 2 (although in this case the carbon analogue is the more potent). The second exception to the generalisation is the ethano-bridged compound **22** (IC_{50} = 31 μM), which is close in potency to phenothiazine **12** (IC_{50} = 12 μM), despite its lack of one of the N-methyl groups beneficial to activity (vide infra).

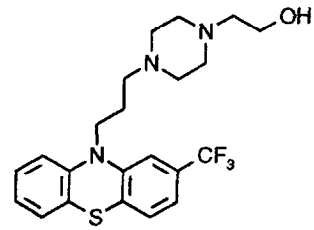
Electronegative substituent. An electronegative substituent at the 2-position of the phenothiazine increases



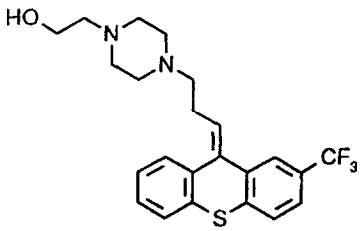
13 (12 μM)



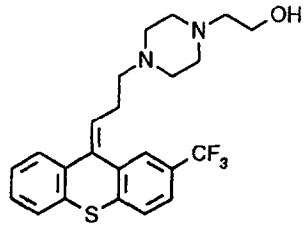
14 (13 μM)



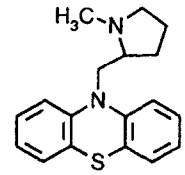
15 (13 μM)



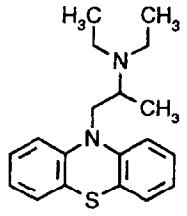
16 (14 μM)



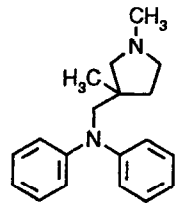
17 (14 μM)



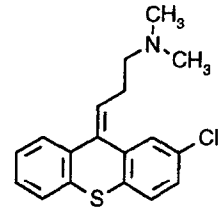
18 (16 μM)



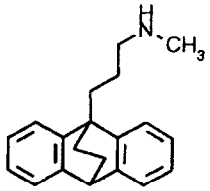
19 (16 μM)



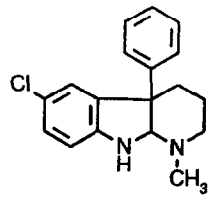
20 (20 μM)



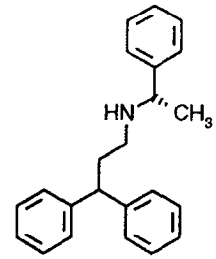
21 (22 μM)



22 (31 μM)



23 (31 μM)



24 (36 μM)

Fig. 4. (*Continued*)

potency. For example, the introduction into **12** (IC_{50} = 12 μM) of either a 2-chloro- (**10**, IC_{50} = 7 μM) or a 2-trifluoromethyl- (**9**, IC_{50} = 7 μM) substituent improves activity. Larger electron-withdrawing groups such as acetyl do not appear to be well tolerated (**35**, IC_{50} = 53 μM). In the thiaxanthene series, the introduction of a 2-substituent gives rise to the possibility of geometric isomers. No difference is found between the activities of the cis- (**17**) and trans- (**16**) isomers.

Basic nitrogen. The presence of a basic nitrogen atom in the side-chain is a key requirement for activity. Reducing the basicity of this centre reduces potency. For example, the tertiary amine **9** (IC_{50} = 7 μM) is more active than the less basic secondary amine **29** (IC_{50} = 45 μM) and the essentially non-basic amide **50** (IC_{50} > 100 μM), whereas piperidine **1** (IC_{50} = 4 μM) is much more potent than the approximately isosteric pyridine **48** (IC_{50} > 100 μM). Compound **34** (IC_{50} = 51 μM) indicates that an amide can be restored to partial activity by the presence of a neighbouring basic nitrogen. In the most active compounds, the basic centre is contained as part of a piperidine (**1, 6**), pyrrolidine (**2, 7, 8**), or piperazine (**3, 4, 5**) ring. For the piperazines, there may be an additive effect for the two nitrogen atoms. For the other rings, it

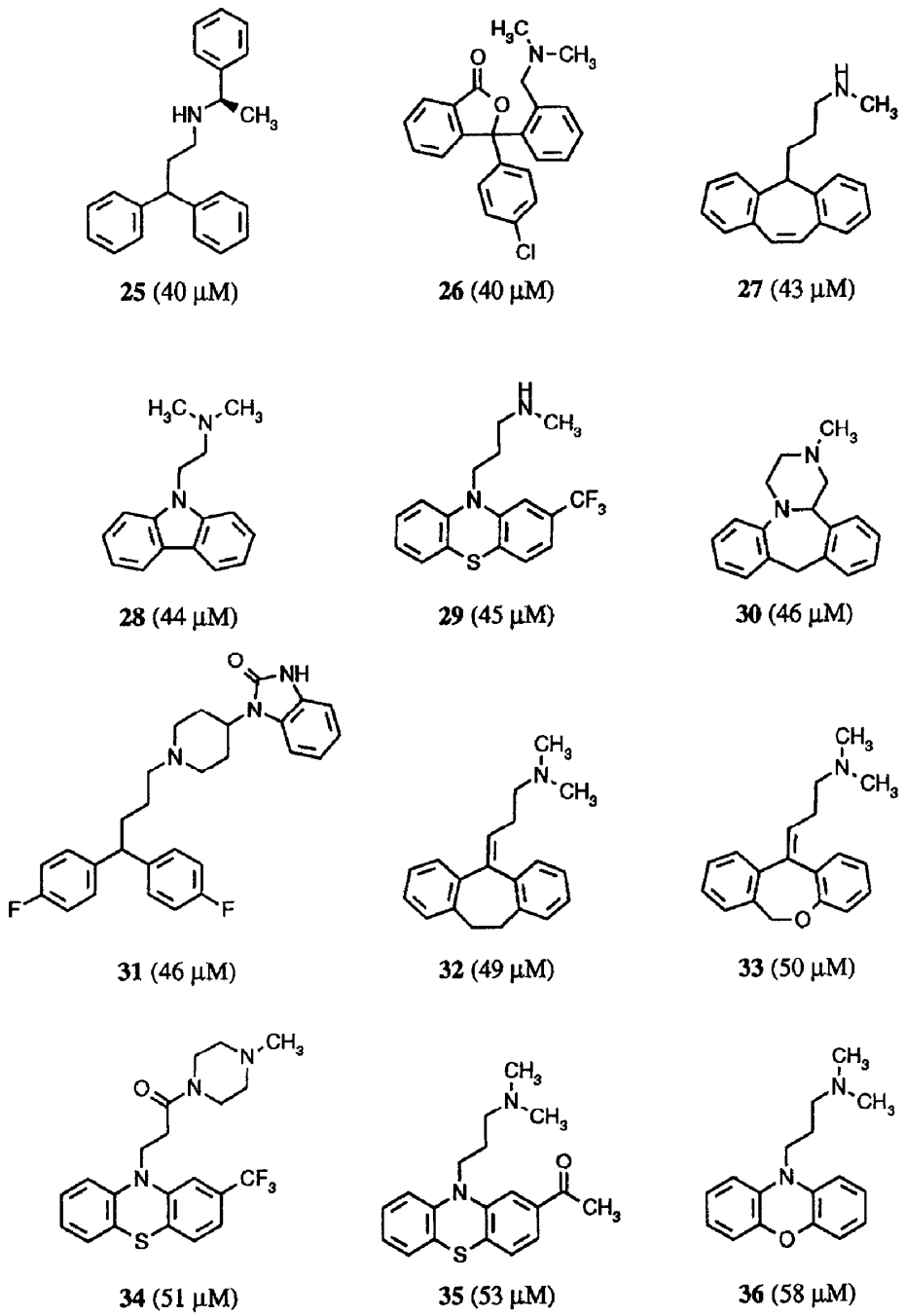


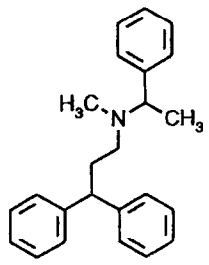
Fig. 4. (*Continued*)

seems probable that the ring is constraining the side chain in a favourable conformation. Substitution around the ring can increase potency, presumably by increasing the degree of conformational restraint. When this part of the molecule is sufficiently constrained, the need for the phenothiazine is reduced. For example, compound **20** (IC_{50} = 20 μM) has a respectable potency (compared with **39**, IC_{50} = 82 μM) despite the presence of a diphenylamino residue replacing the phenothiazine. Not all piperidine and pyrrolidines are as active. Thus, compound **18** (IC_{50} = 16 μM) is 2- to 3-fold less active than the best compounds, whereas compounds **47** and **52** both have IC_{50}s > 100 μM.

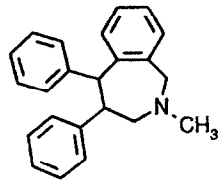
Others. Although the main focus of this study has been on phenothiazine analogues, a number of diverse structural types with activity have been identified. There is no clear evidence that these all act at the same site. However, comparing the two piperidinoindolines **23** (IC_{50} = 31 μM) and **40** (IC_{50} = 89 μM) suggests that the beneficial effects of an electronegative substituent are carried over into this series.

Effects of phenothiazines on actin polymerisation

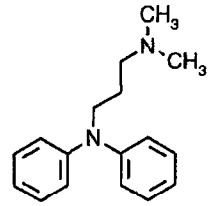
The constant shape-changing necessary for lymphocyte motility involves cycles of actin polymerisation and depolymerisation. The amount of polymerised action (F-



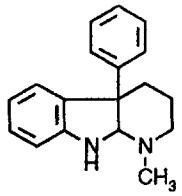
37 (65 μM)



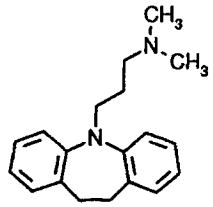
38 (69 μM)



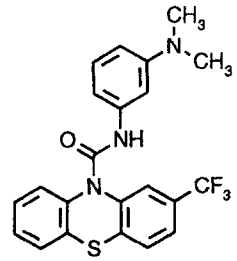
39 (82 μM)



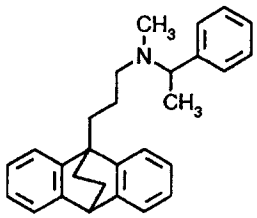
40 (89 μM)



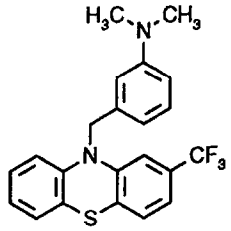
41 (90 μM)



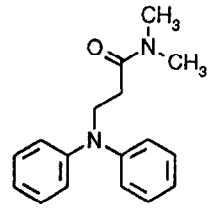
42 (97 μM)



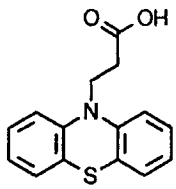
43 (98 μM)



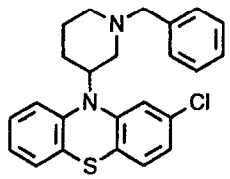
44 (>100 μM)



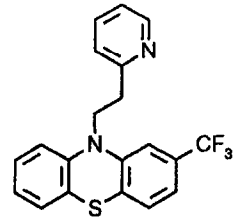
45 (>100 μM)



46 (>100 μM)



47 (>100 μM)



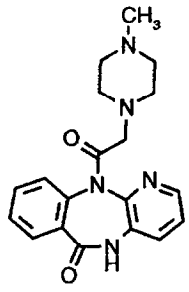
48 (>100 μM)

Fig. 4. (*Continued*)

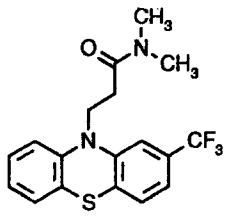
actin) is also increased in motile lymphocytes relative to non-motile cells [13]. As shown in Table 1, inhibition of shape change of MOLT-4 cells with phenothiazines was associated with a reduction in F-actin concentration. Of the four compounds tested, trifluoperazine was the most potent at inhibiting shape-change and at reducing F-actin.

DISCUSSION

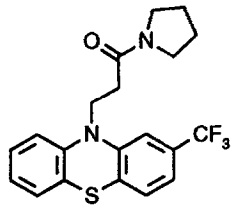
The conventional assays of lymphocyte motility are not suited for large-scale studies. As an alternative, we have used a flow cytometric assay that gives a measure of the relative proportions of spherical and irregularly shaped lymphocytes in a population. Non-motile lymphocytes are spherical cells, whereas motile cells are constantly shape-changing and irregular [7, 8, 9]. Constitutively motile and non-motile MOLT-4 lymphocytes can be distinguished in the flow cytometric assay. Although we have routinely expressed our data in two-dimensional plots of forward versus side scatter, the major differences between motile and non-motile MOLT-4 cells are seen in forward scatter, and a simple histogram of forward scatter distributions would have sufficed. A previous attempt at using light scatter to analyse lymphocyte shape concluded that the method was not sensitive enough to detect a small proportion of peripheral



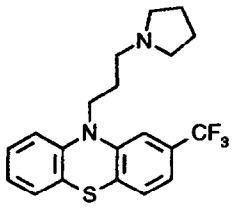
49 (>100 μM)



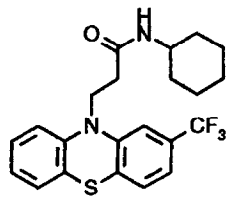
50 (>100 μM)



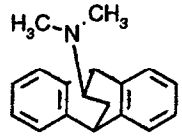
51 (>100 μM)



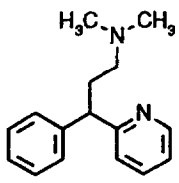
52 (>100 μM)



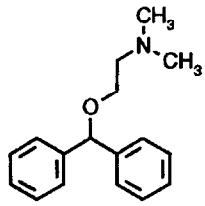
53 (>100 μM)



54 (>100 μM)



55 (>100 μM)



56 (>100 μM)

Fig. 4. (*Continued*)

blood lymphocytes changing shape [14]. The present study differs in that greater changes in the proportion of shape-changing cells are being studied, and that the MOLT-4 cells are larger than peripheral blood lymphocytes with correspondingly increased changes in light scatter. In additional, experiments have confirmed that the assay works for other established T lymphocyte lines, such as CCRF-CEM and BW5147.3. The flow cytometric method has the limitation that it measures only a static parameter rather than dynamic shape changing, and we would not advocate its use as a replacement for more rigorous but time-consuming morphometric measurements [15]. Nevertheless, in the present study inhibition of motility of motile MOLT-4 cells by chlorpromazine as measured in a conventional transmigration motility assay correlated closely with the flow cytometric assay.

Table 1. Effect of phenothiazines on F-actin concentration of motile MOLT-4 cells

Treatment (compound #)	F-actin concentration (mean fluorescence intensity)
Nil	141
Chlorpromazine (**10**)	114
Triflupromazine (**9**)	116
Prochlorperazine (**4**)	89
Trifluoperazine (**3**)	74

Cells were incubated with 12.5 μM compound for 30 min prior to measurement of F-actin. Fluorescence intensity is the mean of 10,000 cells.

This novel assay made it possible to screen a large number of phenothiazine and related compounds for inhibition of lymphocyte motility. The most active compounds were those with the tricyclic phenothiazine nucleus, although the phenothiazine nitrogen could be replaced by a trigonal carbon. At the 2-position, electronegative substituents, such as chlorine or trifluoromethyl, were more potent than no substituent or larger electron-withdrawing groups. As for the side chain, a basic nitrogen is essential, with the most active compounds having the nitrogen as part of a piperidine, pyrrolidine, or piperazine ring. Conformational restraint of the ring induced by ring substitution also increases potency.

The SAR differs from those described previously for phenothiazine inhibition of PKC [2] or calmodulin [6]. For example, for inhibition of lymphocyte motility, com-

pound **3** > **9** = **10**; but in terms of PKC inhibition trifluoperazine (**3**) is much less active (IC_{50} = 100 μM) than **9** (IC_{50} = 28 μM) or **10** (IC_{50} = 50 μM) [2]. The discordancies between calmodulin and motility inhibition include: comparable activity of **12** and **41** for calmodulin but 8-fold difference for lymphocyte motility; high activity of pimozide (**31**) for calmodulin (IC_{50} = 7 μM) but only moderate activity for motility (IC_{50} = 46 μM). For the most active phenothiazines, IC_{50}s are in the range 4–10 μM for inhibition of lymphocyte motility, with much higher concentrations needed for inhibition of PKC or calmodulin (>20 μM). This is especially notable because in these latter systems purified proteins were used, whereas for inhibition of lymphocyte mobility intact cells were used, and compounds presumably have to cross the plasma membrane.

Is there any relationship between phenothiazine inhibition of lymphocyte motility and neuroleptic effects? Some of the most potent inhibitors of motility, such as trifluoperazine (**3**), mellaril (**1**), and mepazine (**6**), are used as anti-psychotic agents. However, cis- and trans-flupenthixol (**16, 17**), which differ 1000-fold in terms of neuroleptic activity [16], show comparable inhibition of lymphocyte motility. Others have found that immunosuppression mediated by phenothiazines could be dissociated from dopamine antagonism [3].

Phenothiazines have been reported to have a bewildering variety of effects in biological systems. Some of these effects, such as inhibition of inositol phosphate metabolism [17] and activation of phospholipases [18], are superficially attractive as mechanisms of modulating motility, but in these studies concentrations of >100 μM were necessary. More recently, chlorpromazine has been found to alter endocytic recycling [19] at concentrations more in keeping with those reported in this study. Although we do not know the exact mode of action in our system, the ultimate effect is inhibition of actin polymerisation, a process that is critical for lymphocyte shape-changing and motility [13].

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