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Biochemical and pharmacological properties of an allosteric modulator site of the human P-glycoprotein (ABCB1)☆

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ABSTRACT

The drug-transport function of the human P-glycoprotein (Pgp or ABCB1) is inhibited by a number of structurally unrelated compounds, known as modulators or reversing agents. Among them, the thioxanthene derivative flupentixol inhibits Pgp-mediated drug transport by an allosteric mechanism. Unlike most other Pgp modulators, the *cis* isomer of flupentixol [*cis*-(*Z*)-flupentixol] facilitates interaction of Pgp with its transport-substrate [^{125}I]iodoarylazidoprazosin (or [^{125}I]IAAP), yet inhibits transport. In this study, we show that the flupentixol site acts as a common site of interaction for the tricyclic ring-containing modulators thioxanthenes and phenothiazines. The allosteric stimulation of [^{125}I]IAAP binding to Pgp occurs independent of the phosphorylation status of the transporter. Stimulation is retained in purified Pgp reconstituted into proteoliposomes, suggesting no involvement of any other cellular protein in the phenomenon. However, perturbation of the lipid environment of the reconstituted Pgp by nonionic detergent octylglucoside abolishes stimulation by *cis*-(*Z*)-flupentixol of [^{125}I]IAAP binding. Extensive trypsin digestion of the [^{125}I]IAAP-labeled Pgp generates a 5.5 kDa fragment with 80% of the stimulated level of labeling associated with it. Sensitivity to inhibition by transport-substrate vinblastine and competitive modulator cyclosporin A suggests that the elevated level of [^{125}I]IAAP binding to the fragment represents a functionally relevant interaction with the substrate site of Pgp. In summary, we demonstrate that allosteric modulation by *cis*-(*Z*)-flupentixol is mediated through its interaction with Pgp at a site specific for tricyclic ring-containing Pgp modulators of thioxanthene and phenothiazine backbone, independent of other cellular components and the phosphorylation status of the protein.

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1. Introduction

Human P-glycoprotein (Pgp or ABCB1), the product of the *MDR*1 (multidrug resistance) gene [1], is an ATP-dependent efflux pump for a large variety of structurally unrelated hydrophobic compounds, including many anticancer and antimicrobial agents [2]. It is a 1280 amino acid plasma membrane protein with two homologous halves (NH-terminal and COOH-terminal) connected by a short linker region [3]. Each half consists of a highly hydrophobic region with six putative transmembrane (TM) α-helices and a consensus nucleotide binding/hydrolysis domain (NBD) also called the ATP site [4]. A close association between the two halves of the protein is necessary for ATP-dependent drug transport [5,6].

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The linker region, connecting the two halves, has multiple sites for phosphorylation, the functional significance of which has yet to be established [4]. Pgp is also glycosylated at the first putative extracellular loop, which directs proper intracellular targeting of the protein [7]. The transmembrane regions from the two halves of Pgp orient themselves to form a single drug-translocating pathway also called the substrate site [8,9].

The most fascinating aspect of Pgp is its broad substrate specificity [2], the molecular basis of which remains unresolved. Substrates of the pump include structurally unrelated hydrophobic compounds ranging from essential metabolites, such as cholesterol and phospholipids, to cytotoxic agents with anti-microbial and anti-neoplastic activity. Evidence suggests that transport substrates are recognized directly from the lipid bilayer [10] before they pass into the cytoplasmic phase. Photoaffinity analogs of Pgp substrates specifically label two discrete regions of the protein [11], one in and around TM 5 and 6 and the other near TM 11 and 12 [12–14]. These sites are most likely two distinct contact points of the substrate molecules with Pgp within the drug-translocating pathway [15].

The drug-transport function of Pgp has a tremendous clinical consequence. Besides being expressed in 50% of all multidrug resistant malignancy, Pgp is also expressed in vital locations of many normal tissues and dictates pharmacokinetics of the therapeutic agents that are substrates of the pump [2]. A number of molecules (such as PSC833 and XR9576) have been identified or developed that block drug transport by Pgp and reverse drug resistance, some of which have advanced through clinical trial. However, the mechanisms of action for most of these compounds remain poorly understood, greatly restricting the rational design of new generation inhibitors. It is apparent, that a more strategic approach to develop effective inhibitors will eventually require a clear knowledge of the mode of action of Pgp and the mechanism by which its function is inhibited.

Some of the modulators, such as verapamil and cyclosporin A (first generation inhibitors), are themselves substrates of the pump and inhibit Pgp-mediated drug transport in a competitive manner without interrupting the catalytic cycle [16–18]. However, for the more recent second and third generation inhibitors, the modes of action are yet to be determined. Based on experimental evidence, there is a growing consensus in favor of an allosteric mode of action for some of these Pgp inhibitors. Independent studies, including our own [15,19,20], suggest the existence of distinct modulator site(s) within Pgp that are linked to substrate site(s) either by negative or positive heterotropic cooperativity. Martin et al. [21] demonstrated that anthranilic acid derivative XR9576 inhibits vinblastine transport without a direct physical competition for the drug-translocating pathway. Similarly, the indolizin sulfone SR33557 affects vinblastine binding to Pgp through interaction with a site distinct from the site of substrate recognition [22]. Boer et al. [23] and Ferry et al. [24] have demonstrated that modulators like dexniguldipine and prenylamine modulate vinblastine interaction with Pgp via a non-competitive mechanism.

However, little is known about the identity of the allosteric site(s) or the molecular mechanism leading to inactivation of drug translocation. Studying interaction of Pgp with its transport-substrate [^{125}I]iodoarylazidoprazosin (or [^{125}I]IAAP) in intact cells or in isolated membrane vesicles, we demonstrated that thioxanthene-based modulator flupentixols inhibit drug transport through interaction at a site functionally distinct from the site of substrate recognition and translocation [15,20]. Among these, the *cis* isomer of flupentixol modulates Pgp function in an unusual manner. *cis*-(Z)-Flupentixol inhibits [^{125}I]IAAP transport by preventing substrate dissociation from Pgp, resulting in formation of a stable Pgp-[^{125}I]IAAP complex. A phenylalanine residue at position 983 of the transmembrane region 12 (TM12) of Pgp plays a crucial role in modulation by flupentixols [20]. Replacement of the F983 with alanine (F983A) abrogates inhibition of [^{125}I]IAAP transport as well as stimulation of Pgp-[^{125}I]IAAP interaction by *cis*-(Z)-flupentixol, without any effect on the basal level of [^{125}I]IAAP binding and transport by Pgp [25]. However, the specificity of the allosteric site toward other Pgp modulators and the role of other cellular components in the modulatory process remained to be determined.

In this study, we determine the pharmacological properties of the flupentixol interaction site and investigate the possible involvement of other cellular factors or events in the modulatory process.

2. Materials and methods

2.1. Chemicals and cell lines

cis-(Z)-Flupentixol was purchased from Research Biochemicals International (presently Sigma). *cis*-(Z)-Clopentixol, *trans*-(E)-clopentixol, and *cis*-(Z)-753, compound 789, *cis*-(Z)-768, *trans*-(E)-768 were generous gifts from Dr. James M. Ford, Stanford University School of Medicine, CA 94305. Cyclosporin A, rapamycin, nicardipine and niguldipine were purchased from Calbiochem. Promethazine, chlorpromazine, trifluperazine, and thioridazine were obtained from Research Products Incorporation. Octyl β-D-glucopyranoside (octylglucoside) was obtained from Calbiochem. [^{125}I]Iodoarylazidoprazosin or [^{125}I]IAAP (2200Ci/mmol) was purchased from NEN Perkin-Elmer Life Sciences. All other chemicals including trypsin were obtained from either Sigma or Bio-Rad. Previously characterized [25] mouse NIH*MDR*1-WT and NIH*MDR*1-F983A cells were used for the studies with intact cells. Cells were maintained as described earlier [25]. N3V2400, N4V2400, and N5V2400 cells [30] were obtained from the laboratory of Dr. Michael M. Gottesman, National Cancer Institute, National Institutes of Health. *Trichoplusia ni* (high five or HF) cells were obtained from Invitrogen (San Diego, CA), and maintained according to Maki et al. [25,26].

2.2. Baculovirus-mediated expression of human Pgp

A recombinant baculovirus harboring the human *MDR*1 cDNA, with 6× His-tag at the C-terminal end, BV-MDR1-(H_6) [27] was used to infect high five insect cells grown in serum free Excell 400 medium as described [28]. For expression of Pgp, cells were propagated at 27 °C in monolayer to 80% confluency and infected with the recombinant baculovirus with multiplicity of infection of 10, and harvested after 72 h of infection.

2.3. Isolation of crude membranes from high five insect cells

Crude membranes were prepared according to Dey et al. [15]. The infected cells were harvested, and washed two times in phosphate buffered saline (PBS) containing 1% aprotinin. Washed cells were incubated on ice for 45 min in homogenization buffer (50 mM Tris–HCl, pH 7.5, 50 mM mannitol, 2 mM EGTA, 1 mM DTT, 1 mM 4-(2-aminoethyl)-bezenesulfonylfluoride (AEBSF), and 1% aprotinin) and disrupted by repeated strokes of a Dounce homogenizer. After homogenization, undisrupted cells and nuclei were removed by centrifugation at $500 \times g$ for 20 min. The supernatant was collected and diluted with resuspension buffer (containing 50 mM Tris, pH 7.5, 300 mM mannitol, 1 mM EGTA, 1 mM DTT, 1 mM AEBSF, and 1% aprotinin) and centrifuged at $100{,}000 \times g$ for 1 h. The pellet was washed once with the same buffer, and resuspended in resuspension buffer containing 10% glycerol by passing through a bent hypodermic needle (gauge size 19 and then 23). Membranes were stored at −70 °C in aliquots. Protein concentration was measured by a modified Lowry method [29] using BSA as a standard.

2.4. Solubilization of Pgp from insect cell membranes

Membranes prepared from insect cells were solubilized using octyl β-D-glucopyranoside (octylglucoside) in the presence of *Escherichia coli* bulk phospholipid, phosphatidylcholine, phosphatidylserine, and cholesterol (all from Avanti Polar Lipids, Albaster, AL) [30]. The Pgp-containing membranes were resuspended in 2.0 mg/ml in 20 mM Tris–HCl (pH 8.0), 20% glycerol, 150 mM NaCl, 2 mM β-mercaptoethanol, 0.4% lipid mixture consisting of *E. coli* phospholipids, phosphatidylcholine, phosphatidylserine, and cholesterol, at 60:17.5:10:12.5 (w/w/w/w), respectively, 2.0% octylglucoside, 1.5 mM $MgCl_2$, 1 mM AEBSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 1% aprotinin and incubated on ice for 20 min. After incubation, insoluble material was removed by centrifugation at $100{,}000 \times g$ for 1 h. The supernatant was saved as solubilized extract.

2.5. Purification of Pgp by metal affinity chromatography

Solubilized 6xHis-tagged Pgp was purified using Talon affinity resin (Clontech) as previously described [19]. A final concentration of 2 mM imidazole was added to the detergent extract (5 mg of protein), and incubated at 4 °C for 30 min on a rotary shaker with 0.5 ml of Talon metal affinity resin (Clontech) that was prewashed once with buffer A composed of 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 20% glycerol, 2.5 mM β-mercaptoethanol, 0.1% lipid mixture, 1.25% octylglucoside, 1 mM $MgCl_2$, 1 mM AEBSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 1% aprotinin. The metal affinity beads were pelleted by centrifugation for 5 min at $500 \times g$ and washed twice by resuspending and incubating in 10 ml of buffer A at 4 °C for 10 min on a rotary shaker. The beads were then resuspended in 1 ml of buffer A and applied to a 4 ml disposable column (Bio-Rad, Hercules, CA). After being washed twice with 5 ml of buffer A containing 500 mM KCl, proteins were eluted stepwise in 2 ml each of buffer B (same as buffer A except with 20 mM Tris–HCl at pH 6.8 instead of pH 8.0) containing 10, 100, 200 mM imidazole. Fractions eluted from the column were concentrated using Centriprep-50 concentrators (Amicon, Beverly, MA) and stored in aliquots at −70 °C. For [^{125}I]IAAP binding, a solubilized and purified preparation (1 μg in 5 μl volume) containing 1.25% octylglucoside and a 0.1% lipid mixture was first diluted to 20-fold in a reaction mixture (100 μl final volume) containing 50 mM Tris–HCl (pH 7.0), 125 mM KCl, and 1 mM DTT, and incubated at room temperature for 10 min with 5 nM IAAP either in the presence or in the absence of indicated concentrations of *cis*-(Z)-flupentixol prior to photocrosslinking at room temperature for 30 min.

2.6. [^{125}I]IAAP photocrosslinking of Pgp in intact cells

For [^{125}I]IAAP photocrosslinking of Pgp in intact cells, 0.5×10^6 cells/well were grown in monolayers in a 24-well plate. Cells were washed once with 1 ml/well of DMEM + 10% FBS and incubated at 37 °C for 60 min with 0.3 ml of IMEM + 10% FBS containing 1.5 nM [^{125}I]IAAP either in the presence or in the absence of indicated concentrations of modulators. Cells were exposed to a UV light (360 nm) (SPECTROLINE, model XX-15A, 365 nm) for 5 min at room temperature. After photocrosslinking, cells were trypsinized and resuspended in 100 μl/well (0.5×10^6 cells/100 μl) of cell lysis buffer containing 10 mM Tris, pH 8.0, 0.1% (v/v) Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM DTT, 2 mM AEBSF and 50 U/ml micrococcal nuclease (*Staphylococcus aureus*). Resuspended cells were lysed by three cycles of freezing (on dry ice) and thawing (at 37 °C), and resolved by SDS–PAGE. The gels were dried and exposed to an X-ray film or to a phosphorimager screen. Wherever mentioned, cells were pre-incubated for 3 min with indicated concentrations of Pgp modulators, prior to addition of [^{125}I]IAAP.

2.7. [^{125}I]IAAP photocrosslinking of Pgp in isolated membranes

Photoaffinity labeling of crude membranes with the Pgp substrate [^{125}I]IAAP was carried out according to Dey et al. [15] with slight modification. Mammalian or insect cell membranes (10–15 μg protein) expressing human Pgp were incubated at room temperature for 10 min under subdued light with 5 nM [^{125}I]IAAP in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 300 mM mannitol and 1% aprotinin (labeling buffer). Following incubation, membranes were exposed to UV illumination at 365 nm (General Electric F15T8-BLB) for 10 min, at room temperature. Following UV cross-linking, 5× SDS–PAGE sample buffer was added to the reaction mixture which was held at room temperature (~23 °C) for another 30 min, and mixed well before analyzing by SDS–PAGE. Wherever indicated, membranes were pre-incubated for 3 min with modulators of Pgp prior to the addition of [^{125}I]IAAP.

2.8. Extensive trypsin digestion of Pgp in insect cell membranes

Pgp-containing insect cell membranes (0.2 mg/ml) were incubated at room temperature for 30 min in labeling buffer

(100 μl) with 5 nM [^{125}I]IAAP plus 25 μM *cis*-(*Z*)-flupentixol, either in the absence or in the presence of 40 μM vinblastine or 40 μM cyclosporin A. Following incubation, membranes were exposed to UV irradiation for 30 min at room temperature. Photoaffinity-labeled samples were incubated at 37 °C for 1 h with 10 mg/ml (10 μl) of trypsin. Trypsin treated membranes were incubated at room temperature for another 30 min with 110 μl of 2× tricine sample buffer. The tryptic fragments were resolved in a 16.5% tricine gel. The radioactivity associated with the intact Pgp and with the tryptic fragments was captured in an X-ray film. Radioactive bands were cut out and quantified in a scintillation counter as previously described [15].

2.9. SDS–PAGE and immunoblot analysis

Electrophoresis and immunoblot analysis were performed as previously described [31].

3. Results

3.1. Thioxanthenes and phenothiazines share a common site of interaction with human P-glycoprotein

The photoactivatable cross-linking agent [^{125}I]IAAP is efficiently transported by Pgp from cells [25]. Both recognition and transport of [^{125}I]IAAP by Pgp are inhibited by cyclosporin A, a competitive modulator of the transporter. However, thioxanthene derivative *cis*-(*Z*)-flupentixol modulates [^{125}I]IAAP transport in a an allosteric manner, in which the F983 of TM12 plays an important role [20]. Unlike other Pgp modulators, inhibition of transport is due to a low rate of substrate dissociation from Pgp, which results in an increased level of Pgp-[^{125}I]IAAP complex formation [25]. Replacement of F983 by an alanine (F983A) abrogates both stimulation of [^{125}I]IAAP binding to Pgp as well as inhibition of transport by *cis*-(*Z*)-flupentixol [25]. The functional correlation of the stimulation of [^{125}I]IAAP binding with the inhibition of transport provides a convenient assay to screen for compounds with similar mode of action as *cis*-(*Z*)-flupentixol.

We studied [^{125}I]IAAP binding to the wild type Pgp as well as to the Pgp F983A mutant in NIHMDR1-WT and NIHMDR1-F983A cells, respectively, in the presence of several Pgp modulators, both structurally related (Fig. 1A and B) and unrelated (Fig. 1C) to *cis*-(*Z*)-flupentixol. A detectable level of [^{125}I]IAAP binding to the wild type and the Pgp F983A mutant was observed (Fig. 2A–C, autoradiogram, none) in the absence of any modulator. The relatively lower level of [^{125}I]IAAP signal in the Pgp F983A mutant was due to a reduced level of the expression of the mutant Pgp in NIHMDR1-F983A cells, as determined by immunoblotting with a Pgp specific antibody PEPG13 (see immunoblots). As evident in Fig. 2A, thioxanthene derivatives *cis*-(*Z*)-clopentixol, *trans*-(E)-clopentixol, *cis*-(*Z*)-753, compound 789, *cis*-(*Z*)-768, and *trans*-(E)-768, all stimulated [^{125}I]IAAP binding to the wild type Pgp (autoradiogram, upper panel), and the stimulatory effect was abrogated in the Pgp F983A mutant (autoradiogram, lower panel). A similar stimulation (of [^{125}I]IAAP binding to Pgp) sensitive to F983 substitution was also induced by phenothiazines such as promethazine, chlorpromazine, trifluperazine and thioridazine (Fig. 2B, autoradiogram). Therefore, the results indicated a common site of interaction for phenothiazines and thioxanthenes in modulation of Pgp function. On the other hand, structurally unrelated compounds such as cyclosporin A, rapamycin, nicardipine, and niguldipine (Fig. 1C) did not stimulate [^{125}I]IAAP binding to Pgp, instead effectively inhibited the interaction (Fig. 2C, autoradiogram, upper panel). The inhibitory potential of these compounds on [^{125}I]IAAP binding remained unaltered in the Pgp F983A mutant (Fig. 2C, autoradiogram, lower panel), suggesting no involvement of F983 in the modulation of Pgp function by those compounds. Therefore, the data suggest that the flupentixol site preferentially interacts with tricyclic ring-containing Pgp modulators (Fig. 1A and B).

3.2. Pharmacological intervention of the allosteric site blocks cis-(Z)-flupentixol-mediated stimulation of [^{125}I]IAAP binding

The F983 → A substitution selectively affected the ability of flupentixol and its structurally related analogs to modulate interaction of Pgp with its transport-substrate [^{125}I]IAAP, without having any noticeable change in the Pgp-[^{125}I]IAAP interaction per se (Fig. 2A and B). This indicated a spatial distinctness of the flupentixol site from the site of substrate ([^{125}I]IAAP) recognition. To better understand the allosteric nature of the flupentixol interaction site, we looked for compounds that have no inhibitory effect on Pgp-[^{125}I]IAAP interaction, but can block the *cis*-(*Z*)-flupentixol-mediated stimulation of [^{125}I]IAAP binding. Inside out vesicles were used to minimize complications due to differential permeability of the modulators across the plasma membrane. The results indicated that thiothixene (a thioxanthene derivative) and thioridazine (a phenothiazine), up to a concentration of 10 μM, had minimal effect on Pgp-[^{125}I]IAAP interaction per se, whereas at the same concentration (10 μM) both compounds effectively blocked the stimulatory action of *cis*-(*Z*)-flupentixol on [^{125}I]IAAP binding (Fig. 3A and B). The effect of thioridazine (Fig. 3B) was more dramatic than that of thiothixene (Fig. 3A), which inhibited about 70% of the stimulated binding without any inhibitory effect on the basal level of [^{125}I]IAAP interaction with Pgp. The results suggested that the interaction of *cis*-(*Z*)-flupentixol with Pgp could be selectively affected by structurally related compounds without any interference to [^{125}I]IAAP binding to the substrate site. This further substantiated the allosteric nature of the modulator site and ability of other tricyclic ring-containing modulators to interact at the site.

3.3. cis-(Z)-Flupentixol stimulates [^{125}I]IAAP binding independent of the phosphorylation state of Pgp

It has been demonstrated that phenothiazines and thioxanthenes are potent functional regulators of calcium-binding protein calmodulin, which regulates phosphodiesterase activity of many enzymes [32,33]. At the same time, modulation of Pgp by compounds like safingol has been attributed to the ability of the drug to inhibit protein kinase C (PKC) activity [34], which is involved in phosphorylation of Pgp [35]. The human P-glycoprotein is phosphorylated in vitro at five different

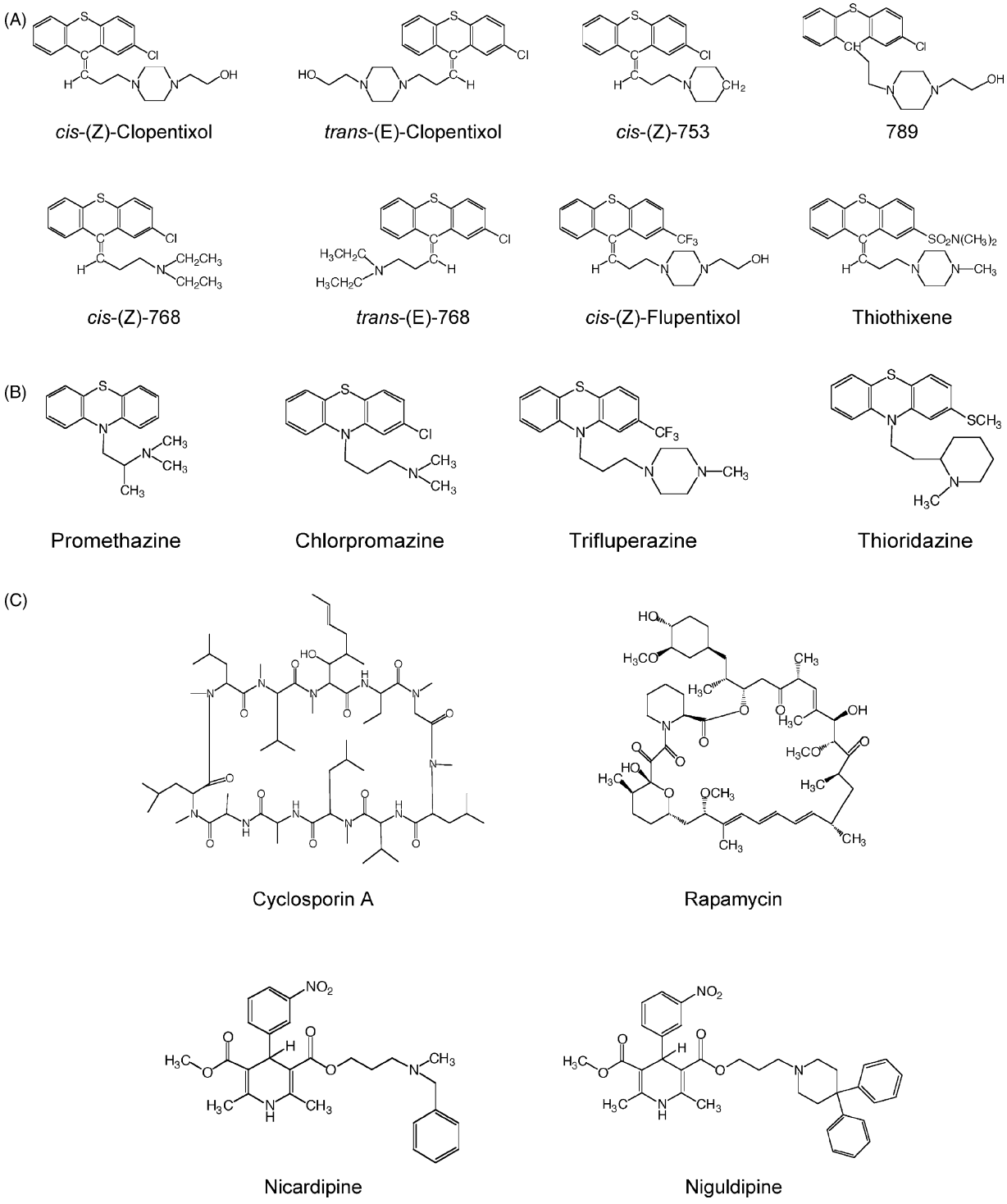

Fig. 1 – Structural representation of Pgp modulators: (A) thioxanthenes; (B) phenothiazines; (C) immunosuppressives and calcium channel blockers.

serine residues, namely Ser-661, Ser-667, Ser-671, Ser 675 and Ser-683, out of which, three of them (Ser-661, Ser-667, and Ser-671) are phosphorylated in vivo [31,36]. Replacement of all five of the serine residues, either with alanine (which cannot be phosphorylated) or with aspartate (which mimics phosphoserine) suggested that the phosphorylation–dephosphorylation mechanism has no regulatory role in the drug-transport function of Pgp [31]. However, a more recent study suggested that phosphorylation of the linker region has a subtle but a detectable effect on the K_m for certain drugs, such as verapamil, vinblastine and rhodamine 123 [36] in stimulating the ATPase activity of Pgp. Therefore, to assess any possible role of phosphorylation on modulation by *cis*-(*Z*)-flupentixol, we studied [^{125}I]IAAP binding in phosphorylation-defective

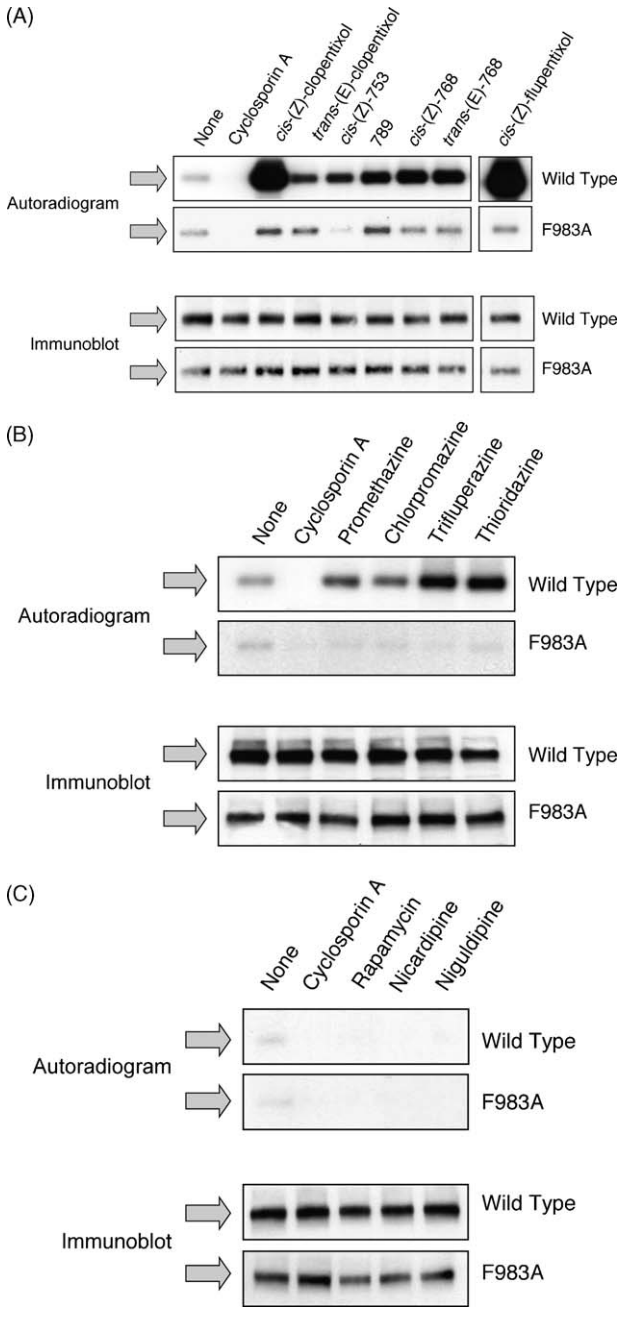

Fig. 2 – (A) Effect of thioxanthenes on Pgp-[^{125}I]IAAP interaction in NIHMDR1-WT (wild type) and NIHMDR1-F983A (mutant F983A) cells. Monolayers of NIHMDR1 cells were incubated at 37 °C with 1.5 nM [^{125}I]IAAP for 60 min either in the presence of 5 μM cyclosporin A (CsA) (lane 2), 10 μM *cis*-(*Z*)-clopentixol (lane 3), 10 μM *trans*-(E)-clopentixol (lane 4), 10 μM *cis*-(*Z*)-753 (lane 5), 10 μM 789 (lane 6), 10 μM *cis*-(*Z*)-768 (lane 7), 10 μM *trans*-(E)-768 (lane 8), 10 μM *cis*-(*Z*)-flupentixol (lane 9) or in the absence of any modulator (none) (lane 1). Following incubation, cells were exposed to UV irradiation for 5 min, lysed, and resolved by SDS–PAGE (80,000 cells per well) as indicated in Section 2; (B) effect of phenothiazines on Pgp-[^{125}I]IAAP interaction in NIHMDR1-WT (wild type) and NIHMDR1-F983A (mutant F983A) cells. Experiment was done as described for (A), except that the modulators added were 5 μM cyclosporin A (CsA) (lane 2), 10 μM promethazine (lane 3), 10 μM chlorpromazine (lane 4), 10 μM trifluperazine (lane 5), 10 μM thioridazine (lane 6), or 1% DMSO (none) (lane 1); (C) effect of other modulators on Pgp-[^{125}I]IAAP interaction in NIHMDR1-WT (wild type) and NIHMDR1-F983A (mutant F983A) cells. Experiment was done exactly the same way as described in (A), except modulators used were 5 μM cyclosporin A (CsA) (lane 2), 5 μM rapamycin (lane 3), 10 μM nicardipine (lane 4), 10 μM niguldipine (lane 5), or in the absence of any modulator (none) (lane 1). In each experiment, radioactivity associated with Pgp was captured on an X-ray film (autoradiogram) and the amount of Pgp present per sample was detected by western analysis using Pgp-specific antibody PEPG13.

Pgp mutants. NIH3T3 cells expressing the wild type Pgp (N3V2400), or the Pgp mutants with all five serine residues changed either to alanine (N4V2400), or to aspartate (N5V2400) were incubated with 5 nM [^{125}I]IAAP either in the presence or in the absence of 25 μM *cis*-(*Z*)-flupentixol as indicated in Section 2. As evident in Fig. 4A and B, *cis*-(*Z*)-flupentixol induced a four- to five-fold stimulation in [^{125}I]IAAP binding by both the wild type Pgp as well as the Pgp phosphorylation mutants N4V2400 and N5V2400 (Fig. 4B). Immunoblot analysis of the labeled samples suggests that the observed effect cannot be attributed to the difference in level of Pgp in the samples (Fig. 4A, lower panels). The reason for a marginally lower level of Pgp in the cells treated with *cis*-(*Z*)-flupentixol is not clear. However, it is less likely to be due to an altered turnover rate of the protein [25], instead, a more likely explanation would be that it is a procedural artifact. Therefore, although phosphorylation modulates interaction of certain Pgp substrate, it does not have any detectable contribution to modulation of [^{125}I]IAAP binding by *cis*-(*Z*)-flupentixol.

3.4. *Modulatory effect of cis-(Z)-flupentixol was retained in purified and reconstituted Pgp*

A direct interaction between the mouse Pgp homolog MDR3 and calmodulin has been demonstrated, and described as the possible mechanism for down-modulation of Pgp function by Ca^{2+}-calmodulin [37]. To explore the possibility of any such cellular protein involved in modulation of Pgp by *cis*-(*Z*)-flupentixol, we studied the effect of the modulator on purified Pgp reconstituted into proteoliposomes. Pgp (with 6-histidine tag) was expressed in insect cells, solubilized with nonionic detergent octylglucoside in the presence of phospholipids, purified by metal affinity chromatography as described in Section 2. Purified protein was reconstituted into proteoliposomes by a rapid dilution procedure and [^{125}I]IAAP labeling was carried out both in the absence and in the presence of varying concentrations of (10, 20, and 50 μM) *cis*-(*Z*)-flupentixol. Consistent with our observation in membrane vesicles (Fig. 3) and in intact cells (Fig. 2A), [^{125}I]IAAP binding to purified Pgp was stimulated by *cis*-(*Z*)-flupentixol in a concentration-dependent manner (Fig. 5A). This result suggests that the stimulatory effect of *cis*-(*Z*)-flupentixol on [^{125}I]IAAP binding is not mediated through other cellular components.

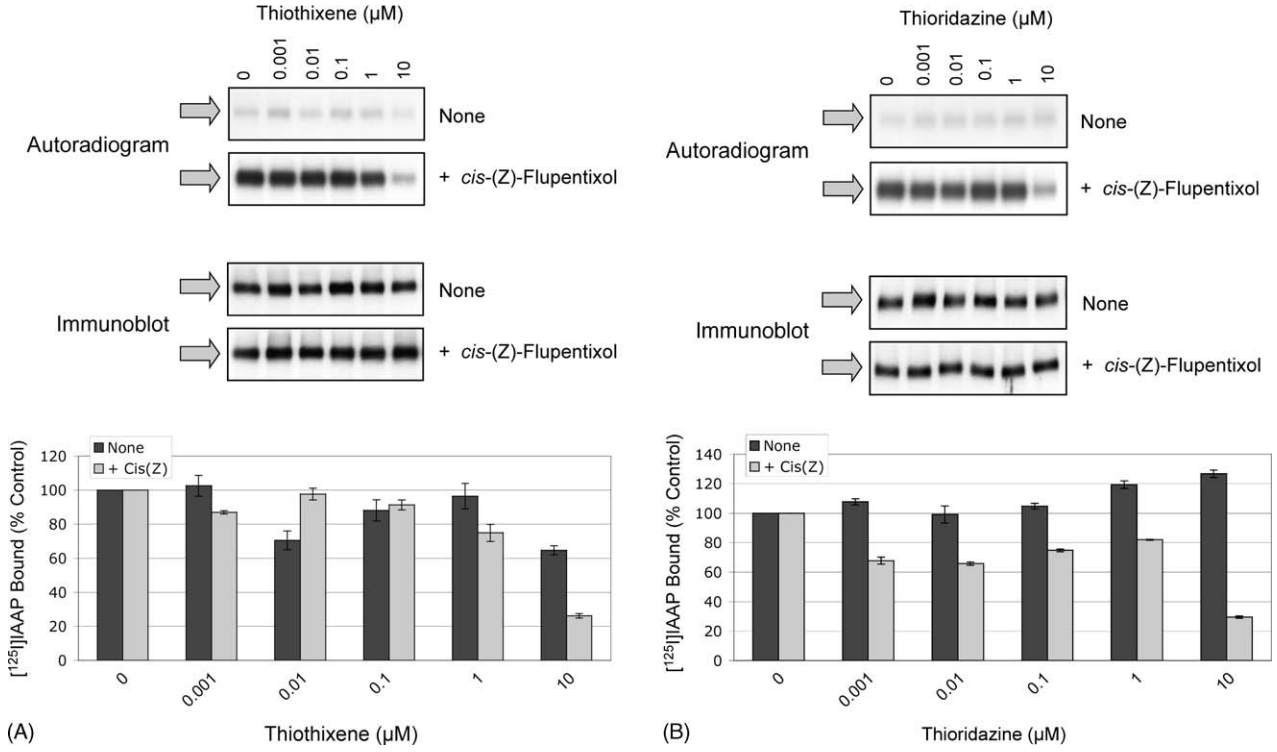

Fig. 3 – Pharmacological intervention of *cis*-(Z)-flupentixol-mediated stimulation of [^{125}I]IAAP binding. Pgp in isolated insect cell membranes (0.1 mg/ml) were photoaffinity labeled with 5 nM [^{125}I]IAAP either in the presence or in the absence of 5 μM *cis*-(Z)-flupentixol following preincubation for 3 min with indicated concentrations of either thiothixene (A) or thioridazine (B). Phhotocrosslinked samples were resolved by SDS–PAGE. The radioactivity associated with Pgp was captured on an X-ray film (autoradiogram) and the amount of Pgp present per sample was detected by western analysis using Pgp-specific antibody PEPG13. Two micrograms of membrane proteins were loaded in each lane.

The nonionic detergent octylglucoside perturbs the native structure of the transmembrane regions of Pgp and prevents stimulation of ATP hydrolysis by transport substrates [27,38]. To understand whether stimulation of [^{125}I]IAAP binding by *cis*-(Z)-flupentixol require the integrity of the transmembrane region, we studied stimulation of [^{125}I]IAAP binding to purified and reconstituted Pgp in the presence of 1.2% sodium octylglucopyranoside. Although a very low level of [^{125}I]IAAP binding to Pgp was detected in the presence of octylglucoside, no stimulation by *cis*-(Z)-flupentixol was observed (Fig. 5B), suggesting that the structural integrity of the transmembrane regions was essential for *cis*-(Z)-flupentixol-mediated stimulation.

3.5. *Stimulation of by cis-(Z)-flupentixol represents increased binding of [^{125}I]IAAP to a functionally relevant site in Pgp*

The stimulatory effect of *cis*-(Z)-flupentixol on [^{125}I]IAAP labeling could be a consequence of two alternative possibilities. It could be due to binding of [^{125}I]IAAP at multiple sites within Pgp that are induced by the action of *cis*-(Z)-flupentixol. Alternatively it might represent an increased [^{125}I]IAAP association with the functionally relevant substrate site of Pgp. To address these possibilities, we have subjected photoaffinity-labeled Pgp to extensive trypsin digestion and resolved the tryptic fragments in a 16.5% tricine gel [39]. Trypsin digestion yielded a single [^{125}I]IAAP-labeled peptide band of approximate molecular weight of 5.5 kDa (Fig. 6). The fragment retained 80% of the total [^{125}I]IAAP bound to the intact Pgp. No other radioactive fragment was detected even after extended exposure of the gel to an X-ray film. Although the amount of total [^{125}I]IAAP bound was considerably less, a tryptic fragment of identical mobility was detected in absence of *cis*-(Z)-flupentixol (data not shown). The association of [^{125}I]IAAP with the 5.5 kDa fragment was completely inhibited in the presence of Pgp substrate vinblastine as well as competitive modulator cyclosporin A (Fig. 6). Therefore, the results suggest that the increase in Pgp-[^{125}I]IAAP association represents a functionally relevant binding, and rules out the possibility of increased photocrosslinking at multiple sites within Pgp (Fig. 6).

4. Discussion

The mechanism of action of Pgp modulators has been a matter of considerable interest from both biochemical and pharmacological standpoints. The wide range of structural diversity among the compounds that interact with Pgp and inhibit its drug-transport function has made it extremely difficult to derive a common structural basis with inhibitory potential.

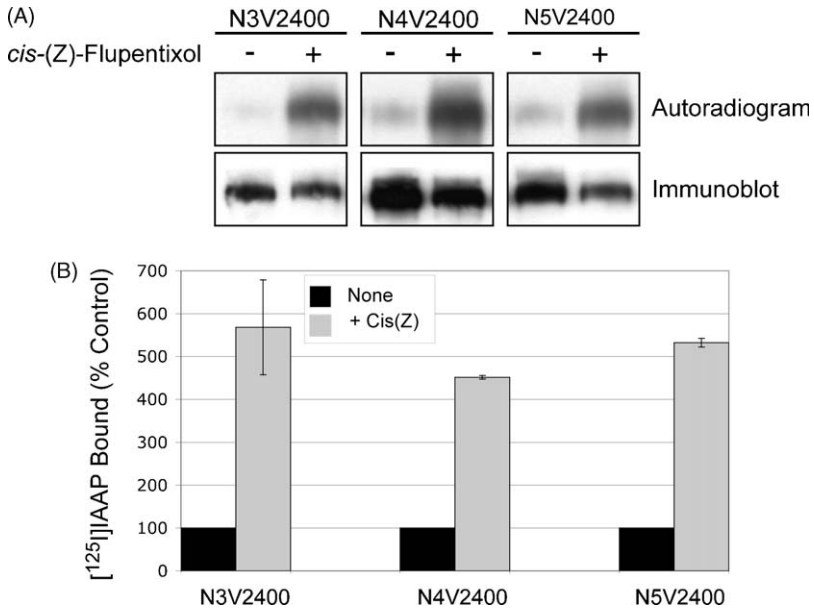

Fig. 4 – Effect of *cis*-(*Z*)-flupentixol on [^{125}I]IAAP interaction with phosphorylation-defective Pgp mutants. NIH3T3 cells expressing wild type Pgp (N3V2400), 5A mutant (N4V2400), or 5D mutant (N5V2400) were grown in monolayers, trypsinized, washed in PBS (plus 1% aprotinin), and incubated at 37 °C with 5 nM [^{125}I]IAAP for 60 min, either in the presence or in the absence of 25 μM *cis*-(*Z*)-flupentixol, UV irradiated for 15 min at room temperature, pelleted, and lysed by repeated freezing and thawing in TD buffer. Lysed cells were resolved by SDS–PAGE (80,000 cells per well), as indicated in Section 2. Radioactivity associated with Pgp was captured in an autoradiogram (A) and quantified using a phosphorimager (B). The amount of Pgp in each sample was detected in an immunoblot using Pgp-specific antibody PEPG13, quantified by densitometry using software Image SXM, and used to normalize the amount of [^{125}I]IAAP bound to Pgp in each sample. The data expressed as a percentage of [^{125}I]IAAP bound to Pgp in the absence of any modulator.

Biochemical characterization of their effects on the functional aspect of Pgp, and identification of amino acid residues involved in the process indicate more than one modes of action for Pgp modulators. Recently, we demonstrated that the dopamine receptor antagonist flupentixol, which also interacts with calcium-sensor protein calmodulin, inhibits Pgp-mediated drug transport in an allosteric manner. Interestingly, the *cis* isomer of flupentixol, although inhibits transport, does not physically compete for the binding of transport-substrate [^{125}I]IAAP, instead stimulates its interaction with Pgp. The allosteric property of *cis*-(*Z*)-flupentixol and its ability to interact with other cellular proteins necessitated a detailed characterization of the mechanism by which it modulates Pgp function. In this report, we provide evidence that the site of *cis*-(*Z*)-flupentixol interaction with Pgp acts as a common site of interaction for tricyclic ring-containing modulators thioxanthenes and phenothiazines, and that modulation of Pgp by these compounds occurs independent of other cellular factors.

The ability of phenothiazines and thioxanthenes including *cis*-(*Z*)-flupentixol to interact with and inhibit the function of calmodulin and protein kinase C has been implicated as a possible mechanism by which these drugs may modulate Pgp function [40]. A direct interaction between calmodulin and the mouse homolog of *MDR*3 (mdr2) has been demonstrated by Schlemmer et al. [37]. On the other hand, staurosporine derivative CGP41215, an inhibitor of protein kinase C, has been implicated to reverse multidrug resistance by modulating the phosphorylation state of Pgp [41]. These findings necessitated a more definitive study to explore any possible involvement of other cellular proteins in modulation by *cis*-(*Z*)-flupentixol. The fact that in purified and reconstituted Pgp, *cis*-(*Z*)-flupentixol stimulates [^{125}I]IAAP binding (Fig. 5A) strongly argues against any involvement of other cellular proteins in the phenomenon. In addition, Pgp mutants that have the potential phosphorylation-site serines substituted either by alanines or by aspartates [31] show no loss of stimulation of [^{125}I]IAAP binding (Fig. 4). Therefore, the result confirms that the modulatory effect of flupentixol on [^{125}I]IAAP binding was not mediated through the protein kinase C pathway. Interestingly the residue F983, located in TM12, plays a crucial role in modulation by *cis*-(*Z*)-flupentixol, which suggests that the allosteric site is part of the TM regions. Consistent with that, in purified Pgp reconstituted into proteoliposomes, perturbation of the lipid environment of the TM regions with nonionic detergent octylglucoside completely abrogates the stimulatory effect of *cis*-(*Z*)-flupentixol (Fig. 5B). Although the basal level of [^{125}I]IAAP binding is severely affected as well, the residual amount of bound [^{125}I]IAAP remain completely insensitive to stimulation. Similar effect of detergent had been observed in stimulation of ATP hydrolysis by Pgp substrates that interact with the TM regions of Pgp [38].

The ability of *cis*-(*Z*)-flupentixol to stimulate [^{125}I]IAAP binding is a unique phenomenon, where inhibition of transport occurs due to a slow rate of dissociation of [^{125}I]IAAP from Pgp, induced by *cis*-(*Z*)-flupentixol [25]. The role of F983 in modulation substantiated the claim for a functionally and

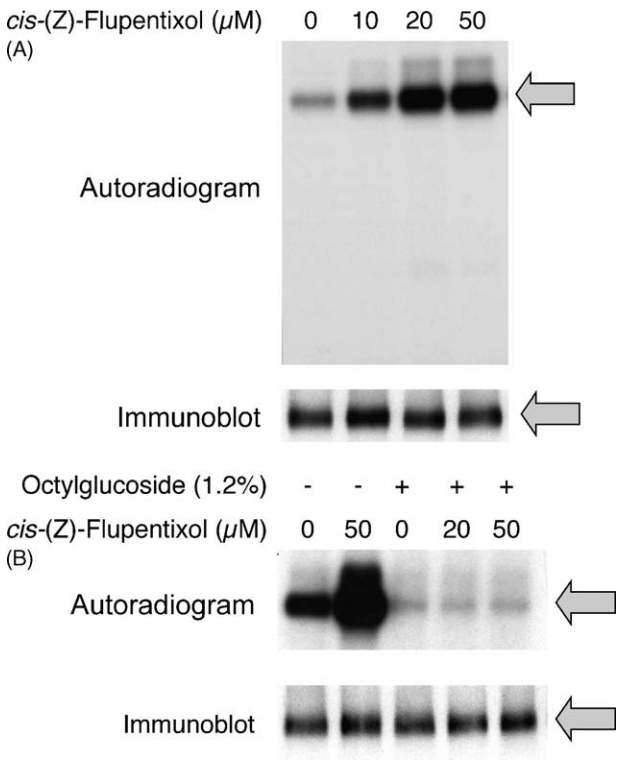

Fig. 5 – [^{125}I]IAAP binding to purified Pgp reconstituted into proteoliposomes: (A) effect of *cis*-(*Z*)-flupentixol. Pgp (1.5 μg) was purified and reconstituted into proteoliposomes following a rapid dilution protocol as described in Section 2. Reconstituted Pgp was photoaffinity labeled with 10 nM [^{125}I]IAAP, in the presence of indicated concentrations of *cis*-(*Z*)-flupentixol (0–50 μM). Pgp (0.75 μg) from each assay was resolved by SDS–PAGE and radioactivity associated was captured in an X-ray film (autoradiogram). The amount of Pgp present per sample was detected by western analysis using Pgp-specific antibody PEPG13 (immunoblot); (B) effect of octylglucopyranoside on [^{125}I]IAAP binding and its stimulation by *cis*-(*Z*)-flupentixol. Purified and reconstituted Pgp were photoaffinity labeled as described in the presence of indicated amount of *cis*-(*Z*)-flupentixol either in the absence (first two lanes) or in the presence (last three lanes) of 1.2% octylglucopyranoside. Samples were resolved by SDS–PAGE and Pgp-associated radioactivity was captured in an X-ray film (autoradiogram). The amount of Pgp present per sample was detected by western analysis using Pgp-specific antibody PEPG13 (immunoblot). Pgp (0.75 μg) were loaded in each lane.

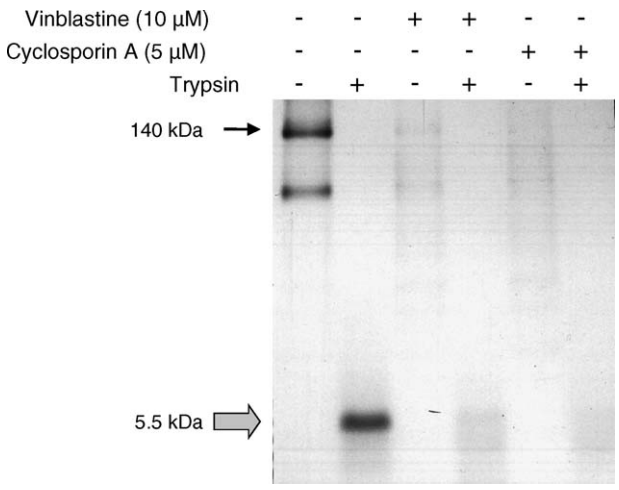

Fig. 6 – Extensive trypsin digestion of [^{125}I]IAAP-labeled Pgp. Pgp in isolated membranes (0.2 mg/ml) was photoaffinity labeled with 5 nM [^{125}I]IAAP in the presence of 25 μM *cis*-(*Z*)-flupentixol, with or without preincubation with either 5 μM cyclosporin A or 10 μM vinblastine. Labeled Pgp was subjected to extensive trypsin (10 mg/ml) digestion for 1 h at 37 °C, and the tryptic fragments were resolved by SDS–PAGE in a 16% tricine gel. The radioactive fragments were detected in an aoutoradiogram. Five micrograms of protein was added per well.

spatially distinct site for the modulator, through which it allosterically inhibits transport. However, to gain insight into the mechanism, it is important to understand the pharmacological property of this allosteric site. The stimulatory effect on [^{125}I]IAAP binding, and its abrogation in the Pgp F983A mutant, provided us with a convenient assay to study the specificity of the allosteric site. The data indicates that the site act as a common site of interaction for tricyclic ring-containing Pgp modulators of thioxanthene and phenothiazine backbone (Fig. 2A and B). The fact that in presence of a less potent stimulator such as thiothixene or thioridazine, the stimulatory effect of *cis*-(*Z*)-flupentixol is attenuated (Fig. 3), suggests a true physical competition between the two tricyclic ring-containing compounds for the same site. The structural determinant for the stimulatory effect on [^{125}I]IAAP binding cannot be predicted at this point, however, the data are indicative of an interaction between the tricyclic ring nucleus of the thioxanthenes or phenothiazines and the phenyl group of F983 side chain. The role of phenylalanine residues of Pgp TM regions in interaction with phenothiazines and thioxanthenes was predicted by Hait and Aftab [42], in which a π orbital overlap between the aromatic groups of phenylalanine residues and the tricyclic ring nucleus of the drugs was proposed. The specificity of the interaction is evident from the fact that substitution of F983 with alanine has no effect on modulation of [^{125}I]IAAP binding by structurally unrelated Pgp modulators, such as calcium channel blockers niguldipine and nicardipine (dihydropyridine derivatives) as well as immunosuppressants cyclosporin A (a dodecapeptide) and rapamycin (a bacterial macrolide) (Figs. 1C and 2C).

It is interesting to note that although at 10 μM concentrations thiothixene and thioridazine compete with flupentixol for the allosteric site, neither of them by themselves stimulated [^{125}I]IAAP binding to Pgp to the same extent as *cis*-(*Z*)-flupentixol (Fig. 3). This indicates that although the tricyclic ring structure is the major determinant for their recognition by Pgp, the stimulatory effect on [^{125}I]IAAP binding requires additional structural components. Both thiothixene and *cis*-(*Z*)-flupentixol are thioxanthene derivatives. However,

thiothixene has a dimethyl sulfonamide group at position 2 of the tricyclic ring nucleus, instead of a trifluoromethyl in *cis*-(*Z*)-flupentixol (Fig. 1A). In addition, the piperizinyl side chain of thiothixene is less hydrophilic compared to that of *cis*-(*Z*)-flupentixol. Therefore, either one of these two components, or both combined, could play a determining role in generating the stimulatory effect on [^{125}I]IAAP binding. Since thioridazine, a phenothiazine derivative (Fig. 1B), has a similar effect as thiothixene in blocking *cis*-(*Z*)-flupentixol-mediated stimulation of [^{125}I]IAAP binding (Fig. 3B), it substantiates the fact that recognition by the allosteric site does not discriminate between a phenothiazine and a thioxanthene ring nucleus.

We demonstrated that stimulation by *cis*-(*Z*)-flupentixol of [^{125}I]IAAP labeling to Pgp represents an increased binding of the transport substrate, and not an improved photocrosslinking [25,26]. Although the increased labeling was found to be selective to the COOH-terminal half of the protein [15], the possibility of an increase in the number of interaction sites in the presence of *cis*-(*Z*)-flupentixol cannot be ruled out. The fact that extensive trypsin digestion of the [^{125}I]IAAP-labeled Pgp yielded a single 5.5 kDa fragment (Fig. 6), that retained more than 80% of the total radioactivity, argues against multiple sites being photoaffinity labeled. The ability of transport-substrate vinblastine and the competitive modulator cyclosporine A to inhibit labeling of the 5.5 kDa fragment (Fig. 6), suggested that the stimulated binding of [^{125}I]IAAP occurs at a functionally relevant site within Pgp. Identification of this [^{125}I]IAAP-labeled fragment could provide an important clue on the mechanism of flupentixol action. We are currently pursuing this goal in our laboratory.

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