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PHENOTHIAZINES IN TREATMENT OF HIV INFECTION

(A review)*

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Introduction

A wide variety of pharmacological effects have been demonstrated for the phenothiazine class of compounds. These effects include antiemetic, neuroleptic, anticholinergic, antihistaminic and sedative effects [1]. A large number of phenothiazine derivatives and related compounds were synthesized in the past, but only a relatively small number are in use or being evaluated in clinics for these indications. Antiplasmid, anticancer and immunomodulating activities of phenotiazines have also been demonstrated [2, 3]. Recently, anti-HIV effects were also reported by Hewlett et al. [4].

Structure-activity studies with phenothiazines revealed the following general trends: substitution on the aromatic ring at position 2 increases activity in order of increasing electron-withdrawing capacity of the substituting group but substitution at other sites of the molecule, except on the N atom, reduces activity [5]. Most of the clinically useful molecules have a side chain attached to the N atom, which separates another N atom by a three-carbon atom chain. The second N atom may be substituted by methyl groups or may be incorporated into a piperidine or a piperazine moiety. Pharmacological activities varied according to these substitutions. Similar structure activity relations were demonstrated when a C atom in the phenothiazine structure, yielding the thioxanthenes, substitutes the N atom. Interestingly, different 7 and/or 8 substituted chlorpromazine derivatives expressed quite different activity against HIV infectivity of H9 cells [4].

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Replication of HIV in cells can be inhibited in several ways. In the clinic, inhibitors of reverse transcriptase, integrase, protease or their combinations are used most frequently [6]. Theoretically, inhibition of HIV binding to the host cell would also prevent virus replication. Such possibility was shown recently in *in vitro* studies with chlorpromazine derivatives [4].

This chapter will focus on the following aspects of inhibition of HIV replication with phenothiazine or closely related compounds:

- Methods for studying inhibition of HIV replication by phenothiazine type of compounds *in vitro*
- *In vitro* studies with single compounds
- *In vitro* studies with combinations of agents
- Intracellular accumulation of phenothiazine or related compounds; P-glycoprotein modulation of intracellular effectiveness
- Clinical trials

Methods for studying inhibition of HIV by phenothiazines *in vitro*

Determination of inhibition of binding the gp120 virus coat protein for the mAb anti-Leu3a by drugs to the CD4 T cell receptor

Human peripheral blood lymphocytes are obtained from donors or blood banks and purified by density gradient centrifugation as detailed by Weaver et al. [7]. To the isolated peripheral blood lymphocytes (106 cells/ml) in serum and indicator-free RPMI medium the drugs are added in suitable solvent (>0.2% v/v) at room temperature. To determine the effect of serum binding of the drug, an experiment with RPMI containing serum is also performed. After 10–15 min incubation, saturation concentration of anti-Leu3a-fluorescent isothiocyanate or rgp120-fluorescein isothiocyanate is added and the cells are incubated for 30 min in the dark. Incubation can be carried out at different temperatures in order to determine the strength of the binding of the drugs to the CD4 receptor. Cells are then washed in the centrifuge twice to three times and fluorescence histograms are obtained on a flow cytometer using 488 nm excitation wavelength.

Determination of inhibition of fusion between CD4 and gp120 expressing cells by drugs

Cell-cell fusion assay is best carried out with TF cells stably expressing gp120/160 virus coat protein and in combination with Sup-T cells stably expressing

CD4 T cell receptors, according to Jonak et al. [8]. For the assay, TF cells (106 cells/well) are introduced into 96 well plates and are incubated at 37 °C in a 5% CO_2 atmosphere to produce uniform monolayers. Sup-T cells are incubated with 5-microM calcein-AM dye for 1 h, are washed in the centrifuge and added to the monolayers (103 cells/well). Drugs are added to the monolayers in suitable solvents (<0.1% v/v) 10 min before introducing the Sup-T cells. Plates are incubated at 37 °C and 5% CO_2 atmosphere for 4 h.

Fusion is assessed by measuring fluorescent areas, resulting in from fusion and syncytia formation of the calcein stained Sup-T cells with the TF cells. A stage-scanning laser microscope is used to determine differences between fluorescent areas of wells containing only TF, only Sup-T, TF and Sup-T or drug plus TF and Sup-T cells. The laser is operated at 488 nm wavelengths. Typically an image of 1×1 mm^2 area is acquired. Negative control is the image of the Sup-T cells alone and positive control is when a saturation concentration of anti-Leu-3a is used instead of drugs. The mAb, Leu-3a inhibits fusion, cell-cell contact and the spreading of the calcein dye.

Alternatively to the stage-scanning microscope assay, counting them under a light microscope at different time points can assess syncytia formation. The light microscope assay has the advantage that shape and size of the formed syncytia can be determined directly and can be compared to controls.

Determination of infectivity of cells by HIV in the presence of drugs

Drug induced inhibition of HIV infectivity can be determined by using the H9 T cell line and the virus HIV MN. H9 cells are grown to a density of 106/ml and divided into 4 aliquots as described earlier [4]. These aliquots are treated with different concentrations of the studied drug for 20 min, followed by pelleting the cells in a centrifuge. Cells are then resuspended in RPMI medium and treated with HIV MN, equivalent to 100 ng p24 virus protein/108 H9 cells. Virus adsorption is allowed to proceed for 30 min and is followed by washing in the centrifuge. Five ml cultures are plated and 1 ml aliquots are removed at days 4, 7 and 11 for p24 and polymerase chain reaction analyses. Viability assays are performed at the same days using Trypan Blue staining. P24 antigen determination is performed with ELISA kit. Each removal of samples is followed by addition of fresh media with the appropriate amount of drugs to maintain treatment concentrations.

The polymerase chain reaction is carried out as described by Dhawan *et al*. [9]. For this purpose DNA is obtained from the cells by a rapid extraction method for nucleic acids. Digestion with proteinase K is carried out at 56 °C for 60 min and is followed by incubation at 95 °C for 10 min. For polymerase chain reaction,

amplification of the gag region of the HIV genome, a primer pair, SK145 and SK431 can be used. Hybridization can be done with 32P-labeled SK102 probe, analogous to the primer set. Gel electrophoresis is conducted on hybridized samples using 12% acrylamide gels.

Alternatively, drug induced inhibition of virus replication can be assessed based on virus induced cytopathicity. For this purpose, the cell lines MT-4 or C8166 can be used. Cells, 104/100 microl, are introduced into flat bottom microtitre plates and drugs are added in appropriate solvents (<0.1% v/v). At this time HIV, 100×ID50, is added in 20-microl volume of media. After 4 days of incubation at 37 °C in a 5% CO_2 atmosphere, the number of viable cells is determined by the MTT dye method [10]. In this assay, the cytotoxicity of the drug used is also determined. This is done using the same cell culture set up, the same concentration of the drug, without the virus and with the MTT test method.

Reverse transcriptase assay

One of the reverse transcriptase assays is based on poly(rA)n-oligo(dT)12 directed incorporation of [3H]dTTP into cDNA. For this purpose, 20–30 microl solution of 500 mM Tris buffer containing 20 microg/ml template, 5 microM dTTP precursor, 7 kBq tritiated precursor and the test compound is prepared. To this solution 2 international units of reverse transcriptase is added. After 40 min incubation at 37 °C an aliquot is transferred to a Whatman filter paper disc. The disc is washed then with 5% disodium hydrophosphate buffer for 3×3 min followed by water and 96% ethanol washes. The disc is dried and placed into scintillation cocktail for radioactivity measurement. Incontrols no enzyme and no drug are added.

In vitro **studies with single compounds**

One cell biological effect of phenothiazines, specifically chlorpromazine, the direct action on nucleic acids of cells and viruses was suggested by Lialiaris *et al*. [11]. These authors concluded that chlorpromazine could cause scissions and fragmentation to DNA, RNA and plasmid. These effects may be the reasons for the observed cell division delay of human lymphocytes *in vitro* and increased sister-chromation exchange *in vivo*. The observed effects are further increased in combination treatments with adriamycin and caffeine. Whether this mode of action of phenothiazines is operating with HIV replication in cells is not fully understood. Experiments showing that promethazine and chlorpromazine can eliminate F=lac and hemolysin plasmids from *E. coli* JF 2571/Rm98, albeit at relatively high concentrations, tends to support

the action of these compounds on nucleic acids [2]. A recent review of the literature by Glocke concludes that chlorpromazine and related phenothiazines are not genotoxic under mutagenicity test conditions [12]. However, under certain conditions, phenothiazines can be photomutagenic.

Other types of effect of chlorpromazine derivatives were shown on cellular receptors of HIV [4]. It was postulated by the authors that chlorpromazine or its derivatives could affect surface receptors, specifically the CD4 receptor of T4 cells, because of the known charge transfer propensity of this type of chemical structure [13]. The CD4 molecule of T4 cells is known to be a receptor for most HIVs. The receptor-function of the CD4 molecule for the HIV coat protein, gp120 is based on electrostatic interactions [14]. This fact was suggested based on computational simulation experiments, using calculation of electrostatic interactions between charged amino acids of the CD4 receptor and the gp120 viral coat protein. The postulation was proven by flow cytometric binding assays. 7,8-dihydroxy- and 7,8-dioxo-chlorpromazine hindered the binding of the CD4 specific mAb, Leu3a. Other similar chlorpromazine derivatives, for example 7,8-diacetoxy-chlorpromazine was practically ineffective in this assay. Further experiments along this line have shown that chlorpromazine derivatives, found active in the binding assay, also inhibit cell-cell fusion between cells constitutively expressing CD4 or gp120 molecules on their plasma membrane. Fusion of these cells results in giant cells and syncytia formation. 7,8-dioxo-chlorpromazine hindered the formation of syncytia in a dose dependent fashion. This fact was interpreted as action of this phenothiazine on the surface of cells, hindering the fusion process possibly by the postulated electrostatic interactions with the CD4 receptor. In addition to these, polymerase chain reaction assay carried out on the target H9 cells indicated no viral DNA in these cells [4]. This finding also supports the argument that the test compounds act on the surface of the cells and no viral genome penetrates the cells.

Trifluoperazine was shown to inhibit reverse transcriptase of Moloney murine leukemia virus at concentrations of 2 to 8 microM. Interestingly a tin complex of this drug inhibited this enzyme more effectively than the parent compound [15]. Other substituted phenothiazines, for example 10-(3-phtalimido)propyl-2-chloro-10H-phenothiazine and 1-(2-chloroethyl)-3-(2-chloro)-10H-phenothiazine-10yl-butyl-1-urea also inhibited reverse transcriptase at concentrations around 100 microM [16].

Other metal complexes of phenothiazines, such as chlorpromazine and promazine were investigated for their ability to prevent binding of the mAb, Leu3a to its epitope on the CD4 receptor of human peripheral blood lymphocytes [17]. The palladium and gold complexes of both chlorpromazine and promazine were effective while the copper complex as well as the parent compounds were not. However, the

inhibition of binding of Leu3a to the CD4 receptor was shown to be nonspecific and could be related to conformational changes of the epitope of the receptor molecule. Namely, it could be demonstrated that not only the binding of Leu3a was hindered but also the binding of mAbs to epitopes what are unrelated to gp120 binding sites, such as OKT3 and Leu8. Further support of this conclusion was obtained by cell-cell fusion assays. Syncytia formation was hindered by the metal complexes, including the copper complexes, but only by concentrations when visible morphological changes of the cells could be observed. This finding was interpreted as general conformational changes of molecules on the surface of the cells. One should point out, for comparison purposes, that specific hindering of binding of Leu3a or rgp120 to the CD4 receptor could be demonstrated by certain sulfonated molecules unrelated to phenothiazines [18]. These molecules induced no morphological or viability changes in human peripheral blood lymphocytes but hindered the binding of the Leu3a mAb.

In vitro studies with combinations of agents

Combination therapy of AIDS has the advantage that the frequency of simultaneous mutations at more than one point in the viral genome is less likely to occur than at a single point. Therefore, the administration of more than one drug, acting at different sites of the viral replication sequence is recently the preferred chemotherapy of AIDS [6]. With these reasons in mind, agents found active against HIV alone are usually investigated in combinations with other anti-AIDS drugs. The 7,8-dioxo-chlorpromazine, found active against HIV replication by Hewlett *et al*. [4], was studied in combinations with AZT. 7,8-dioxo-chlorpromazine was shown to act at cell receptor level, as discussed above in *in vitro* studies with single agents, and AZT is a reverse transcriptase inhibitor. These two compounds block the HIV replication sequence at different points. The study revealed that 7,8-dioxo-chlorpromazine and AZT act synergistically. A concentration of 7,8-dioxo-chlorpromazine of 2 microg/ml resulted in a p24 titer of 950,000 pg/ml and a concentration of 0.05 microg/ml AZT a titer of 11,900 pg/ml, at time of 11 days of incubation. The combination of these concentrations of the two drugs yielded only 1200 pg/ml p24. A concentration of 6 microg/ml 7,8-dioxo-chlorpromazine alone resulted in a titer of 24,000 pg/ml at 11 days of time and with combination with 0.05 microg/ml of AZT a 0 pg/ml p24 titer. The p24 assay results were supported by polymerase chain reaction assay of viral mRNA formation. The two assays indicated essentially the same conclusions. Therefore, it was suggested, based on this study, that blocking the replication of HIV at viral binding site and at the reverse transcriptase site could lead to reasonable chemotherapy of AIDS. In addition to the favorable synergistic effect, another

advantage of this approach would be that mutations occurring at the binding sites of gp120 could affect results of the drug treatment at a lower level.

Table I

Effect of antifusion peptide, T20 and the chemokine – receptor antagonist, SDF-1 alpha on HIV-MN infection in H9 cell culture

SDF-1μg/ml / T20 μg/ml	0.125	0.25	0.5
0.0125	NA	9	-3
0.025	66	6	2
0.05	78	21	4

Values show the difference in percent between the "calculated titer" of HIV p24 and the found one. "Calculated titer" is a multiplication of found titers after T20 or SDF-i alpha administration alone.

Other investigators also recommended the use of combinations of anti-HIV drugs, which drugs intercept HIV replication at different sites of the replication cycle. For example Brennan *et al*. [19] recommended combinations of two different reverse transcriptase inhibitors with either a protease inhibitor or with a glucosidase 1 inhibitor. Other drug combinations intercepting HIV replication at quite different sites of the replication cycle were also suggested. With this principle in mind, we have studied the possible synergism between a peptide called T20, part of the sequence of the p41 fusogenic peptide of HIV, and an agent, SDF-1, a derivative of the chemokine Rantes. T20 can block HIV fusion but not bind to cell receptors [20], while SDF-1 blocks T-cell tropic HIV attachment to the CXCR4 co-receptor of T cells [21, 22]. We have found significant synergism between these two agents in an *in vitro* system using H9 cells and HIV-MN. Table I shows the difference between expected additive and the found p24 assay results.

Intracellular accumulation of phenothiazine or related compounds: P-glycoprotein contribution to intracellular effectiveness

Fluphenazine and some drugs closely related to phenothiazines, such as amitriptyline, maprotiline, trimipramine, imipramine and doxepin were found modulators of the efflux pump, P-glycoprotein [23]. P-glycoprotein is a 170 kDa membrane bound ABC cassette type pump responsible for efflux of compounds with a variety of structures from cells. This pump, and several others, can cause cancer cells to resist chemotherapy. Interestingly, several phenothiazines and related compounds bind to P-glycoprotein and modulate its ability to efflux other drugs, such as doxorubicine.

This fact was demonstrated with the above listed phenothiazine and related drugs ex vivo with resistant blood cells of acute myeloid leukemia patients [23]. One can speculate that phenothiazine type compounds are nonspecific towards protein binding, especially if we consider the involvement of some phenothiazines in blocking HIV binding to the CD4 receptor, as discussed above. One can also predict that because some of the phenothiazines are used as antipsychotics, in simultaneous treatment, for example acute myeloid leukemia patients with anticancer drugs may facilitate chemotherapy of P-glycoprotein expressing resistant cancer.

Two compounds, N-phtalimido-alkyl- and 2-chloraoethyl- substituted phenothiazine derivatives were also found to block P-glycoprotein function [2]. Interestingly, the cytotoxicity of vincristine increased in the presence of trifluoperazine in low concentration doxorubicin resistant mouse leukemia L1210 cells, but not in high concentration doxorubicin resistant cells. However, accumulation of vincristine in all these cells was increased in the presence of 5-microM trifluoperazine. This seemingly contradiction was interpreted so that trifluoperazine also modulated other than P-glycoprotein resistant factors in these cells [24].

The importance of P-glycoprotein in treatment of AIDS was realized recently. It was shown that most HIV protease inhibitors are substrates of P-glycoprotein [25]. Also, it is known that CD4 and CD8 cells, targets of most HIV-1, express functional P-glycoprotein [26]. These facts may explain the failure of some patients to respond to protease inhibitors, such as ritonavir, saquinavir and indinavir. The finding that some phenothiazines and related drugs inhibit P-glycoprotein, and that these drugs may be used simultaneously with anti-AIDS protease inhibitors, raises an interesting possibility; namely, the introduced antipsychotic phenothiazine or related drugs may potentiate the effectiveness of the anti-AISDs protease inhibitors. Similar possibilities were raised for anticancer drugs in use with multidrug resistant cancers, as discussed above. However, potential host toxicity of such combinations of drugs as mentioned below should be anticipated and avoided.

Clinical trials

Phenothiazines and structurally related drugs are often used for prevention of nausea and vomiting secondary to radiation, cancer treatment and other drug toxicities [1]. As antipsychotic drugs - phenothiazines, such as chlorpromazine - and antidepressants - imipramine and nortriptiline - are frequently used in AIDS patients [27]. The possible drug-drug interactions of these antiemetic and antidepressant phenothiazine drugs with anti cancer and anti-HIV drugs were mentioned above. These

drug-drug interactions at the P-glycoprotein level can affect the metabolism, distribution and pharmacokinetics of drugs [28].

We have determined the concentrations of several phenothiazines and related drugs blocking the function of P-glycoprotein in human Caco-2/MDR1 and MDCK/MDR1 cells. We then compared these concentrations with the therapeutic blood concentrations for these drugs. We found that perphenazine, chlorpromazine, trifuopromazine, imipramine, clomipramine, doxepin, maprotiline and protriptiline block P-glycoprotein at usual clinical blood concentrations. Our findings may explain, at least in part, that 17% of patients treated with protease inhibitors and simultaneously with tricyclic antidepressants developed severe, sometimes life-threatening side effects [29]. One can speculate, in connection with these clinical findings that the protease inhibitors open up the blood-brain barrier by bloocking P-glycoprotein in the capillary endothelial cells and allow unusual amounts of phenothiazine to enter the CNS compartment, resulting in the side effects. Our findings may also be relevant to the results of the clinical trials of Elliott and coworkers [30]. These investigators found that in HIV-positive patients, the coadministered imipramine resulted in more side effects than paroxetine. Imipramine is a P-glycoprotein blocker while paroxetine is not. Imipramine may have made the blood brain barrier of the patients more permeable, resulting in CNS penetration of more than usual amounts of drugs and manifested in side effects. One should mention here that other drug-drug interactions at the cytochrome P450 enzyme level could also play a role in these side effects [31].

An interesting question is whether concomitantly used phenothiazine or chemically related drugs affect the replication of HIV in AIDS patients. Drugs with phenothiazine like structures can have several mode of action and therefore may add to the anti-HIV effect of anti-AIDS drugs. It was mentioned above that some chlorpromazine derivatives act synergistically with AZT *in vitro* by blocking attachment of HIV to its cellular receptor. Phenothiazines can block the function of P-glycoprotein and can facilitate entry of protease inhibitors into infected cells. Other modes of action for phenothiazines are also known as mentioned in the introduction. For these reasons it would be interesting to conduct clinical studies assessing HIV replication with and without the concomitant administration of phenothiazine type drugs.

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