The essential role played by PDI in HIV-1 entry provides new opportunities for drug development

Keynote review:

Progress in targeting HIV-1 entry

Hugues J.-P. Ryser and Rudolf Flückiger

Current HIV entry inhibitors target the binding of the viral envelope glycoprotein gp120 to cellular CD4 and co-receptors, or block a late stage of the fusogenic activation of adjacent gp41. New targets are suggested by the role of cell surface protein disulfide isomerase (PDI), which attaches to the primary receptor CD4 close to the gp120-binding site. This could enable PDI to reduce gp120 disulfide bonds, which triggers the major conformational changes in gp120 and gp41 required for virus entry. Inhibiting cell surface PDI prevents HIV-1 entry. The new potential targets outlined are PDI activity as well as the sites of PDI–CD4 and PDI–gp120 interaction.

Exciting progress has been made in preventing the entry of HIV-1 into target cells. Agents acting on three basic steps of the process have been developed. Several of them are in clinical trials and one of them has been approved by the FDA for clinical use. These developments are reviewed in the context of newer findings. An unanswered question was the molecular nature of the initial mechanism that triggers the sequence of events leading to virus–cell fusion. A chance finding revealed that a host cell enzyme, surface-associated protein disulfide isomerase (PDI), reduces disulfide bonds in proteins attached to the cell, including those of the receptor-bound envelope glycoprotein gp120. By reducing structure-stabilizing disulfide bonds, PDI causes major conformational change in gp120 that could activate the fusogenic properties of adjacent gp41. The primary receptor CD4 that binds the virus to the cell has separate and adjacent binding sites for gp120 and PDI, suggesting that PDI can access gp120 disulfide bonds. Inhibition of the surface enzyme prevents HIV-1 entry. These findings broaden our understanding of the entry process and open new opportunities for drug development.

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Hugues Ryser received his MD and Dr Med. from the University of Berne, Switzerland. In 1972 he became professor of Pathology and Pharmacology at Boston University, where he is now Professor Emeritus. His laboratory developed drug-macromolecular conjugates that release anti-cancer drugs inside cells. The observation that disulfide bonds in drug conjugates are cleaved upon attachment to cells led to the finding that some gp120 disulfide bonds are reduced upon binding of HIV-1 to its surface receptor.

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Mechanism of HIV-1 entry

It has long been known that entry of HIV-1 into human lymphoid cells requires the cooperation of the viral envelope glycoproteins gp120 and gp41, and of two host-cell proteins, the primary receptor CD4 and a chemokine co-receptor (CCR5 or CXCR4) [1]. More recently a third cell-surface protein was found to play a critical role in HIV-1 entry: the oxidoreductase protein disulfide isomerase (PDI) [2–6]. Gp120 attaches the virus to the cell by binding to CD4. It was found that CD4 also has a binding site for PDI and forms a PDI–CD4–gp120 complex. In that complex, PDI can reach and reduce gp120 disulfide bonds [4–6], causing major conformational changes in gp120. These changes are transmitted to gp41 and activate the fusogenic potential of the viral envelope.

The envelope glycoproteins gp120 and gp41 are derived from the trimeric precursor gp160, which is cleaved by a cell endoprotease. The gp120 and gp41 subunits associate noncovalently and homotrimers of gp120–gp41 are incorporated in the viral envelope. Gp41 is bound to the virus through its C-terminal *trans*-envelope domain, whereas gp120 is not anchored to the viral envelope. CD4 and co-receptors are transmembrane proteins, whereas PDI is loosely attached to the cell surface, where it interacts with several proteins besides CD4 [7]. PDI is relatively abundant at the cell surface. It is shed and rapidly replaced from intracellular sources in a way that is subject to regulation [7].

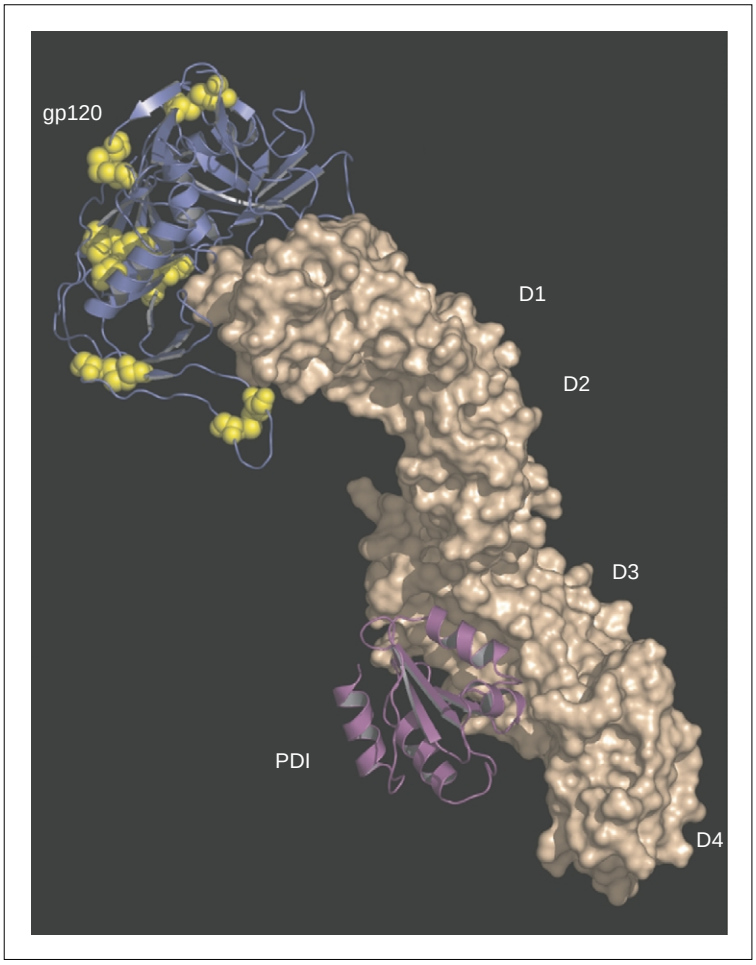

FIGURE 1

Model of PDI–CD4–gp120 complex revealed by molecular docking analysis. Truncated PDI (2B.JX) was docked with CD4 (Chain A of 1WIP). Shown is the model with the tightest PDI–CD4 binding. The gp120 core resolved in [9] is added to D1. CD4 (light brown), PDI (purple), gp120 (blue). The Cys residues on gp120 (yellow) are the potential substrates for PDI. There is a short flexible 'hinge' region between D2 and D3 that could bring gp120 in contact with PDI. (Protein docking data were provided by S. Comeau, and S. Vaida, Boston University).

Primary clinical infections are caused by HIV-1 strains that utilize CCR5 (R5 tropism) but, in the course of infection, the virus can adapt to bind also to CXCR4 (dual tropism) or to CXCR4 only (X4 tropism). The co-receptor usage of gp120 is determined essentially by the primary structure of a variable loop region in the outer domain of gp120 (V3 loop). It has been shown that gp120–co-receptor engagement can influence the kinetics of gp41 activation [8]. The molecular basis of this effect, however, has not been elucidated.

CD4 has a linear ectodomain consisting of four immunoglobulin-like regions (D1–D4) and binds to gp120 through its outermost domain (D1). Molecular docking indicates that PDI binds to the CD4 domain D3 (Figure 1). Binding of gp120 to sCD4, the isolated soluble ectodomain of CD4, induces some conformational changes in gp120 that expose new gp120 epitopes recognized by human neutralizing antibodies; the new conformation also enhances the interaction of gp120 with co-receptors. Binding of gp120 to the cell surface PDI–CD4 complex, however, induces more profound conformational changes through the access of PDI to gp120 and the reduction of structure-stabilizing gp120 disulfide bonds (Figure 2a and b). The ensuing structural rearrangements enable the fusogenic activation of gp41 (Figure 2 b–e). Efforts to develop agents that block HIV-1 entry have focused on three basic steps of this sequence, namely the gp120-CD4 interaction (Figure 2a), the gp120–co-receptor interaction (Figure 2b) and the terminal stages of gp41 activation (Figure 2e). They have led to a drug approved for therapeutic use and to several promising agents undergoing clinical trials, as will be described below. The knowledge required to exploit these targets has been provided by the structure of CD4, by co-receptor modeling, and by the landmark resolutions of the X-ray structure of a modified gp120 core [9] and of the X-ray structure of the activated form of gp41 [10,11].

The crystal structure of a gp120 core, complexed with the D1D2 domains of CD4 and with the Fab domain of an anti-gp120 antibody, identified the amino acid residues of gp120 that make contact with the receptor. Several of these residues are situated in a functionally important pocket of gp120 (Phe 43 pocket) [9,12,13]. It now appears that this pocket harbors the binding site of a potent entry inhibitor [14]. This raises the intriguing possibility that the pocket might be the site of a PDI–gp120 interaction.

The X-ray structure of crystallized components of gp41 revealed features similar to those previously attributed to

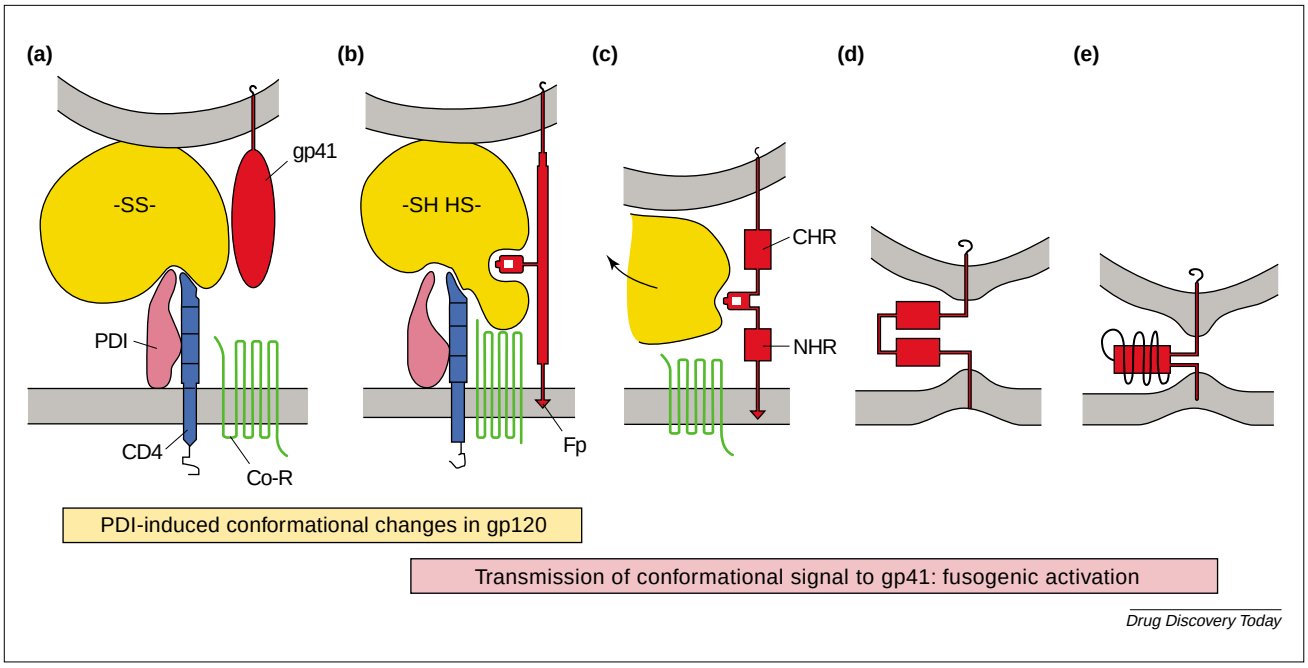

FIGURE 2

Model of PDI-induced conformational changes leading to activation of gp41. (a) PDI present at the surface of target cells binds to domain D3 of CD4, while CD4 (D1) binds to a conserved pocket of gp120. PDI makes contact with gp120 in the region of the CD4-gp120 binding site. The disulfide bonds of gp120 are intact (-SS-) and both envelope glycoproteins are in their native conformation. **(b)** Structure-stabilizing disulfide bonds have been reduced (-SH + HS-) leading to conformational changes that increase the gp120 interaction with co-receptors and with a small conserved loop of gp41. That interaction generates major conformational changes in gp41 that elongate the molecule on both sides of the small loop and form the N-terminal fusion peptide (Fp) that inserts into the cell membrane. **(c, d)** activation of gp41 includes the formation of helices in the heptade repeat section of NHR and CHR that bend to assume an antiparallel position. The distance between virus and cell is decreased and gp41 becomes the only link between them. **(e)** The CHR helix winds itself around the NHR helix. In the trimeric state of gp41 (not shown) the three CHR helices wind themselves around the three NHR helices that form a central coiled-coil, giving rise to a six-helix-bundle (only one-third of that bundle is shown).

the fusogenic state of the envelope of the Influenza virus that belongs to the orthomyxoviruses. This similarity led to the conclusion that the X-ray data derived from these components reflect the fusogenic state of gp41. It is now generally accepted that in the course of its activation gp41 displays two regions containing heptad repeats (HR) with helical potential, separated by a sequence that forms a small conserved disulfide loop (Figure 2b and c), the integrity of which is required for infection. The small loop region and the facing gp120 region are critical sites of gp120-gp41 interaction, as amino acid substitutions in these regions impaired gp41activation [15,16] (Figure 2b). The N-terminal and C-terminal halves of gp41 on each side of the loop give rise to the heptad repeat helices NHR and CHR, respectively (Figure 2c). It is known that gp120 is shed loose of its binding to the virus envelope and to CD4. Although the timing of this disengagement has not been clearly identified, the model of Figure 2 proposes that it occurs shortly after the transmission of the gp120 conformational changes to gp41 and that PDI is shed at the same time (Figure 2c). The constant shedding and replacement of PDI at the cell surface is a physiological process [7].

The end of the N-terminal section of gp41 forms a fusion peptide that inserts into the cell membrane. The two halves of gp41 bend upon each other to form a hairpin that is positioned in a plane parallel to the cell and viral surfaces and brings the two closer together (Figure 2d). Note, however, that gp41, and therefore NHR and CHR, are present as trimers. (For clarity, Figure 2 represents the activation of one gp120-gp41 monomer). A critical structural change occurs when the triple NHR and CHR helices assume an antiparallel position. The three NHR helices form a central coiled-coil and the three CHR helices wrap themselves around the central coil to form a six-helix bundle. This bundle (only one-third of which is shown in Figure 2e) represents the ultimate activation state of the gp41 trimer. There is evidence that the merger of viral and cell membranes occurs before completion of the six-helix bundle, that fusion is facilitated by the C- and N-terminal sub-domains and their interaction with the respective membranes, and that the completed six-helix bundle contributes to the pore formation [17–21]. It appears likely that, after their disengagement from PDI and gp120, CD4 and co-receptor undergo internalization by the target cell. As their transmembrane domains are close to the membrane insertion site of the gp41 fusion peptide, one may postulate that CD4/co-receptor internalization might facilitate virus-cell fusion and the translocation of the HIV-1 core. Such a correlation has not been established, but deserves investigation.

Comparing the activation of HIV-1 and Influenza virus, it is of note that the conformational changes causing the

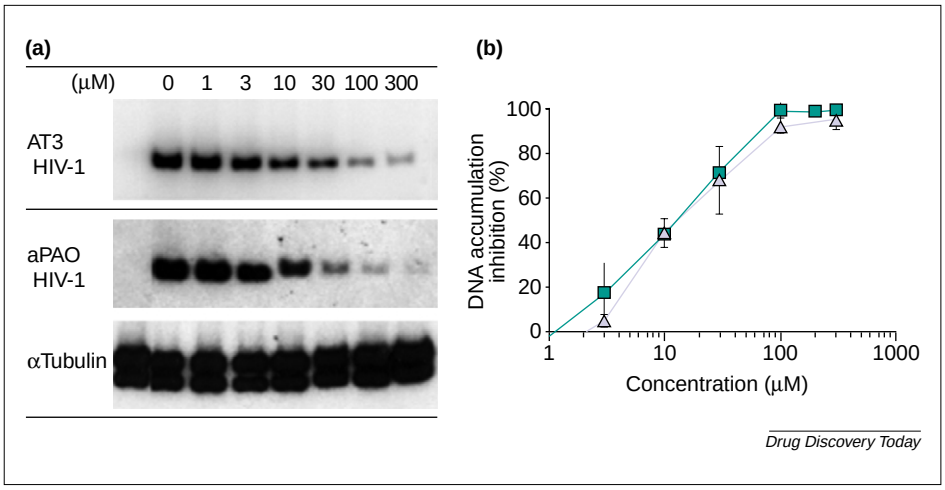

FIGURE 3

Inhibition of surface PDI activity prevents HIV-1 entry (a): HIV-1_{NL4-3} entry into SupT1 cells assayed by measuring the accumulation of PCR-amplified strong-stop DNA. Auto radiograms of amplified reversed transcripts show decreased accumulation in cells exposed to increasing concentrations of PDI inhibitors. The inhibitors do not affect the accumulation of the reverse transcript of the α-tubulin gene used as control. **(b):** Dose-related inhibition of cDNA accumulation derived from **(a)** in the presence of *para*-aminophenylarsine oxide (■: aPAO) and acetyl triiodothyronine (▲: AT3), (reproduced from [4]).

fusogenic activation of Influenza haemagglutinin are initiated by the acidic pH of the intracellular vesicles to which the virus is exposed following its endocytosis. In HIV infection, however, the viral envelope is not exposed to an acidic milieu and no equivalent trigger of fusogenic activation had been singled out. The conformational changes initiated by gp120 binding to sCD4 do not appear to be sufficient to activate gp41 [22][1] Reduction of structure-stabilizing disulfide bonds in gp120 by surface PDI might represent a mechanism capable of triggering the profound conformational changes in gp120 needed to activate gp41.

Evidence for the participation of PDI in HIV-1 entry

Inhibition of the activity of PDI at the surface of target cells prevents the activation of gp41 [6], the entry of HIV-1 strains into target cells [4] (Figure 3), and envelope-mediated cell-cell fusion [3,4]. The following facts and experimental data explain how surface PDI might exert such a critical role. (1) At the cell surface PDI acts as a reductase that cleaves disulfide bonds of proteins attached to the cell, whereas inside the cell PDI forms disulfide bonds in nascent proteins (Figure 4). Both functions are general properties of mammalian cells [7]. (2) Several of the nine conserved disulfide bonds of gp120 are vulnerable to reduction by CD4-bound PDI as they are situated in the immediate vicinity of the gp120–CD4 binding site [4,5,9]. (3) Purified PDI reduces disulfide bonds in a membrane-bound model peptide (Figure 4). The reduction of the model substrate is inhibited in dose-dependent fashion by PDI inhibitors of different modes of action [4]. (4) PDI inhibitors prevent the reduction of disulfide bonds in gp120 attached to target cells [4–6]. (5) Concentrations of PDI inhibitors that prevent the reduction of disulfide bonds in cell-bound recombinant gp120 also prevent HIV entry when present at the time of virus-cell interaction. They have no effect on the entry of Murine Leukemia Virus envelope-pseudotyped HIV-1 [4], indicating that the reductive activity of the target cell surface, although important for the entry of HIV-1, is not required for the entry of other retroviruses. (6) PDI strongly binds to CD4 as shown by various co-precipitation experiments [4]. This view is confirmed by molecular docking studies indicating that PDI interacts strongly with the D3 domain of CD4 (Figure 1) and less strongly with D2. In either case PDI can reach gp120 by direct proximity or through the flexibility of CD4 between D2 and D3. (Figure 1, Figure 2a and b). (7) A PDI-CD4-gp120 complex in which PDI was affinity-labeled on its thiols was isolated from lysates of target cells pre-exposed to gp120 using thiol-specific HRP–NeutrAvidin [4]. PDI and CD4 were readily immuno-detected in the same surface areas of target cells, although evidence of their immuno-co-localization was sparse in the absence of gp120 [3], suggesting that gp120 might enhance PDI–CD4 interaction and the formation of a PDI–CD4–gp120 complex. PDI was also isolated as part of a PDI-CD4–gp120–CXCR4 complex using an anti-CXCR4 monoclonal antibody and the four components of the complex were detected at the cell surface by immuno-co-localization [6]. The puzzling occurrence of CD4-independent infections, however, must be kept in mind[2] [8]. In experiments focusing on the activation of gp41 by using a monoclonal antibody recognizing the six-helix bundle, recognition was abolished by the presence of PDI inhibitors at the time of envelope-receptor–co-receptor interaction [6]. This finding could be viewed as providing a causal link between the

[1]Addition of sCD4 to 293T cells engineered to express gp120/gp41 increased the binding of a CHR-derived inhibitory peptide to gp41 [22]. This finding suggests a possible effect of sCD4 on the formation of the six-bundle helix (see also [51,52]). This effect, however, might be explained by the binding of sCD4 to surface PDI, followed by the binding of CD4-PDI to gp120 present at the surface of engineered 293T cells. As part of the resulting gp120-CD4-PDI complex, PDI could reach and reduce gp120 disulfide bonds and activate gp41. This view is supported by the observation that the changes in gp41 caused by adding sCD4 are abolished by BMS 806 [22].

[2]The critical role of PDI-CD4 interaction in HIV-1 entry is difficult to reconcile with the observation that certain CD4-independent HIV-1 strains can cause infection. As infection can not occur without attachment of the virus to target cells, it is assumed that these HIV-1 strains use substitute primary receptors. Our scant knowledge about such surrogate receptors leaves open the possibility that they may bind both gp120 and PDI and, like CD4, bring the enzyme into contact with gp120. Testing whether CD4-independent infections are inhibited in the presence of PDI inhibitors offers a direct experimental approach to verify this hypothesis.

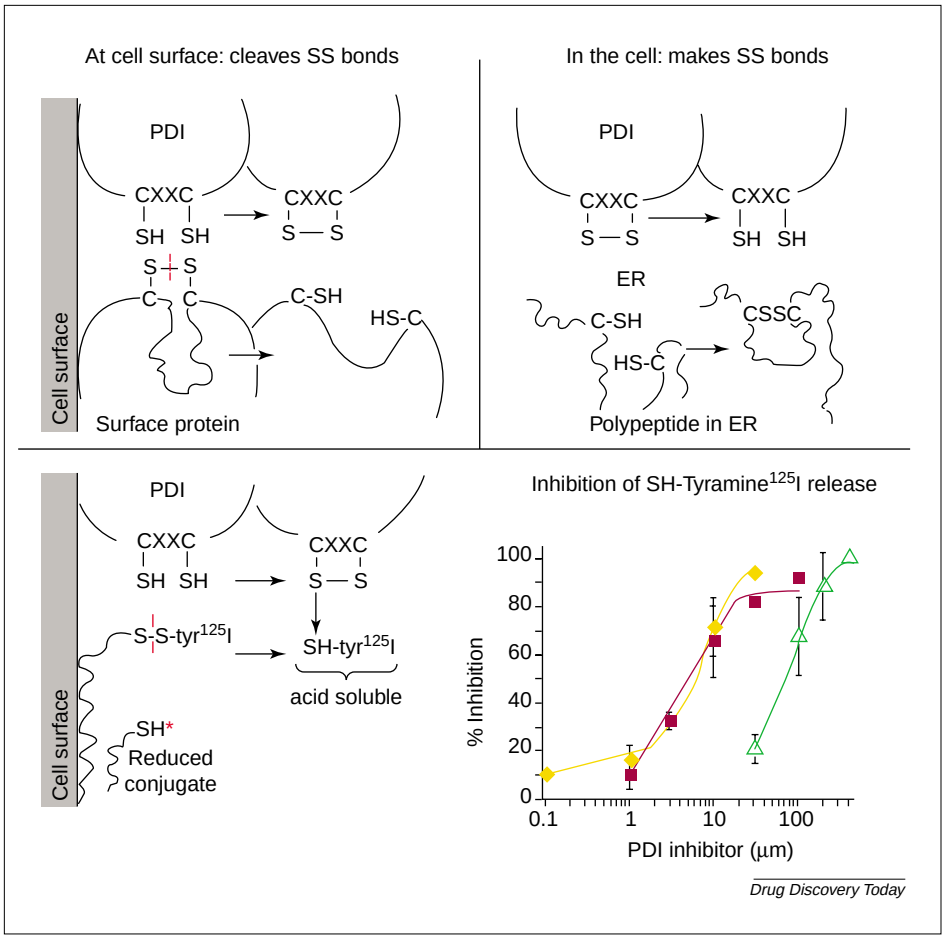

FIGURE 4

Functions of PDI at the cell surface and inside the cell. Top: Model showing the effect of PDI at the cell surface (left), where it acts as a reductase cleaving disulfide bonds of proteins attached to the cell membrane (grey), and inside the cell (right) where it forms disulfide bonds in nascent polypeptides reaching the endoplasmic reticulum (ER). The -CXXC- motif of the PDI active site is oxidized upon reducing a protein disulfide bond (left), and is reduced upon forming a disulfide bond in a nascent polypeptide (right). Bottom: Reduction of the disulfide bond in a cell-bound model conjugate (poly-D-lysine-SS-^{125}I-tyramine) by surface PDI (left) and its dose-dependent inhibition (right) by three PDI inhibitors: (◆ DTNB; ■ *para*-amino-phenylarsine oxide, aPAO; △ N-acetyl-triiodothyronine, AT3) (reproduced from [4]).

enzymatic activity of surface PDI and the fusogenic activation of gp41. This activation results in the formation of the six-helix bundle. As completion of this bundle is required for HIV-1 entry, it appears that the PDI-induced reduction of gp120 disulfide bonds is likely to be the biochemical event that triggers HIV-1 entry.

Inhibitors of HIV-1 entry in use or under development

The search for agents that prevent HIV-1 entry has focused on blocking (a) the interaction of gp120 with the cellular receptor CD4, (b) the secondary interaction of gp120 with cellular co-receptors CCR5 or CXCR4 and (c) the formation of the six-helix bundle of fusion-active gp41. The first two approaches led to promising inhibitors that are under active clinical investigation. The third led to a potent inhibitor of HIV-1 entry that has been approved by the FDA for the treatment of AIDS [FUZEON®]. These achievements have been extensively reviewed [23–30] and are briefly discussed below. Selected entry inhibitors are listed in Table 1.

a) Agents that prevent the interaction of gp120 and CD4

Preventing the virus from binding to its primary receptor is the most obvious and direct way to prevent infection. This approach has been quite successfully exploited. The small molecular entry inhibitor BMS 378806 also called BMS-806 was derived from an active compound identified using a virus-infection-based screen [31]. BMS-806 binds selectively to gp120 [32]. It was initially thought to block the binding of gp120 to CD4 but was shown more recently to bind to a contiguous domain different from the gp120–CD4 binding site. This view is based on the finding that the domain contains eleven amino acids residues that upon mutation decrease the effect of BMS-806 [14]. It was also pointed out that the short V1/V2 stem of gp120 formed by two disulfide bonds is likely to contribute to the drug binding site [14]. The presence of a disulfide bond in, or close to, the BMS 806 binding site is intriguing, considering the facts that BMS 806 prevents HIV-1 entry and that HIV entry requires the reduction of gp120 disulfide bonds. Close examination of Fig 5B in reference [14] and of the spatial relationship to gp120 disulfide bonds reveals that nine of the eleven amino acid residues believed to participate in BMS 806 binding are positioned in the vicinity of four gp120 disulfide bonds[3].

Several HIV-1 strains are strongly inhibited by BMS-806 at nM concentrations, as tested in a large number of clinical isolates that included the most common HIV-1 group (M) and sub-type (B) [32]. Interestingly, infection of CD4-independent strains are prevented by BMS 806 [22]. This compound has undergone clinical testing

[3]Using as a basis the original linear amino acid sequence of gp120 that identified the site of the nine conserved gp120 disulfide bonds [47], it is of note that nine of the eleven amino acid residues participating in the binding of BMS 806 (see Figure 5B in Ref. [14]) are situated in close proximity of four disulfide bonds. Three of them (Lys121, Leu125, Asn197) are part of the short V1/V2 stem formed by Cys/Cys 119/205 and 126/196; three others (Phe382, Met426, Lys 429) are within the V4 loop formed by Cys/Cys 368/445 and 385/418. Furthermore, Trp112 and Asp 113 are close to Cys119, and Ser375 is next to Cys 378. This spatial correlation indicates that BMS 806 binds to a gp120 site rich in disulfide bonds, suggesting that its binding might protect gp120 disulfide bonds from being reached and reduced by PDI, an enzyme brought to the scene by its association with CD4. Interestingly the V1/V2 stem- and the V4 loop disulfides had been previously singled out as likely substrates of PDI on the basis of their proximity to the CD4-gp120 binding site [4, 5].

TABLE 1

Characteristics of selected HIV-1 entry inhibitors

Class/Compound	Manufacturer	Identity	Mechanism	Phase[a]	Refs
Gp120-CD4 binding					
BMS-378806 (BMS-806)	Bristol-Myers Squibb	indole derivative	binds to gp120		[32]
BMS-488043	Bristol-Myers Squibb	2nd generation compound	binds to gp120	II	
Pro 542	Progenics	CD4-IgG2	binds to gp120	II	[33]
CD4 M33		27-aa CD4 mimic	binds to gp120		[34]
TNX-355	Tanox/Biogen	anti-CD4 mAb IgG4	binds to CD4	II	[35]
Gp120-co-receptor binding					
SCH-C	Schering-Plough	oximino-piperidino-piperidine amide	binds to CCR5	discontinued	[37,38]
SCH-D	Schering-Plough	not disclosed	binds to CCR5	II	[49]
Pro-140	Progenics	monoclonal antibody	binds to CCR5	I	[40]
Tak-779	Takeda	quaternary ammonium anilide	binds to CCR5	discontinued	
Tak-220	Takeda	2nd generation compound	binds to CCR5	preclinical	
GW873140	Glaxo SmithKline	Spirodiketopiperazine	binds to CCR5	II	
UK-427,857	Pfizer		binds to CCR5	II/III	[23]
AMD3100	AnorMED	Bicyclam	binds to CXCR4	discontinued	[39,53]
KRH-2731	Kureha	Arginine derivative	binds to CXCR4	preclinical	[42]
Fusion inhibitors					
Fuzeon®	Trimeris	36 aa peptide	CHR mimic	in clinical use	[44]

[a]For up to date information on clinical trials see www.clinicaltrials.gov

that demonstrated potent anti-viral effects such as a decrease in viral load and a reversal of $CD4^+$ blood cell depletion. BMS-806 was found to inhibit infection of HIV-1 strains resistant to agents that target virus replication. It induces drug resistance in tissue culture at rates similar to those observed with inhibitors to HIV-1 replication [32]. Several escape mutants were found to map to five regions of gp120 and two residues of gp41 [14], indicating that the effects of the drug can be compromized by mutations occurring at sites other than the drug binding site and suggesting that the conformational signal believed to be transmitted from gp120 to gp41 can be interrupted within gp41. BMS-806 has recently been replaced with BMS-488043, a compound derived on the basis of structure-activity-relationship studies [Lin *et al.* #534 at http://www.retroconference.org/2004/Home.htm].

Although BMS-806 is no longer considered to interfere essentially with the gp120-CD4 interaction, several macromolecules have been shown to act at that level. The first one tested was sCD4 itself. It inhibited the infection of laboratory strains but was less effective on primary HIV-1 isolates. It encouraged the search for more effective macromolecules that would similarly act as decoys by binding to gp120. Two such rationally designed constructs are PRO 542 [33] and CD4M33 [34]. PRO 542 is a fusion protein containing four IgG2 molecules in which the variable fragments of both light chains are substituted with the D1/D2 domains of CD4. It has eight sites capable of binding gp120. It markedly inhibits the infection caused by clinical isolates, is well tolerated and has antiviral effects in preliminary clinical testing that include patients with advanced disease. CD4M33 is a 27-amino acid peptide mimicking the CD4 domain D1 that binds to gp120. It is specific for gp120 and induces conformational changes in gp120 similar to those initiated by sCD4. Both agents are effective *in vitro* at nM concentrations. Another macromolecule developed for the same purpose is the IgG4 monoclonal antibody TNX 355 [35] targeted to the D2 domain of CD4. It prevents the infection by laboratory strains of HIV-1 and displayed anti-viral effects in preliminary clinical trials, in which it was found to cause some adverse effects. Its exact mode of action is not known. Because of its large size, this antibody might be able to interfere with binding of gp120 to D1 or of PDI to D3, a point that awaits experimental determination. Inhibition of HIV-1 entry should occur in either case, as TNX 355 might prevent the virus from binding to the cell or the access of CD4-bound PDI to gp120 substrates. The three above mentioned agents are not orally bioavailable.

b) Agents that prevent the interaction of gp120 with CCR5 or CXCR4

The co-receptors CCR5 and CXCR4 are seven-transmembrane G proteins with signal transduction functions. The natural ligands of CCR5 (RANTES and MIP-1α and 1-β) and of CXCR4 (SDF-1α) inhibit infection by R5- and X4-HIV-1 strains, respectively. This observation led to the rational design of small molecular agents that mimic natural ligands and block gp120 binding, ideally without inhibiting

the co-receptor signaling function and without inducing co-receptor internalization. An agent has been recently shown to have a dual action as CCR5/CXCR4 inhibitor [36]. As a rule, however, a given agent prevents gp120 binding to only one of the two co-receptors and is effective only as long as the viral strain maintains its original cytotropism. The V3 loop that determines the cytotropism is prone to mutations that could enable the virus to bind to a site different from that occupied by the co-receptor antagonist. Resistance might also occur when an HIV-1 strains become able to infect cells that express abnormally low levels of co-receptor, or when target cells up-regulate co-receptor expression [27]. Small molecular antagonists of CCR5 and CXCR4 have been the subject of a separate review [29].

Anti CCR5 agents

TAK-779, a complex quaternary amine specific for CCR5, was the first compound proposed as CCR5 antagonist to display potent antiviral effect *in vitro*, but its development was discontinued.

It was replaced by the orally administered and structurally unrelated TAK 220 [Iizawa *et al*. #11 at www.retroconference.org/2003]. This agent inhibits the infection of primary isolates at nM concentrations, including isolates that are resistant to reverse transcriptase and protease inhibitors. It has favorable pharmacokinetic properties and is being further investigated. SCH-C is an oximino-piperidine analog effective against a broad range of primary isolates at nM concentrations [37,38]. It demonstrated anti-viral activity *in vivo* in a mouse model and showed synergism with the virus replication inhibitor AZT or the fusion inhibitor T20 [39]. It has been replaced by SCH-D, a more potent second-generation compound with similar pharmacokinetic properties currently in Phase II clinical trials. GW-873140, also known as ONO-4128 and AK-602, is a potent *in vitro* inhibitor of numerous primary HIV-1 isolates [Demarest #139 at www.retroconference.org/2004/Home.htm]. It is well tolerated and has favorable pharmacokinetic properties and is undergoing clinical testing. UK 427,857 is a promising small molecular agent that prevents the binding of gp120 to CCR5. It is effective against clinical isolates at low nM concentrations [Dorr *et al*.#12 at www.retroconference.org/2003] has favorable pharmacokinetic characteristics and is undergoing clinical trials. PRO-140 is an anti-CCR5 monoclonal antibody that binds to several sites of the CCR5 ectodomain. It has been shown to prevent HIV-1 entry in a mouse model carrying infected human lymphocytes, where it significantly reduces the viral load [40].

Anti-CXCR4 agents

The opposite electrostatic properties of the V3 loop and of the ectodomain of CXCR4 led to the development of cationic agents blocking anionic residues of the co-receptor. Among them are small cationic peptides and arginine conjugates. The bicyclam AMD3100 was the first CXCR4 antagonist to reach clinical trials. It suppressed infection by X4-strains in a majority of subjects, but its development was halted because of unfavorable pharmacokinetic properties, negligible bio-availability, and cardiac side effects. AMD070 is a second-generation CXCR4 antagonist of lower molecular weight than AMD3100 but with a similar binding site. It is highly potent against CXCR4 and its natural ligand and is effective against a broad range of clinical isolates at low nM concentrations. It is orally administered and is currently the leading AMD derivative under clinical investigation [41]. KRH-1636 is a potent new agent that is bio-available after duodenal administration. It is effective against clinical isolates and reduced infection in a mouse model carrying infected human lymphocytes. In a short-term *in vivo* test it inhibited viral replication and prevented the fall of circulating $CD4^+$ blood cells of infected subjects [42]. This compound is under further investigation.

c) Agents preventing viral-cell fusion by direct action on gp41

Knowledge of the fusogenic structure of gp41, in particular the finding that the ultimate active state of gp41 is characterized by the formation of a six-helix-bundle, opened new opportunities to prevent HIV-1 entry. A 36-amino acid peptide, T20, based on the sequence of the CHR helix of gp41 was found to bind to the NHR coiled-coil. It prevents the peripheral attachment of the three CHR helices to the NHR coiled-coil, thereby preventing the formation of the six-helix bundle [43]. T20 attaches to a short-lived intermediate of gp41 exposed during gp41 activation. Surprisingly, the kinetic window during which T20 can bind to CHR is influenced by the affinity of gp120 for CCR5. Increased affinity accelerates the formation of the six-helix bundle, shortens the window and decreases the sensitivity to T20. The concentrations needed to inhibit viral isolates can vary by two logs [8]. T20 does not interact with native gp41 or with the formed bundle, but was reported to bind to gp120 in a way that interferes with the gp120-CXCR4 interaction and might increase its effectiveness against CXCR4 strains [44]. In clinical trials T20 reduced viral RNA in the blood of infected subjects and reversed the loss of blood $CD4^+$ lymphocytes. It is administered by infusion. T20 has been approved by the FDA for therapeutic use in the treatment of AIDS under the name of Enfuvirtide [Fuzeon®]. The development of an agent with a similar mode of action, T2149, was halted. Peptides based on the sequence of NHR have also been shown to prevent HIV-1 entry [45]. Surprisingly, some mutations that developed in the CHR or NHR peptides of patients treated with T20 were found to enhance virus infectivity, making the virus drug dependent [46]. To overcome the limitations of parenteral administration of T20, efforts are under way to develop orally bio-available low molecular compounds with similar modes of action.

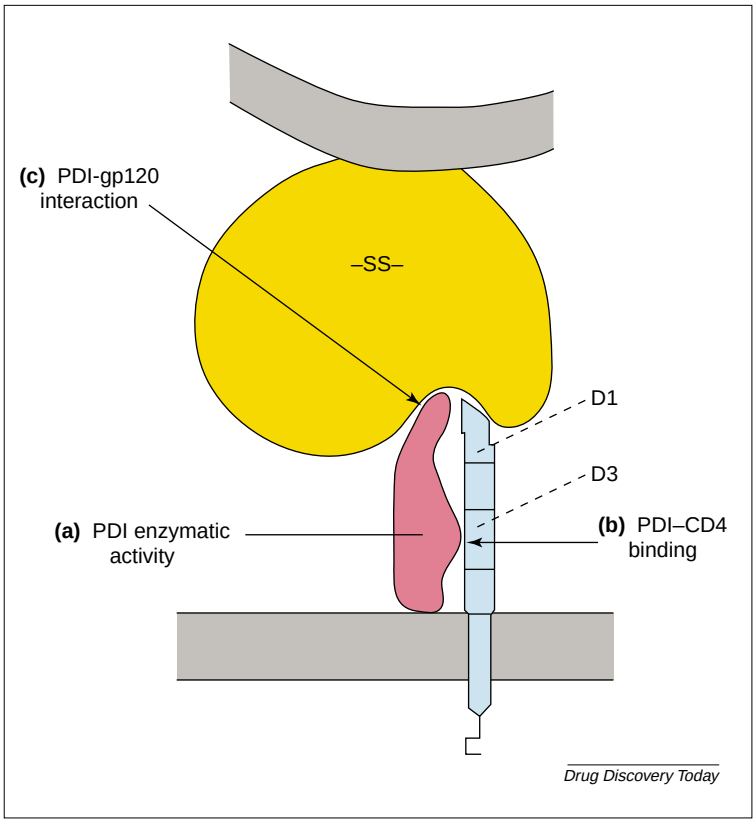

FIGURE 5

Targets defined by participation of PDI in HIV-1 entry. Target **(a)**, PDI activity; target **(b)**, PDI-CD4 binding; target **(c)**, PDI gp120 interaction. Both CD4 and PDI reach gp120 in the same pocket. Gp120 and PDI are shown to bind to CD4 domains D1 and D3, respectively.

New targets uncovered by the participation of PDI in virus entry

The involvement of PDI in HIV-1 entry identifies three new targets (Figure 5) that could be exploited to prevent HIV-1 entry: (1) the activity of surface PDI; (2) the binding of PDI to CD4; (3) the access of CD4-bound PDI to gp120 disulfide bonds. All three processes are essential for infection and differ from those used in past drug developments (Figure 5).

1) Targeting the enzymatic activity of PDI

Investigational agents that target the activity of PDI have been most useful to demonstrate the role of PDI in HIV-1 entry. Their potential as therapeutic agents, however, is limited by the physiological functions of PDI both inside the cell (Figure 4) and at the cell surface [7]. Attempts to knock out the gene of PDI in mammalian cells have resulted in lethal mutations. Only targeting cell surface PDI by using membrane impermeant inhibitors holds any therapeutic promise and is itself limited by the other physiological functions of surface PDI [7]. Investigational membrane impermeant PDI inhibitors include thiol blockers such as DTNB and PCMBS, blockers of the CXXC vicinal thiols of the PDI active site such as para-amino-phenylarsine oxide (aPAO) and its glutathione conjugate (GSAO) [48], the acetylated form of triiodothyronine (AT3) [4] and anti-PDI monoclonal antibodies [2,3,6]. The mode of action of the antibiotic bacitracin, one of the first agents used as PDI inhibitor, has not been clearly established. AT3 strongly inhibits PDI, presumably through an allosteric mechanism, but like aPAO is not strictly membrane impermeant. Anti-PDI antibodies are difficult to raise and are not very potent as enzyme inhibitors, although they very effectively detect the protein in western blots [2,3,6]. The above agents are not likely to be of therapeutic use.

It was suggested that an enzyme related to PDI, thioredoxin, might play a role in HIV-1 entry [49]. The suggestion was based on the observation that thioredoxin activates T-lymphocytes by reducing a disulfide bond in the D2 domain of CD4 and that inhibition of the activation with a trivalent arsenical prevented HIV-1 entry. Thioredoxin and PDI activities are inhibited by trivalent arsenic. Knowing that such compounds prevent HIV-1 entry (Figure 3 and [4]), and that thioredoxin does not reduce gp120 disulfide bonds and does not impair gp120 binding to CD4 [49], it appears likely that, in this case, the observed inhibition of HIV-1 entry was due to the inhibition of PDI. Yuan *et al.* [50] described in several HIV-1 strains the occurrence of disulfide bonds between subunits of envelope glycoprotein trimers. Such bonds modestly enhanced the antigenicity of trimers, but did not influence the entry of affected HIV-1 strains into target cells, nor the dose-dependent inhibition of entry by PDI inhibitors.

2) Targeting the PDI-CD4 interaction

This approach is very promising as the two separate CD4 binding sites for gp120 and PDI are close enough for the two proteins to reach each other (Figures 1 and 2). Preventing that contact has the potential of preempting the PDI-induced reduction of gp120 disulfide bonds and its conformational consequences. Molecular docking studies suggest that PDI binds to the D3 domain of CD4 (Figure 1). It can be anticipated that an antibody directed against D3 would block PDI-CD4 binding. Closer identification of the CD4-docking site should make it possible to rationally design small molecular agents that interfere with HIV-1 entry. Inhibiting HIV-1 entry with such agents or with an anti-D3 antibody should provide the proof-of-concept that the PDI-CD4 interaction is a valid target for drug development. As the interacting proteins PDI and CD4 are stable cellular proteins, they are less subject to mutations that would impair drug binding. By contrast, even small mutations in gp120 can prevent or circumvent drug binding. Blocking the site of PDI that binds to CD4 is also a possibility, as that domain is different from the PDI active site. Once identified, it might be masked without affecting the physiologic functions of surface PDI.

3) Targeting disulfide bonds of gp120 that are substrates for PDI

This approach is made possible by the description of Phe43 pocket of gp120 harboring the CD4-gp120 binding

site [9,12]. It is made particularly attractive by the identification in the same region of a BMS 806 binding site [14]. Recent structural studies confirm that the gp120 pocket is a potential binding site for entry inhibitors [13]. As mentioned previously[3] the presence of four disulfide bonds at or near that site suggests that BMS 806 binding might prevent PDI from reaching and reducing gp120 disulfide substrates, thereby preventing the initiation of the important conformational changes that lead to gp41activation and HIV-1 entry. If such is the case, BMS 806 would antagonize PDI by competing for binding to a site located in the Phe43 pocket of gp120. Blocking that site with BMS 806 might prevent access of the enzyme to its gp120 disulfide substrates, thereby preventing the reduction of structure-stabilizing disulfide bonds and the initiation of the conformational changes required for gp41 activation and HIV-1 entry. BMS 806 would thus antagonize PDI by competing with the enzyme for binding to the same site of the Phe43 pocket that happens to be disulfide-rich. This would make BMS 806 the first entry inhibitor that targets gp120 disulfide bonds and directly prevents a critical PDI–gp120 interaction. This hypothesis supports the view that the Phe43 pocket of gp120 is an attractive target for therapeutic intervention [8,14]. The reduction of cell-bound gp120 and its prevention by PDI inhibitors has been demonstrated in tissue culture [4–6]. These references describe the methodology to test whether BMS 806 prevents disulfide cleavage in gp120. If it does, it would establish a leading mode of action of BMS 806 and would provide the proof-of concept that regions surrounding gp120 disulfide bonds are appropriate targets for agents preventing HIV-1 entry.

As PDI catalyses a true disulfide interchange, two thiols are generated in the substrate and a disulfide bond is formed in the PDI active site (Figure 4). The conformational changes resulting from the first disulfide reduction might bring the newly generated thiols close to a second disulfide bond that can get reduced non-enzymatically. It has indeed been shown that gp120 undergoes several disulfide reductions upon its binding to cellular CD4 [5]. The presence of free thiols in an environment that is unstable and rich in disulfide bonds could start a cascade of reductions. It is conceivable that disulfide bonds present in the ectodomains of co-receptors might also be involved. The occurrence of a conformational cascade suggests that additional potential drug targets are present along the road to gp41 activation.

Summary

Conformational changes in receptor-bound gp120 profound enough to activate virus-cell fusion can be initiated by the reduction of structure-stabilizing disulfide bonds, carried out by the receptor-associated enzyme PDI. This view suggests three novel targets for anti-HIV-1 drug development. Inhibiting the activity of surface PDI, the first potential target, is not a favored option because the enzyme serves other physiological functions at the cell surface. The sites of PDI–CD4 and PDI–gp120 interactions, however, are two attractive targets. PDI–CD4 binding involves two stable cellular proteins, all but eliminating the possibility that drug-induced mutations would abolish drug binding. Any event preventing PDI–CD4 interaction is likely to preclude infection.

The feasibility of preventing a critical interaction of PDI with gp120 will no doubt be addressed by further research on the mode of action of BMS 806, a known inhibitor of HIV-1 entry. Blocking that third target is likely to lead to the type of escape mutations that have been observed after the administration of BMS 806. Such mutations were mapped not only to the drug binding site but also throughout gp120 and even in gp41. This is consistent with the view that HIV-1 entry is the end point of a cascade of conformational events. The transmission of a conformational signal from gp120 to gp41 is a fast and complex process that holds the promise that virus-infection-based screening could identify inhibitors that interrupt the transmission at many unpredictable sites. The success of T20 [Fuzeon®], which acts on a short-lived phase of gp41 activation, indicates that even transient targets are of therapeutic interest. Clearly, therapeutic success will continue to improve with the diversity and number of available drug targets.

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