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Drug–Antibody Conjugates with Anti-HIV Activity

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ABSTRACT. Human immunodeficiency virus (HIV)-specific peptide antibody–brefeldin A conjugates and antibody–glaucarubolone conjugates directed to cell surface viral glycoprotein epitopes were prepared and tested for antiviral activity. A selective response was observed both on survival of cell lines permanently infected with lentiviruses and on HIV infectivity. With human peripheral blood mononuclear cells (PBMCs), the conjugate also was effective in reducing virus titers. The effectiveness of an HIV-specific peptide antibody–brefeldin A conjugate was enhanced by combination with 3′-azido-3′-deoxythymidine (AZT) and was effective against AZT-resistant isolates in combination with AZT. The conjugates reduced virus production in MOLT-4 cells and in HIV-1-infected PBMCs without affecting the viability of uninfected cells. BIOCHEM PHARMACOL **58**;11: 1781–1790, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. human immunodeficiency virus (HIV); glaucarubolone; quassinoids; MOLT-4 cells; peripheral blood mononuclear cells (PBMCs); drug conjugates; AZT

In previous reports, the inhibitors of a cell surface NADH-protein disulfide reductase (NADH oxidase) (NOX), brefeldin A [1] and glaucarubolone [2], were shown to selectively inhibit growth of CFK¶ cells permanently infected with FIV or human MOLT-4 cells permanently infected with HIV-1 [1, 2]. In an effort to direct the drug to a putative target at the cell surface with increased efficacy and improved therapeutic index, drug–antibody conjugates were prepared and tested. The drugs were conjugated with antibodies specific for surface epitopes of viral glycoproteins expressed at the surface of infected cells. Even during early stages of viral replication, virus-infected cells transport and express viral glycoprotein antigens at their cell surface ([3–6]; early literature reviewed in Ref. 7). The expression of these glycoprotein antigens provides opportunities (a) for drug targeting to virus-infected cells, and (b) to immobilize the drugs to restrict their action to cell-surface targets.

MATERIALS AND METHODS

Cell Culture

CFK cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS, 1% MEM non-essential amino acids, 1% BME vitamins, 1% sodium pyruvate, 1% penicillin (100 U/mL), 1% streptomycin (100 μg/mL), and 50 mg gentamicin/L.

FIV Infection

Infected CFK cells were maintained from a CFK cell stock that was chronically infected with the Petaluma strain of FIV [8].

PBMCs

PBMCs were derived from freshly prepared pooled buffy coats from approximately 15 healthy blood donors (German Red Cross). The PBMCs were isolated by Ficoll density gradient centrifugation and cultured (stimulated with phytohemagglutinin) for 48 hr in RPMI-1640 medium with 20% FBS. The time course of infection and drug addition with PBMCs is summarized in Fig. 1.

MOLT-4 Cells

The MOLT-4 cells, obtained from the ATTC, were a permanent cell line derived from a patient with acute lymphoblastic leukemia. The MOLT-4 cells were propagated in RPMI-1640 medium with 10% FBS.

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¶ *Abbreviations*: CFK, Crandall Feline Kidney; PBMCs, human peripheral blood mononuclear cells; FIV, feline immunodeficiency virus; HIV-1, human immunodeficiency virus Type 1; PDI, protein disulfide isomerase; TMSOTf, trimethylsilyltrifluoromethanesulfonate; FBS, fetal bovine serum; and AZT, 3′-azido-3′-deoxythymidine.

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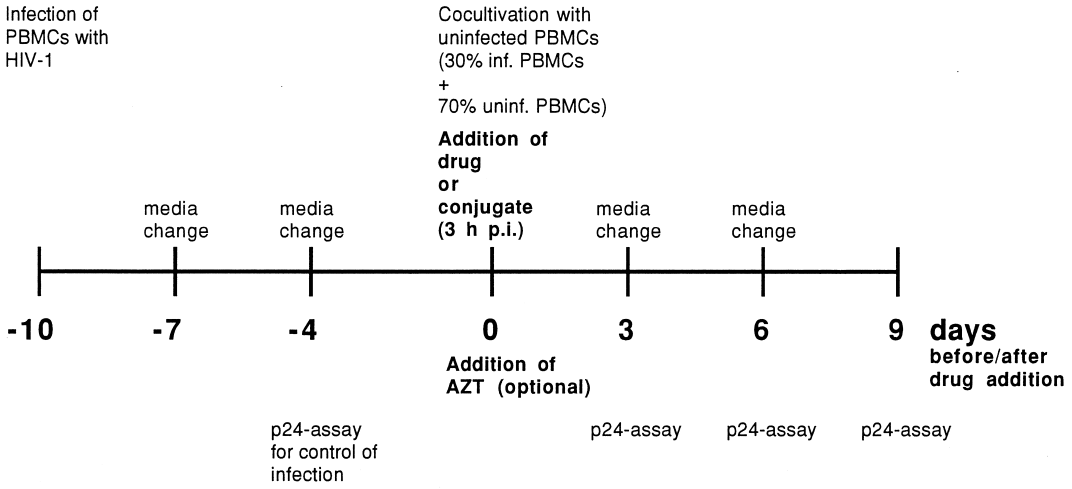


FIG. 1. Time course of infection and drug addition with PBMCs.

HIV-1 pp

The virus strain was a clinical isolate from an AIDS patient hospitalized in Berlin in 1990. The virus was isolated by cocultivation of PBMCs from the patient together with pooled buffy coat derived PBMCs from healthy blood donors. HIV-1 pp is an AZT-sensitive strain that induces syncytia formation with PBMCs and MOLT-4 cells.

Determination of Virus Reproduction and Efficacy of Drug–Antibody Conjugates

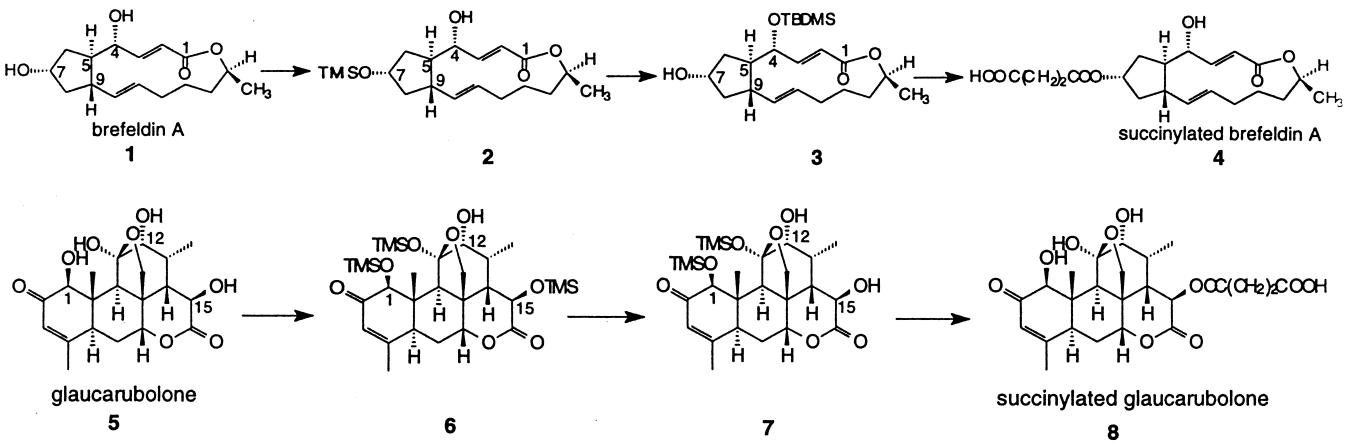
Virus reproduction was determined by detection of p24 antigen of HIV-1 (Du Pont) in a quantitative assay [9].

Preparation of Succinylated Brefeldin A Derivative for Conjugation

Compound 4 (Scheme 1) was prepared by a synthetic strategy that allowed us to readily differentiate the two hydroxyl groups of commercially available brefeldin A (compound 1). Exposure of brefeldin A to trimethylsilyl chloride in tetrahydrofuran containing triethylamine gave rise (90% yield) to the C(7) silylated compound 2. Silylation of compound 2 with tert-butyldimethylsilyl chloride in methylene chloride containing 2,6-lutidine and subsequent treatment [0°] with dilute hydrochloric acid in tetrahydrofuran generated (75% overall) the C(4) protected substrate (compound 3). Exposure of compound 3 to succinic anhydride followed by treatment with HF/CH_3CN resulted in succinylated brefeldin A (compound 4) in 80% yield. Structures were verified from spectral properties using methods described previously (^{1}H NMR, IR) [10].

Preparation of Succinylated Glaucarubolone

We prepared compound 8 (Scheme 1) for conjugation with antibodies based on a general procedure developed for the differentiation of the C(1), C(12), and C(15) hydroxyl groups in glaucarubolone (compound 5). Exposure of glaucarubolone to TMSOTf in pyridine containing triethyl-



SCHEME 1. Preparation of succinylated brefeldin A (compound 4) and succinylated glaucarubolone (compound 8) for conjugation to antibodies. Abbreviations: TBDMS, *tert*-butyldimethylsilyl; and TMS, trimethylsilyl.

amine (0° – RT, 1 hr) gave rise (75% yield) to tris-trimethylsilylated compound 6. Exposure of compound 6 to tetrabutylammonium fluoride in tetrahydrofuran cleaved exclusively in excellent yield the C(15) OTMS ether (compound 7). Treatment of compound 7 with succinic anhydride in methylene chloride containing triethylamine and 4-dimethylaminopyridine gave rise (80%) after treatment with HF/CH_3CN to compound 8, which was conjugated to antibodies. Note that the C(12) hydroxyl group was sufficiently hindered to allow rapid stereospecific acylation at the C(15) hydroxyl group to give rise exclusively to compound 8. The structure of derivatized glaucarubolone was verified from spectral properties (^1H NMR, IR) [10].

HIV gp120 Peptides for Preparation of Antisera

Three criteria for the selection of peptide sequences used for antibody production were: (a) hydrophilicity as calculated according to the algorithm of Hopp and Woods [11], (b) surface probability as calculated according to a formula of Emini *et al.* [12], and (c) the antigenic index measuring the probability that a region was antigenic as calculated by summing several weighted measures of secondary structure [13].

Peptides capable of generating high titer antisera that recognized surface epitopes of gp120 of HIV-1 were identified as follows:

Ab 75. CEESQNQQEKNEQEL
Ab 76. CNRVRQGYSPLSFQT
Ab 77. CEGIEEEGGERDRDR

Antibody Conjugate Preparation

Succinylated drugs were conjugated to affinity-purified antibodies as previously described for Adriamycin™ (doxorubicin) [14].

Affinity columns were prepared using peptides against which the antibodies were generated. The antibodies were bound to the affinity column and were conjugated with compounds 4 or 8.

For immunoaffinity purification of the antibody, the antigen peptide, derived from HIV envelope glycoprotein gp120 and conjugated to bovine serum albumin (10 mg/mL), was coupled to cyanogen bromide- (CNBr) activated Sepharose 4B (Pharmacia) by incubating the peptide along with swollen Sepharose in 0.1 M $NaHCO_3$, pH 8.3 (coupling buffer), at 4° for 16 hr. The remaining active groups were quenched by transferring the gel to a solution of 0.2 M glycine, pH 8.0, and incubating for 2 hr at room temperature. The column was washed with coupling buffer, then with 0.1 M acetate containing 0.5 M NaCl at pH 4.0, and finally with the coupling buffer. Then the peptide–Sepharose conjugate was incubated with the antisera at 4° for 16 hr. The immunoaffinity-purified antibody was eluted from the gel using 100 mM triethylamine, pH 11.5, and the eluted fraction was dialyzed against 20 vol. of PBS.

For the preparation of drug–antibody conjugates, affinity-purified antibodies were bound to the peptide column. The antibody bound to the peptide affinity column was incubated with succinylated drug in the presence of a 10 mM concentration of the coupling reagent 1-ethyl-3(3-diethylaminopropyl)carbodiimide (EDAC) (Sigma). The unbound drug was removed by washing three times with PBS. Then the drug conjugated with antibody was eluted from the peptide affinity column using 100 mM triethylamine, pH 11.5, and the eluted fraction was dialyzed against 20 vol. of PBS.

The number of drug molecules per immunoglobulin was estimated to be 20 per 150-kDa IgG heterooligomer. Succinylated glaucarubolone (compound 8) (0.27 mg) or succinylated brefeldin A (compound 4) (0.18 mg) was reacted with *ca.* 1.5 mg of affinity-purified antibody bound to the peptide affinity column. Approximately 50% of the succinylated compounds bound, based on what passed through the column plus what was bound nonspecifically to the column material in the absence of antibody. The starting antibody–drug conjugates were adjusted with PBS to give 35 μM drug equivalent concentrations and were diluted further with cell culture medium to give the drug equivalent concentrations for each of the antibody conjugates tested.

FIV Major Envelope Glycoprotein Peptides for Preparation of Polyclonal Antisera

Parallel procedures were employed for conjugation of drugs to peptide antibodies specific for the major envelope glycoprotein of FIV. Antisera to two different putative extracellular domains of the major FIV envelope glycoprotein were prepared and affinity purified.

S-I CKWEEAKVKFHCQRT
S-II CARFRIRCRWNVGSD

The specificity of the peptide antisera for the major envelope glycoprotein of FIV was confirmed by western blot analysis of whole cell lysates of FIV-infected CFK cells (not shown). The peptide antisera to gp120 were checked against authentic gp120 obtained through the AIDS Research and Reference Program of the National Institutes of Health.

RESULTS

Drug–Antibody Conjugates for Targeting Drugs to Surface Sites of Infected Cells

In previous studies, CFK cells infected with FIV as well as MOLT-4 cells infected with HIV-1 exhibited differential killing of infected cells as compared with uninfected cells by the quassinoid glaucarubolone [2]. HIV-1-infected MOLT-4 cells were differentially killed at a lesser amount (two log orders) of glaucarubolone than were uninfected cells. The growth inhibition by glaucarubolone has been correlated with inhibition of a cell surface hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity [15, 16] as the basis for the hypothesis that the

TABLE 1. Response to glaucarubolone conjugated with FIV antibody S-II or HIV-1 antibody 75

Response	EC_{50} (μM)
Glaucarubolone–FIV Ab S-II conjugate	
FIV-infected CFK cell viability (day 4)	1
Uninfected CFK cell viability (day 4)	>100*
Glaucarubolone–HIV-1 Ab 75 conjugate	
p24 production in HIV-1-infected PBMCs (day 6)	0.2
PBMC viability (day 6)	>1*

Values are based on three to four determinations.
*Highest concentrations tested due to limited availability of antibody-conjugated drug.

drugs need not enter cells to be effective in differential killing of the infected cells.

To target drugs to the surface of infected cells as an approach to improve both efficacy and therapeutic index, a series of drug–antibody conjugates were prepared. The drugs were conjugated to antibodies specific for surface epitopes of viral glycoproteins expressed at the surface of infected cells. Even during early stages of viral replication, well before mature virions are budded from the cell surface, virus-infected cells transport and express viral glycoprotein antigens at their cell surface ([3–6]; early literature reviewed in Ref. 7). Antibody–drug conjugates were prepared as described to both brefeldin A and glaucarubolone by reacting the drugs with peptide antibodies, as described in Materials and Methods, against both FIV envelope glycoproteins and to gp120 of HIV-1.

Peptide Antibodies to Major FIV Glycoproteins

With peptides to the major FIV envelope glycoprotein, polyclonal antisera were generated against two 15-mer sequences. Uninfected cells treated with the antibody–glaucarubolone conjugates were not killed at concentrations up to 100 μM, whereas FIV-infected CFK cells were killed at concentrations as low as 10^{-8} M (EC_{50} = 1 μM) (Table 1, Fig. 2). Conjugates with brefeldin A killed infected cells at a 1 μM concentration (Fig. 3), which was not toxic for uninfected cells.

Peptide Antibodies to Conserved Regions of gp120 HIV-1

The experience with brefeldin A and glaucarubolone linked to antibodies directed against the major FIV envelope glycoprotein was followed by trials with the same drugs linked to peptide antibodies raised against conserved regions of gp120 of HIV-1. Three different peptide antisera (numbered 75, 76, and 77 in Materials and Methods) conjugated with brefeldin A were designated as S-3, S-4, and S-5, respectively. Activity was observed with both human MOLT-4 cells and with HIV-1-infected PBMCs with antibody conjugates of either glaucarubolone (Table 1) or brefeldin A (Figs. 4–7).

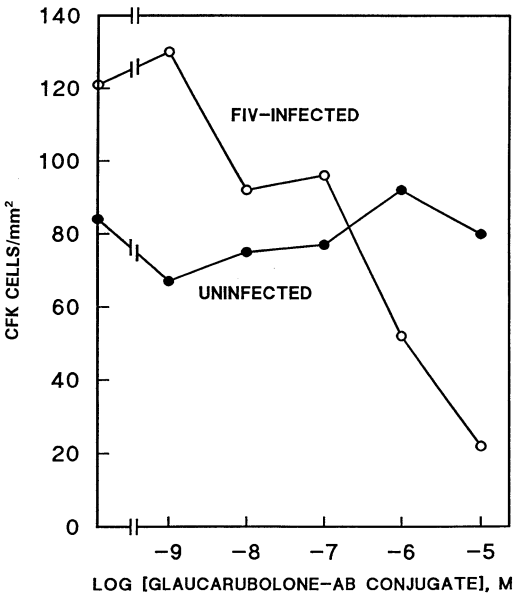


FIG. 2. Selective growth inhibition and killing of FIV-infected CFK cells with glaucarubolone conjugated to peptide antibody S-II. Each value is the average of four determinations. The uninfected cells varied ± 11 cells/mm², and the infected cells varied ± 14 cells/mm².

Results for selective growth inhibition and killing of HIV-1-infected MOLT-4 cells are given in Fig. 4. After 65 hr of drug treatment, all three conjugates (S-3, S-4, and S-5) reduced the growth of infected cells by 35–65% without loss of viability of uninfected cells. The conjugates also reduced virus production by the MOLT-4 cells after 48 hr, based on a p24 assay with an IC_{50} of about 1 nM (Fig. 5).

With PBMCs prepared by mixing 70% uninfected cells

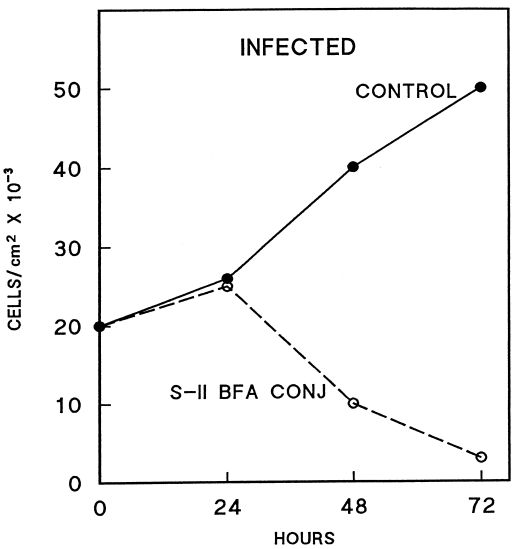


FIG. 3. Selective killing of FIV-infected CFK cells with or without (control) *ca.* 1 μM brefeldin A (BFA) equivalents conjugated to a peptide antibody (S-II) directed to a surface epitope of the major FIV glycoprotein. Results are from a typical experiment. The observations were repeated three times with similar results. The time for killing of 50% of the cells was estimated to be 48 ± 6 hr.

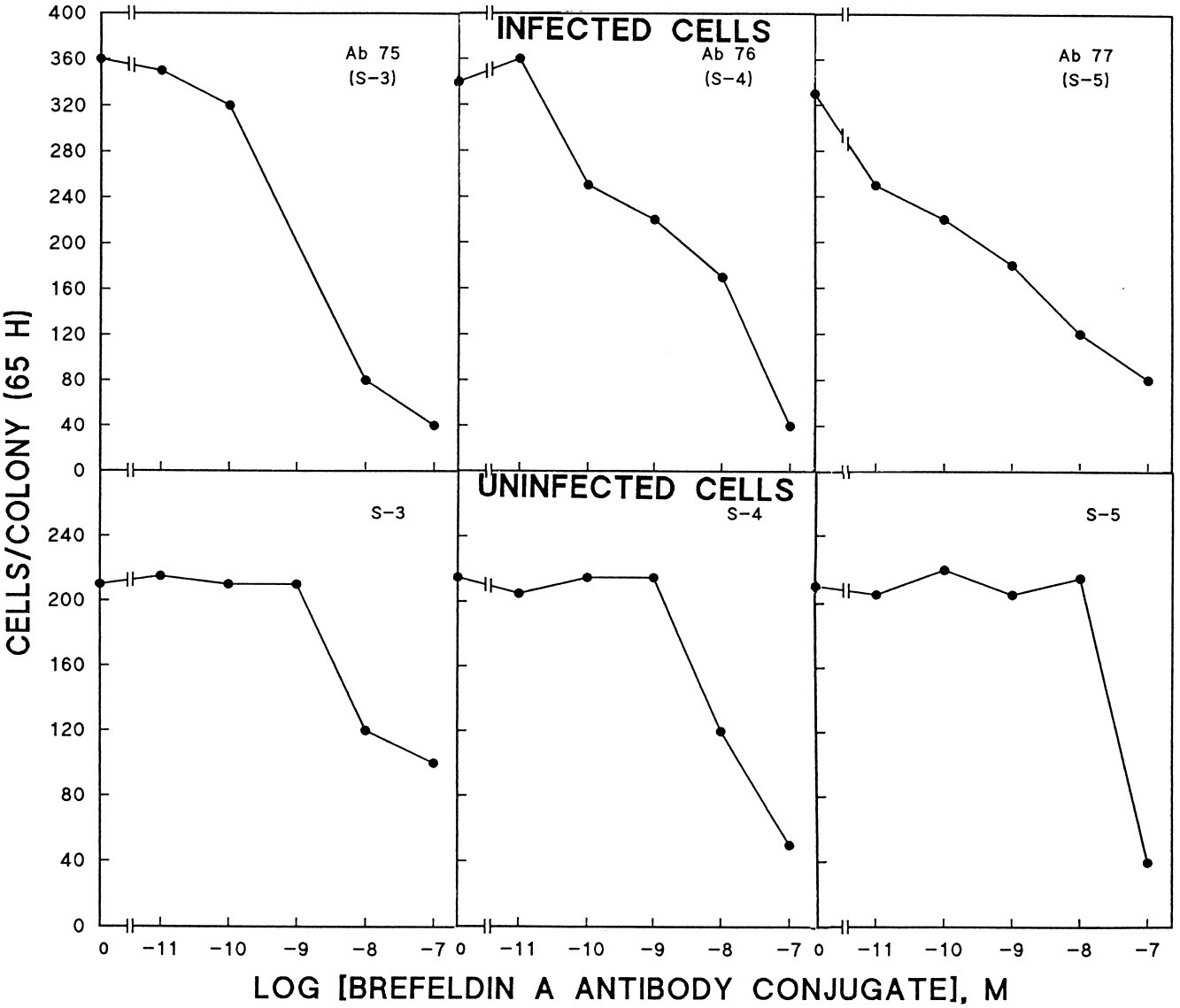


FIG. 4. Selective growth inhibition and killing of HIV-1 infected human MOLT-4 cells after 65 hr, comparing brefeldin A conjugated to three different peptide antibodies directed to surface epitopes of gp120 (conjugates S-3, S-4, and S-5). The upper three panels are from infected cells. The lower three panels are from uninfected cells tested in parallel with drug conjugated to antibody exactly as described for infected cells. Values are averages of duplicate determinations and exhibited an average deviation from the mean of ±7%.

with 30% HIV-1-infected cells (Fig. 1), conjugates S-3 (antibody 75), S-4 (antibody 76), and S-5 (antibody 77) killed 20–30% of the infected cells by 18 hr after treatment under conditions where uninfected cells remained completely viable (upper panels, Fig. 6). S-3 appeared to be somewhat more effective in slowing virus production (based on p24 assays; lower panels, Fig. 6) and was tested further.

S-3 at 10 nM reduced p24 levels in cell supernatants with HIV-1-infected PBMCs without affecting cell viability of uninfected cells (Fig. 7). Antibody alone was without effect. Brefeldin A alone was cytotoxic to uninfected cells at 10^{-8} M, whereas the conjugate was not (Fig. 7). A physical mixture of antibody and brefeldin A in the approximate ratios as present with the conjugate exhibited cytotoxicity equivalent to brefeldin A alone (data not shown). The succinylated brefeldin A also failed to exhibit selectivity and was not tested further.

In the PBMC system, the most promising conjugate, S-3, was tested further in combination with AZT using AZT-sensitive and AZT-resistant isolates of HIV-1. S-3 (10 nM) also was active when combined with 10 and 100 nM AZT (Figs. 8 and 9, Tables 2–4) according to the infection schedule summarized in Fig. 1. In the presence of 10 nM S-3 plus 10 nM AZT, production of virus was inhibited by 90% over the 9-day observation period (Fig. 8). Approximately 10 μM AZT alone was required to reduce p24 levels to the same degree as the combination of 10 nM AZT with 10 nM S-3. Antibody 75 plus AZT were equivalent to AZT alone. Brefeldin A plus AZT were not tested.

On day 3 following infection, the combination of 1 nM

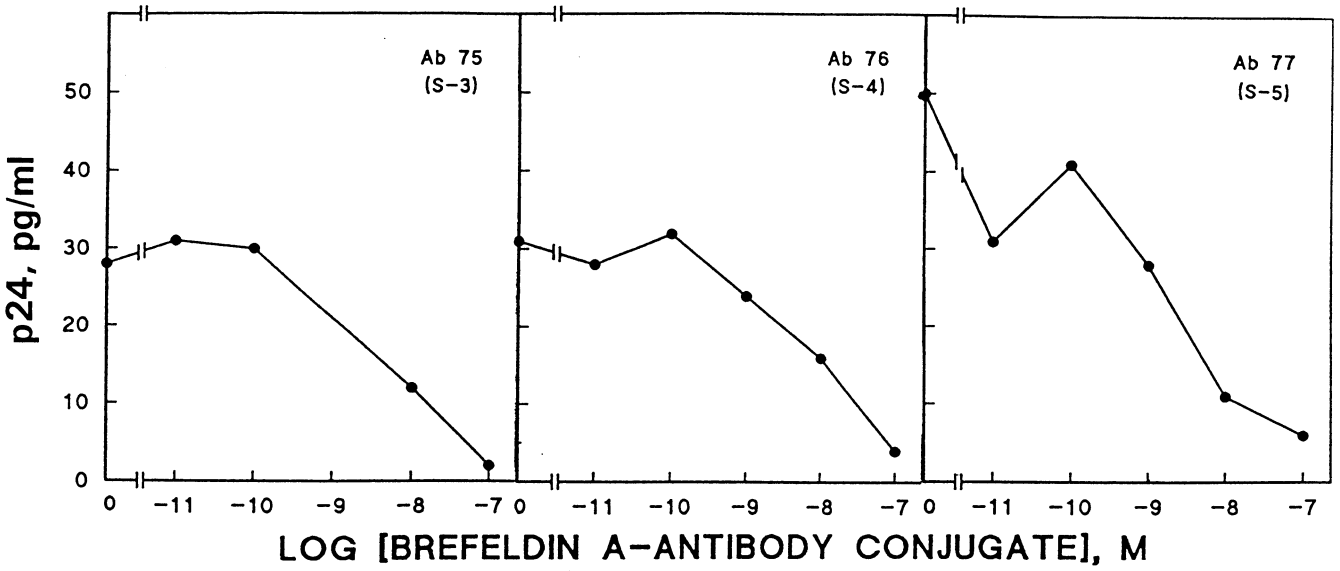


FIG. 5. ELISA of supernatant p24 as a measure of mature virus production after 4 days with HIV-1 infected human MOLT-4 cells treated with various concentrations of brefeldin A–antibody conjugates. Results are based upon quadruplicate determinations. A background blank of 10 pg/mL was subtracted. Values varied, on average, ±10%.

S-3 plus 10 nM AZT was more effective than either S-3 or AZT alone in blocking p24 production by PBMCs infected with an AZT-sensitive HIV-1 strain (Table 2). By days 6 or 9 following infection, S-3 alone or S-3 combined with 10 nM AZT were equivalent in effectiveness but more effective than AZT alone.

The therapeutic index for the S-3 antibody conjugate showed that 4–10-fold higher concentrations were required to alter cell viability than to reduce p24 production. In addition, the combination of S-3 and AZT was effective against an AZT-resistant virus strain (Table 4). With the AZT-resistant strain of HIV-1, the concentration of AZT required to reduce p24 production by 50% was reduced 3- to 5-fold by combination with 10 nM S-3.

In the presence of 10 nM S-3, 10 nM AZT resulted in a markedly enhanced reduction of p24 production equivalent to 10 μM AZT and with only a 10% loss of cell viability (Fig. 8).

DISCUSSION

The approach that we followed represented a novel use of antibodies. They were used neither to neutralize virus nor to deliver a toxic drug into virus-infected cells but to generate a drug–antibody conjugate directed to a novel cell surface target to reduce virus infection and the number of virus-infected cells. The conjugates were toxic to permanently infected cell lines and with PBMCs led to a reduction of HIV reproduction, exhibited enhanced activity as mixtures when combined with AZT, and reduced virus reproduction of AZT-resistant HIV-1 at drug concentrations not toxic to uninfected cells. Free brefeldin A has been reported to exert antiviral activity [17–21] but alone was cytotoxic and unselective with HIV-1-infected PBMCs in our studies.

The antibodies were bound to an affinity column during conjugation. This ensured that the antibody binding site was protected during conjugation and that little or none of the drug was conjugated through the portion of the molecule involved with antigen binding. This was an important consideration in the design of the conjugation strategy and one of the principal reasons that peptide antibodies were utilized. The binding of the drug–antibody conjugates to the surface of infected cells was the product of the affinity of binding of the drug portion to its target and the affinity of binding of the peptide antibody portion. The primary purpose of using the antibodies toward cell surface epitopes was to provide selectivity to ensure binding of the drug–antibody conjugates to virus-infected cells with greater avidity and selectivity than to uninfected cells.

The conjugates were relatively stable and appeared not to require entry into cells to be effective. Due to the large size of the antibody–drug conjugate, cell entry would be restricted to endocytotic release of free drug by enzymatic hydrolysis of the conjugates. While it is not known if the conjugates affect virus replication *per se*, they did interact synergistically with AZT and were effective against AZT-resistant virus isolates. We suggest that the conjugates block at a novel cell surface target. They appear to exert antiviral activity by killing infected cells and by interfering with either reinfection or budding and release of mature virus or both.

Enveloped mammalian viruses generally enter cells via fusion of viral and cellular membranes [22]. Thiol-disulfide interchange reactions occur during the interaction of viruses with cells, and these interactions may be necessary for

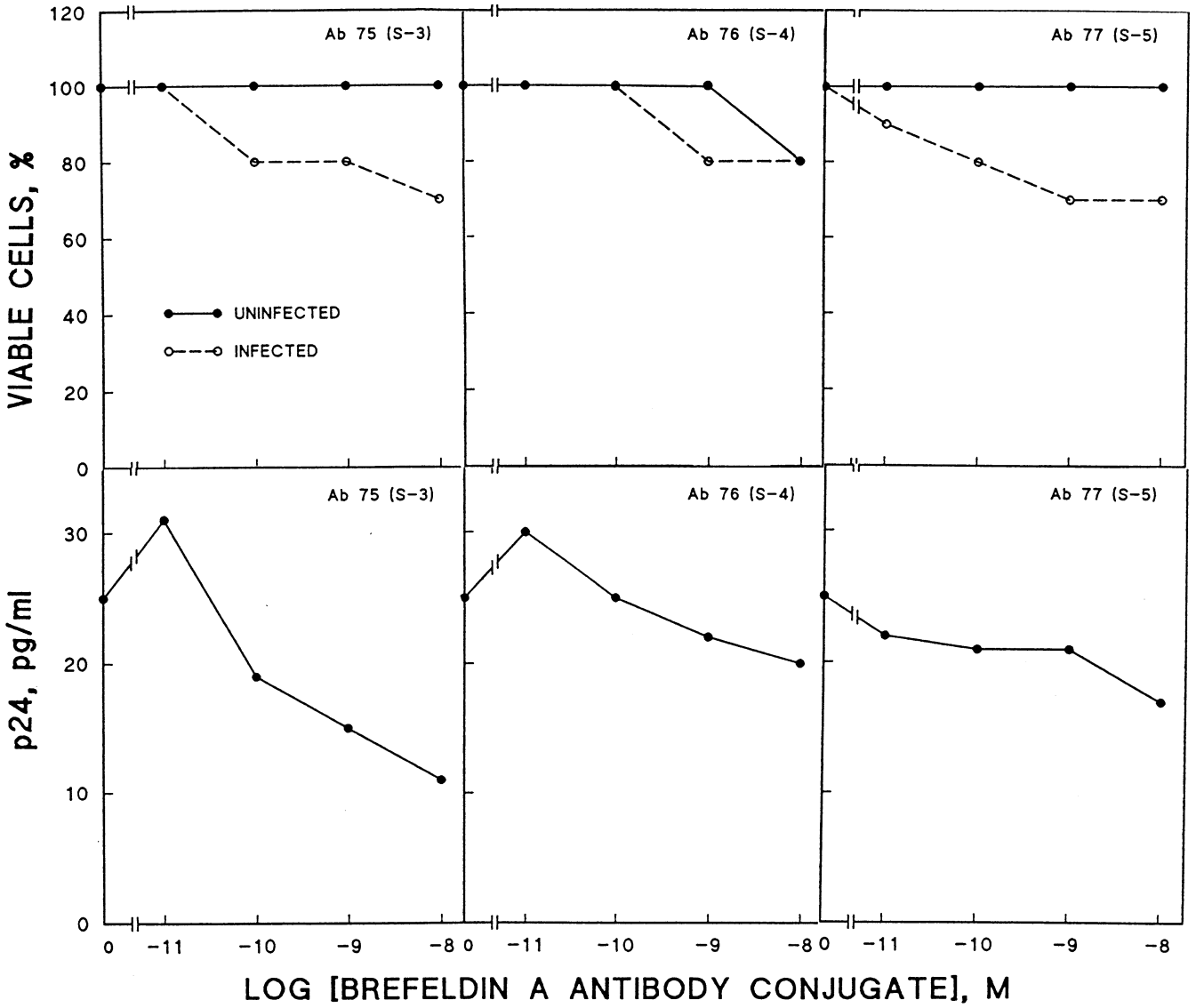


FIG. 6. Viability (upper panels) and HIV-1 pp reproduction (p24 production; lower panels) in human peripheral blood monocyte cultures. The cultures were prepared by mixing 70% uninfected cells with 30% infected cells followed by treatment with brefeldin A–antibody conjugates. The upper panels are percent cells after 18 hr of treatment. Infected (open symbols) but not uninfected (solid symbols) cells appear to be killed selectively. Values are averages of six replicate determinations. The lower panels are virus production after day 4 determined according to the scheme of Fig. 1. The average for the untreated cells was 25 ± 3 ng/mL after subtraction of a background blank of 8 pg/mL.

fusion of viral and cellular membranes [23, 24]. For example, Sindbis virus-induced fusion of cells was enhanced by exogenous reducing agents and inhibited by thiol alkylating agents [24]. The explanation offered was based on a model in which virion binding to cells led to reduction of critical disulfide bonds in the Sindbis envelope proteins. The result was suggested to be an increased flexibility required for fusion of viral and cellular membranes.

Infection of lymphoid cells by HIV-1 was inhibited by membrane impermeant sulfhydryl blocking reagents and by inhibitors of cell surface PDI [23]. Implicit in the findings of Ryser *et al.* [23] was the interpretation that the PDI-like activity mediates a thiol-disulfide exchange with HIV-1 envelope proteins, triggering changes in conformation required for HIV-1 entry.

We have reported previously a hydroquinone (NADH) oxidase (NADH-protein disulfide reductase) with protein disulfide-thiol interchange activity of the plasma membrane [16]. Activity measured as the oxidation of NADH is inhibited by both glaucarubolone [15] and brefeldin A [1] and their conjugates (unpublished results) and is the putative drug target for the studies reported here.

The first demonstrations of the thiol interchange activity used as the principal criterion were: the restoration of activity to reduced, denatured, and oxidized (scrambled) yeast RNase through reduction, refolding under non-dena-

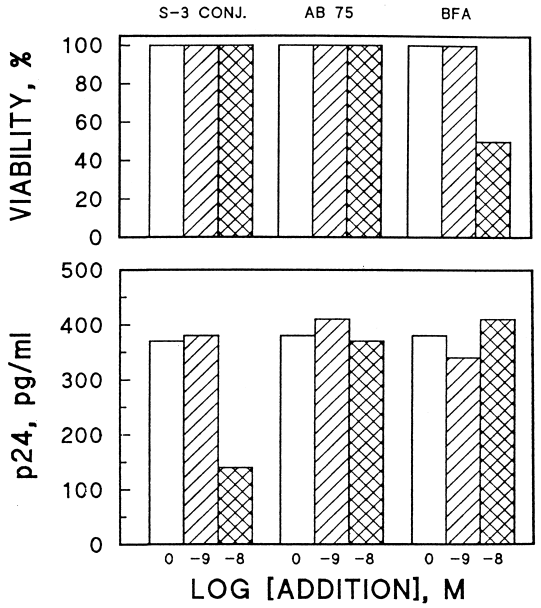


FIG. 7. Viability of uninfected human peripheral blood monocyte cultures (PBMCs) (upper panel) and HIV-1 pp reproduction (p24 production) in infected PBMCs (lower panel). Determinations were as for Fig. 4 except after 9 days of treatment, comparing the S-3 conjugate of HIV-1 peptide antibody to gp120 No. 75 (Ab 75) on a brefeldin A (BFA) equivalent basis and BFA alone at 1 and 10 nM final concentrations. Ab 75 alone was tested at 75 and 750 ng/mL protein in the assay corresponding to the amount added as the S-3 conjugate.

turing conditions, and reoxidation to form a correct secondary structure stabilized by internal disulfide bonds. The activity resembled that of a classical PDI of the endoplasmic reticulum [25] but clearly was due to the activity of a different protein. With cancer cells, the plasma membrane activity was responsive to a small cadre of potential antitumor drugs, and the drug-responsive protein disulfide-thiol interchange activity was not altered by the presence of two different antisera to PDI [26]. One antisera tested was mouse monoclonal antibody (SPA-891, StressGen Biotechnologies) to PDI from bovine liver (cross-reactive with PDI from human, monkey, rat, mouse, and hamster cell lines). The other was a peptide antibody of our own derivation directed to the characteristic cys-X-X-cys motif common to most, if not all, members of the PDI family of proteins [27, 28].

The drug-inhibited NADH oxidation site of the cell surface-located drug-inhibited protein disulfide-thiol interchange protein postulated to be essential to virus entry also

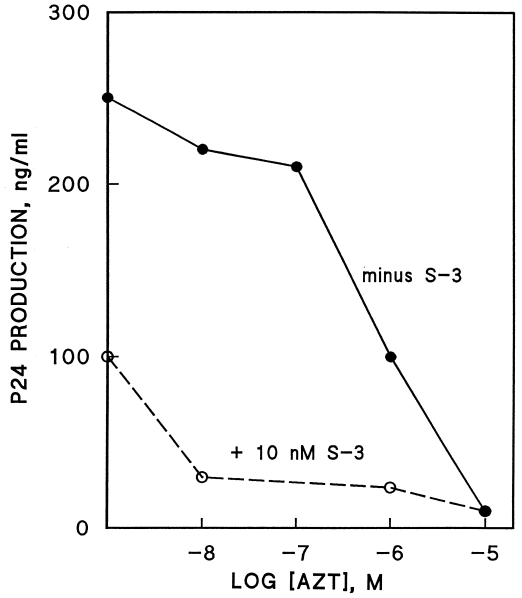


FIG. 8. AZT dependence of p24 production after 9 days in the presence of various concentrations of AZT by PBMCs infected with HIV-1 pp in the absence (solid symbols) or presence (open symbols) of 10 nM conjugate S-3. Values are averages of four determinations ± 5 ng/mL.

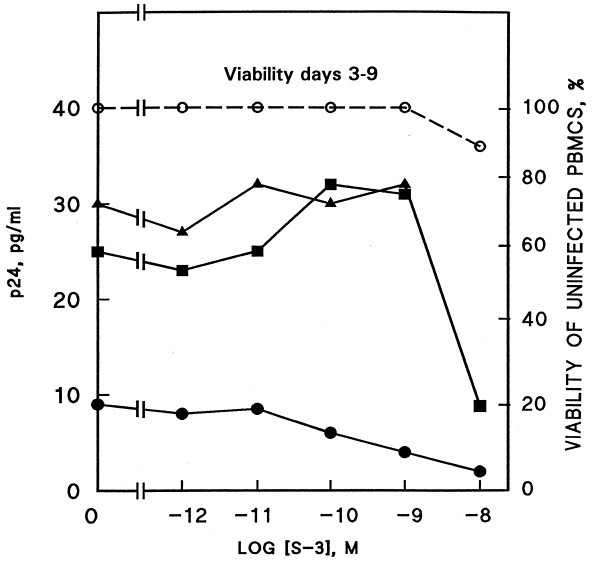


FIG. 9. Effect of the drug–antibody conjugate S-3 in combination with 10 nM AZT on viability of PBMCs as for Fig. 4 and on HIV-1 pp reproduction (p24 production) as for Fig. 5 except that a background blank (about 5 pg/mL) was not subtracted. Results are from a single experiment. Closed circles: p24 production, day 3. Closed squares: p24 production, day 6. Closed triangles: p24 production, day 9. Open symbols and dashed line: viability of uninfected PBMCs at days 3–9. Viability of uninfected cells was determined by light microscopy.

TABLE 2. Effect of the antibody conjugate S-3 and AZT alone and in combination on p24 production by PBMCs infected with AZT-sensitive strain HIV-1 pp

	IC_{50} based on p24 production (nM)		
Drug	**Day 3**	**Day 6**	**Day 9**
AZT	300	500	900
S-3	10.0	4.4	6.1
S-3 combined with 10 nM AZT	0.4	6.5	5.3

Values are based on quadruplicate determinations with five concentrations of AZT alone (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M), S-3 alone (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M), or S-3 combined with 10 nM AZT for each treatment and time point.

TABLE 3. Effect of the antibody conjugate S-3 and AZT alone and in combination on viability of uninfected PBMCs

	LC_{50} (μM)		
Drug	**Day 3**	**Day 6**	**Day 9**
AZT	180	32	32
S-3	0.038	0.038	0.034
S-3 combined with 10 nM AZT	Not toxic at 10 nM*		

Values are based on means of quadruplicate determinations with five concentrations of AZT alone (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M), S-3 alone (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M) or S-3 combined with 10 nM AZT for each treatment and time point.

*Higher concentrations of S-3 were not tested in combination with AZT. AZT was without effect on cell viability.

is external [29]. Therefore, based on the considerable correlative evidence summarized below, we postulate that the drug-responsive protein disulfide-thiol interchange protein of the mammalian surface is the target responsible for the antiviral activities of the conjugated drugs and, therefore, represents a novel new target for drug prevention of viral infection as well as an opportunity to expand understanding of how enveloped viruses gain entry into cells during the infective process.

The NADH oxidase with protein disulfide-thiol interchange activity of the plasma membrane was first described as growth factor- and hormone-responsive [16, 30]. In transformed cells, the activity was constitutive [31] and no longer hormone- and growth factor-responsive [32]. Additionally, the activity was inhibited by thiol reagents [33]. The cancer form of the activity was inhibited specifically by a small cadre of antitumor drugs whose sites of action, until then, had remained elusive. These drugs included the antitumor sulfonylureas [34], the antitumor quassinoids [15], and the vanilloid capsaicin (8-methyl-*N*-vanillyl-6-noneamide) [31, 35]. The target protein was at the external cell surface [29], and drugs directed to the protein [36] did not need to enter the cells to be effective [14, 37]. A cell surface target was indicated previously from studies where an antitumor sulfonylurea was conjugated to an impermeant cyclodextrin [37]. Similarly, the derivatized glaucarubolone when linked to aminopolyethyleneglycol (aminoPEG) reduced infectivity under conditions where 70% uninfected cells were mixed with 30% HIV-1-infected cells. When compared in the presence of various concentrations of AZT, the effectiveness of conjugated drug showed an even greater enhancement of activity, essentially preventing virus infection as evidenced by a constant low titer of p24 over a 9-day period (Fig. 9). These findings, although preliminary, indicate a potential cell surface target involved in virus infection that is sensitive to immobilized drugs. The immobilized drugs also inhibit, in infected cells, an NADH oxidase with protein disulfide thiol interchange activity located at the cell periphery.

TABLE 4. Effect of the antibody conjugate S-3 and AZT alone and in combination on p24 production by PBMCs infected with AZT-resistant strain HIV-1 p214

	IC_{50} based on p24 production (μM)		
Drug	**Day 3**	**Day 6**	**Day 9**
AZT	4.0	4.6	9.0
S-3	>0.01	>0.01	>0.01
AZT combined with 10 nM S-3*	1.5	1.6	1.8

Values are based on quadruplicate determinations with five concentrations of AZT alone, S-3 alone, or AZT combined with S-3 at each time point. The concentrations of drug tested are those given for Tables 2 and 3.

*S-3 was not toxic at 10 nM to infected cells.

References

1. Morré DJ, Paulik M, Lawrence JM and Morré DM, Inhibition by brefeldin A of NADH oxidation activity of rat liver Golgi apparatus accelerated by GDP. *FEBS Lett* **346**: 199–202, 1994.
2. Morré DJ, Zeichhardt H, Maxeiner HG, Grünert HP, Sawitzky D and Grieco P, Effect of the quassinoids glaucarubolone and simalikalactone D on growth of cells permanently infected with feline and human immunodeficiency viruses and on viral infections. *Life Sci* **62**: 213–219, 1998.
3. Owens RJ and Compans RW, Expression of the human immunodeficiency virus envelope glycoprotein is restricted to basolateral surfaces of polarized epithelial cells. *J Virol* **63**: 978–982, 1989.
4. Stephens EB and Compans RW, Assembly of animal viruses at cellular membranes. *Annu Rev Microbiol* **42**: 489–516, 1988.
5. Saraste J and Kuismanen E, Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell* **38**: 535–549, 1984.
6. Tooze J, Tooze SA and Warren G, Replication of coronavirus MHV-A59 in sac$^-$ cells: Determination of the first site of budding of progeny virions. *Eur J Cell Biol* **33:** 281–293, 1984.
7. Morré DJ and Ovtracht L, Dynamics of the Golgi apparatus: Membrane differentiation and membrane flow. *Int Rev Cytol Suppl* **5:** 61–188, 1977.
8. Phillips TR, Talbott RL, Lamont C, Muir S, Lovelace K and Elder JH, Comparison of two host cell range variants of feline immunodeficiency virus. *J Virol* **64**: 4605–4613, 1990.
9. Japour AJ, Mayers DL, Johnson VA, Kuritzkes DR, Beckett LA, Arduino JM, Lane J, Black RJ, Reichelderfer PS, D'Aquila RT, Crumpacker CS, the RV-43 Study Group and the AIDS Clinical Trials Group Virology Committee Resistance Working Group, Standardized peripheral blood mononuclear cell culture assay for determination of drug susceptibilities of clinical human immunodeficiency virus type 1 isolates. *Antimicrob Agents Chemother* **37**: 1095–1101, 1993.
10. Fleck TJ and Grieco PA, Synthetic studies on quassinoids: Total synthesis of (±)-glaucarubolone and (±)-holacanthone. *Tetrahedron Lett* **14**: 1813–1816, 1992.
11. Hopp TP and Woods KR, Prediction of protein antigenic determinants from amino acid sequences. *Proc Natl Acad Sci USA* **78**: 3824–3828, 1981.
12. Emini EA, Hughes JV, Perlow DS and Boger J, Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J Virol* **55**: 836–839, 1985.
13. Jameson BA and Wolf H, The antigenic index: A novel algorithm for predicting antigenic determinants. *Comput Appl Biosci* **4**: 181–186, 1988.
14. Morré DJ, Kim C, Paulik M, Morré DM and Faulk WP, Is the drug-responsive NADH oxidase of the cancer cell plasma

membrane a molecular target for adriamycin? *J Bioenerg Biomembr* **29**: 269–280, 1997.
15. Morré DJ, Grieco PA and Morré DM, Mode of action of the anticancer quassinoids—Inhibition of the plasma membrane NADH oxidase. *Life Sci* **63**: 595–604, 1998.
16. Morré DJ, Hormone- and growth factor-stimulated NADH oxidase. *J Bioenerg Biomembr* **26:** 421–433, 1994.
17. Whealy ME, Card JP, Meade RP, Robbins AK and Enquist LW, Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. *J Virol* **65**: 1066–1081, 1991.
18. Zhou X, Glas R, Momburg F, Hammerling GJ, Jondal M and Ljunggren HG, TAP2-defective RMA-S cells present Sendai virus antigen to cytotoxic T lymphocytes. *Eur J Immunol* **23**: 1796–1801, 1993.
19. Kuzushima K, Isobe K, Morishima T, Takatsuki A and Nakashima I, Inhibitory effect of herpes simplex virus infection to target cells on recognition of minor histocompatibility antigens by cytotoxic T lymphocytes. *J Immunol* **144**: 4536–4540, 1990.
20. Tucker SP, Thornton CL, Wimmer E and Compans RW, Vectorial release of poliovirus from polarized human intestinal epithelial cells. *J Virol* **67**: 4274–4282, 1993.
21. Sreenivasan V, Ng KL and Ng ML, Brefeldin A affects West Nile virus replication in Vero cells but not C6/36 cells. *J Virol Methods* **45**: 1–17, 1993.
22. Battini J-L, Danos O and Heard JM, Receptor-binding domain of murine leukemia virus envelope glycoproteins. *J Virol* **69**: 713–719, 1995.
23. Ryser HJ, Levy EM, Mandel R and Di Sciullo GJ, Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. *Proc Natl Acad Sci USA* **91**: 4559–4563, 1994.
24. Abell BA and Brown DT, Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. *J Virol* **67:** 5496–5501, 1993.
25. Freedman RB, Protein disulfide isomerase: Multiple roles in the modification of nascent secretory proteins. *Cell* **57**: 1069–1072, 1989.
26. Morré DJ, Jacobs E, Sweeting M, de Cabo R and Morré DM, A protein disulfide-thiol interchange activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl) urea (LY181984). *Biochim Biophys Acta* **1325**: 117–125, 1997.
27. Chivers PT, Laboissiere MCA and Raines RT, The CXXC motif: Imperatives for the formation of native disulfide bonds in the cell. *EMBO J* **15**: 2659–2667, 1996.
28. Bardwell JCA and Beckwith J, The bonds that tie: Catalyzed disulfide bond formation. *Cell* **74**: 769–771, 1993.
29. Morré DJ, NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl) urea (LY181984) at an external site. *Biochim Biophys Acta* **1240:** 201–208, 1995.
30. Brightman AO, Wang J, Miu RK-M, Sun IL, Barr R, Crane FL and Morré DJ, A growth factor- and hormone-stimulated NADH oxidase from rat liver plasma membrane. *Biochim Biophys Acta* **1105**: 109–117, 1992.
31. Morré DJ, Chueh P-J and Morré DM, Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc Natl Acad Sci USA* **92**: 1831–1835, 1995.
32. Bruno M, Brightman AO, Lawrence J, Werderitsh D, Morré DM and Morré DJ, Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membranes. *Biochem J* **284**: 625–628, 1992.
33. Morré DJ and Morré DM, Differential response of the NADH oxidase of plasma membranes of rat liver and hepatoma and HeLa cells to thiol reagents. *J Bioenerg Biomembr* **27**: 137–144, 1995.
34. Morré DJ, Wu L-Y and Morré DM, The antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N′*-(4-chlorophenyl) urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membranes. *Biochim Biophys Acta* **1240**: 11–17, 1995.
35. Sun IL, Sun E, Crane FL, Morré DJ, Lundgren A and Löw H, Requirement for coenzyme Q in plasma membrane electron transport. *Proc Natl Acad Sci USA* **89**: 11126–11130, 1992.
36. Morré DJ, Morré DM, Stevenson J, MacKellar W and McClure D, HeLa plasma membranes bind the antitumor sulfonylurea LY181984 with high affinity. *Biochim Biophys Acta* **1244**: 133–140, 1995.
37. Kim C, MacKellar WC, Cho N, Byrn SR and Morré DJ, Impermeant antitumor sulfonylurea conjugates that inhibit plasma membrane NADH oxidase and growth of HeLa cells in culture. Identification of binding proteins from sera of cancer patients. *Biochim Biophys Acta* **1324**: 171–181, 1997.